Solanum okadae: A wild diploid potato species with the potential to improve cultivated potato

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ABSTRACT

Crop breeding is critical for the production of high-quality food and, as a result, for increasing global food security. The cultivated potato (*Solanum tuberosum*) is the world's most important tuber crop and the fourth most important crop farmed for human consumption, is a prime candidate crop for addressing global food security. Wild potato species are a significant source of genes for novel traits and resistances to abiotic and biotic stresses that are lacking from most commercial cultivars. Diploid potatoes account for over 70% of wild and landrace potato species, and their tremendous diversity has not been properly defined or fully utilised in past breeding projects. *Solanum okadae* is one such wild diploid potato species from western Bolivia that has not been extensively studied.

This thesis focuses on different aspects of wild potato species (*Solanum okadae*) research. These included *in vitro* micropropagation and regeneration, *Agrobacterium*-mediated transformation, nutritional analysis, *in vitro* screening for salinity tolerance as well as role of melatonin as a salt stress mitigator. This project also involved the identification of SLF sequences for three putative S-locus F-Box protein (SLF) from S_2 haplotype of *Solanum okadae* which are first to be sequenced in diploid potato species.

The *in vitro* micropropagation and regeneration experiments in this study showed a two-step plant regeneration process, which eliminated the need for sub-culturing the regenerated shoots on rooting media and may be utilised to develop an unique potato plant breeding programme and facilitate diploid potato plant transformation research. As compared to *Solanum tuberosum* 'Celandine,' the tubers of glasshouse grown *Solanum okadae* showed to be nutritionally superior, with significantly higher amount of carbohydrates, sugars, proteins, fatty acids, vitamins (C and B12), amino acids, and minerals.. Using *in vitro* screening, this study not only screened *Solanum okadae* for salt tolerance, but also unraveled the crucial role of melatonin in salt stress mitigation and thus can be used in the management of salinity in potato cultivation. *Solanum okadae* was recalcitrant to *Agrobacterium*-mediated transformation, which could be attributed to several factors including genotype, explant, culture conditions. Finally, based on *Petunia* SLF sequences as references, and using consensus PCR, three putative partial SLF sequences (type 7, 12 and 23) from *Solanum okadae* were identified.

The results from this thesis show that *Solanum okadae* can be used as a potential donor candidate for desirable traits in improving cultivated potatoes due to its superior nutritional value and tolerance to abiotic stresses like drought and salinity.

Thesis content and Structure

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General introduction

Chapter 2

Nutritional analysis of *Solanum okadae*

Chapter 3

In vitro screening of *Solanum okadae* salt stress tolerance, and the role of exogenous Melatonin in severe salt stress responses

Chapter 4

Optimizing protocol for micropropagation, callus induction and plant regeneration in *Solanum okadae*

Chapter 5

Designing CRISPR-Cas9 constructs for *S-RNase* knockout in *Solanum okadae*

Chapter 6

Agrobacterium-mediated transformation of *Solanum okadae*

Chapter 7

Self-incompatibility in *Solanum okadae*: Identification of putative S-Locus F-Box gene sequences

Chapter 8

General discussion

Due to the multifaceted nature of this thesis, each chapter has been written in individual research article format and therefore has its own individual figure/table referencing.

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LIST OF ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
BA	6-Benzylaminopurine
Car	Carotenoid
CAT	Catalase
Chl	Chlorophyll
CIM	Callus induction medium
CNSC	Coarse Container Nursery Stock
DAB	3,3′-Diaminobenzidine
DMRT	Duncan’s multiple range test
EAA	Essential amino acid
GC-FID	Gas Chromatography with flame-ionization detection
Geni	Geniticin
GSI	Gametophytic self-incompatibility
Hyg	Hygromycin
HV	Hypervariable
IC-PAD	Ion chromatography with Pulsed amperometric detection
ICP-MS	Inductively coupled plasma mass spectrometry
Kan	Kanamycin
KIN	Kinetin
LC-UV/DAD	Liquid Chromatography with UV/Diode Array Detection
MEGA	Molecular evolutionary genetics analysis
ML	Maximum likelihood
MT	Melatonin
NAA	1-Naphthaleneacetic acid
NEAA	Non-essential amino acid
NMR	Nuclear Magnetic Resonance
PAM	Protospacer adjacent motif
PGR	Plant growth regulator

RDA	Recommended dietry allowance
Rif	Rifampicin
RM	Regeneration medium
SC	Self-compatibility
SCF	Skp, Cullin, F-box
SFB	S haplotype-specific F-box protein
sgRNA	Single guide RNA
SI	Self-incompatibility
SLF	S-Locus F-box protein
Sli	S-Locus inhibitor
SPM	Stock plant medium
SSI	Sporophytic self-incompatibility
STI	Salt tolerance index
STTI	Salt tolerance trait index
WUE	Water use efficiency

CHAPTER 1: General Introduction

1.1 Family Solanaceae

The Solanaceae, also known as nightshades, is a family of flowering plants that includes a variety of edible, medicinal, and ornamental plants as well as annual and perennial vines, herbs, shrubs, lianas, and trees. Several members of the family contain potent, highly poisonous alkaloids, but some, like potatoes, tomatoes, aubergines, capsicums and chilli peppers, are eaten as food. The family belongs to the class Magnoliopsida (dicotyledons), asterid group, and order Solanales (Olmstead *et al*., 1999). The Solanaceae are a large family of flowering plants (angiosperms) with over 100 genera and over 3000 species (Morris and Taylor, 2017).

The family has spread all over the world and can be found on every continent except Antarctica. Central and South America have the most different kinds of *Solanum* species. In 2017, scientists said that a fossil tomatillo they found in the Patagonian region of Argentina and studied was from 52 million years before present. This new information has pushed back the date of the first Solanaceae plant (Wilf *et al*., 2017).

The *Physalis* genus produces the cape gooseberry, tomatillo (*Physalis philadelphica*), Chinese lantern, and so-called ground cherries. Among other genera, *Nicotiana* includes tobacco. Other noteworthy Solanaceae genera include *Datura*, *Mandragora* (mandrake), and *Atropa belladonna*, as well as several ornamental plants like *Browallia*, *Petunia*, and *Lycianthes* and sources of psychoactive alkaloids (deadly nightshade). Some species are well known for their therapeutic applications, psychoactive properties, or toxicity.

Important food species found in the Solanaceae family include the tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), aubergine (*Solanum melongena*), and pepper (*Capsicum annuum*). Originally from South America, tobacco (*Nicotiana tabacum*) is now grown all over the world. In some regions of the world, some Solanaceae species like *Solanum elaeagnifolium* are necessary weeds. Their importance comes from the fact that they may carry pathogens or diseases that affect the cultivated plants; as a result, their presence reduces harvest losses and improves the quality of the gathered crops. The Solanaceae family includes a wide range of plant species and cultivars that are grown as ornamentals, annuals, and herbaceous perennials. Examples include *Candida x Brugmansia* ("Angel's Trumpet"), which is cultivated for its large, pendulous trumpet-shaped flowers, and *Brunfelsia latifolia*, whose flowers are highly aromatic and undergo a 3-day colour change from purple to white. The *Petunia* (*Petunia*

hybrida), *Lycium, Solanum, Cestrum, Calibrachoa hybrida,* and *Solandra* are additional solanaceous species and genera that are grown as ornamentals. There is even an ornamental hybrid of *Petunia* and *Calibrachoa* available for purchase (Shaw, 2007).

Some members of this family's species, including tobacco, *Petunia*s, and tomatoes, serve as model organisms for studies into fundamental biological issues at the cellular, molecular, and genetic levels. The International Potato Genome Sequencing Collaboration generated and published the potato genome sequence in 2011. The assembled potato genome is that of the doubled monoploid *S. tuberosum Group Phureja DM1-3* (AEWC00000000); the genome assembly is 726 MB in size, with 86% anchored to the genetic map and 39,031 annotated genes (PGSC, 2011).

1.2 Potato as a crop

Currently, there needs to be enough food to feed over 8 billion people worldwide. However, productivity varies widely around the world and is at risk from both increased land competition and related environmental deterioration as well as climatic change. Additionally, the increase in dietary diversity and human population density is placing an even greater strain on agriculture. The maximum of this burden is met by the domestication of a few species, mostly in places that differ from their source of domestication. Current technological improvements have raised the probability of *de novo* domestication of wild plants as a practical solution for designing perfect crops while preserving food security and a more maintainable low-input agriculture.

For at least the past 190 years, *Solanum tuberosum L.*, more also known as the common potato, has ranked in the top three most important food crops grown and consumed around the world (FAO, 2013). Landrace populations found in both lowland Chile and the high Andes are known by the scientific name *Solanum tuberosum*. It is also used to refer to the potato cultivars that have been cultivated in locations other than South America since the late sixteenth century. The modern cultivars are the result of intensive breeding between different types of cultivated plants and wild species. Wild potato species and landrace potatoes, both of which belong to the *Solanum* section *Petota*, are frequently used in potato breeding (Spooner *et al*., 2014).

Globally, potato is the most important tuber crop. The potato plant is an auto tetraploid which is propagated via tubers, unlike various other major crops. The genetic profit from population enhancement in most important crops like maize is roughly about 1% per annum (Duvick, 2005). In contrast, breeding has been proved insignificant to escalate the harvests of potato.

Some hundred-year-old potato varieties, like Russet Burbank (released in 1902) and Bintje (bred in 1904) (Ye *et al.*, 2018) are still commonly cultivated. Therefore, an increasing number of studies have been dedicated to redeveloping potato as an inbred line established diploid crop that is propagated by seed (Lindhout *et al.*, 2011). Diploids are ubiquitous amongst domesticated as well as wild species of potato, accounting for about 70% of the germplasm (Spooner *et al.*, 2014). Re-domestication of these diploid potatoes with the help of contemporary agricultural tools would signify a revolution in potato breeding. However, most diploid potato species bearing tubers are self-incompatible, which has hindered inbred line development.

1.2.1. *Solanum okadae*

Solanum okadae Hawkes et Hjert (oka, 2n = 2x = 24) is a wild diploid potato species native to Bolivia and Argentina (Hawkes and Hjerting 1983; Spooner and Salas 2006). It grows in moist mid-elevation areas. Plants are about one-foot-tall with stolon that are at least two feet long. Tubers are round to ovoid and reach a diameter of about 2 cm. Berries are round and about an inch and a half across (Figure 1). *Solanum okadae* is a member of the *Solanum* sect. *Petota Dumort*., the cultivated and wild potatoes with tubers. It is a member of a diverse clade related to the domesticated potato within sect. *Petota*. On a higher taxonomic level, it is a member of the Potato Clade, a group of approximately 200-300 species that includes the tomato and its wild relatives (Bohs, 2005).

At least some accessions of this species, according to Subramanian (2017), have an unusually high dry matter content. According to Ochoa (1990), this species is unusually susceptible to late blight. In contrast, a survey of markers for the late blight resistance genes *R2, R8, R9*, and *Rpi-blb2* in this species revealed that some plants possess all these genes (Muratova 2019). Some accessions of this species carry the *Rpi-oka1* gene, which confers resistance to late blight. Also, Watanabe (2011), reported that *Solanum okadae* is resistant to drought. This indicates that *Solanum okadae* should be a good source of resistance to late blight and drought.

According to Camadro (2008), this species' seed production is difficult even though it produces fertile pollen. There are only a few records of genetic research conducted on this species, despite its potential breeding importance (USDA, 2002), and its cross relations with *Solanum tuberosum* are not completely known. Moreover, accessions from the two countries of origin obtainable at the Potato Germplasm Bank at Balcarce, Argentina, have diverse morphological

phenotypes (Clausen *et al*, 2005). In this location, these accessions flower abundantly and make fertile pollen but in controlled crosses are difficult to reproduce sexually (Clausen *et al*., 2005).

Figure 1: Selected images of different parts of *Solanum okadae* grown in glasshouse conditions. (**A.**) 8 weeks old plants, (**B.**) Flower with prominent yellow anthers and a protruding stigma, (**C.**) Green fruit/berry (**D.**) Freshly harvested tubers.

A.

B.

C.

D.

1.3 Overview of Self-incompatibility (SI)

The laws of Hammurabi (1750 BCE), which reflect human understanding of the relationship between pollination and seed production as integral components of agriculture development, show that humans have been aware of this connection since the Neolithic period (Weiss, 2015). Several Assyrian reliefs from Ashurnasirpal II's rule (884–859 BCE), which depict gods and priests carrying out date palm fertilisation rituals, illustrate this fundamental understanding (Weiss, 2015). Although sexual reproduction has been advantageous to humans for thousands of years, the male and female reproductive organs of plants were not explicitly described until Rudolf Jakob Camerarius's "De sexu Plantarum epistole" was published in 1694 (Abrol, 2012). In 1764, Joseph Gottlieb Kölreuter documented the occurrence of self-sterility in *Verbascum* and performed cross-pollinations to produce interspecific hybrids (East and Park, 1917). In his writings, 'The Effects of Cross and Self-fertilization in the Vegetable Kingdom' (1876) and 'The Different Forms of Flowers on Plants of the Same Species' (1877), Charles Darwin established the influence of self-incompatibility (SI) in plants.

Botanists later proved that self-sterility follows Mendelian inheritance in *Reseda* and *Nicotiana* (East and Park, 1917). The multi-allelic S-locus was later demonstrated to genetically regulate self-sterility in *Nicotiana*, resulting in compatibility classes for intra-sterile and inter-fertile reproduction (East and Yarnell, 1929). These results led to the term "self-incompatibility" gradually replacing the term "self-sterility" (McClure, 2009). By that time, users of SI as a tool came from both public institutions and private businesses. Since 1911, the John Innes Horticultural Institution has researched incompatibility and sterility in cherries, apples and pears (Muñoz-Sanz *et al.*, 2020). Cross-pollination, for example, was used to define intercompatible groups in sweet cherry (*Prunus avium L.*) cultivars (Crane and Brown, 1937), and a pollen irradiation programme produced the first self-compatible (SC) cultivars within this strictly SI species (Lewis and Crowe, 1954). The Sakata Seed Company first released the SI-produced F_1 hybrid Suteni Kanran cabbage variety in 1940. The introduction of the cabbage (*Brassica oleracea L.*) cvs. Choko-1c and Choko-1cc by Takii & Co. Ltd. in 1950 came shortly after this success (Watanabe et al., 2008).

Angiosperms display an extensive variety of floral exhibition and design which comprises of morphoditism, gynandromorphism, and others that display sexual polymorphism with dioecism and gynodioecism being most prominent. Being hermaphroditic generally, the reproductive parts of most of the flowering plants are frequently situated in close vicinity.

There is an increase in the probability of pollen grain from one flower to fall on the stigma of the same flower due to this arrangement. It might be assumed that this arrangement will encourage flowering plants to self-pollinate resulting in self-fertilization, a phenomenon which would then have been generally destructive for the evolution of angiosperms. But flowering plants have developed numerous tactics to evade self-fertilization, one of which is the process of self-incompatibility (Gaude *et al*, 2006).

During the process of self-incompatibility, the pistil, the female reproductive structure of the flower, differentiates between the non-self and the self-pollen thus permitting only the non-self-pollen to achieve fertilization (Kao and McCubbin, 1996). Self-incompatibility is the most prevalent phenomenon by which flowering plants encourage outcrossing and avert inbreeding, thus assuring genetic diversity within a plant population. The development of various processes to prevent inbreeding in flowering plants is partially responsible for their reproductive achievement, thus making them the most successful land-dwelling groups of plants (Silva and Goring, 2001).

In several species displaying self-incompatibility, it has been observed that self-incompatibility is influenced during development and is not expressed in juvenile flowers but only in mature ones. Therefore, using mature pollen to pollinate the immature buds of pistil resulted in fertilization and the production of viable seeds. For the creation plants for homozygous S-allele, young bud pollination has often been used (Bernatzky *et al*., 1988). This method has made it useful to prevent self-incompatibility in the members of the *Petunia* family paving the way for the creation of homozygous stocks (Robbins *et al*., 2000) but the method is unachievable in few families like Rosaceae and Plantaginaceae.

Several mechanisms of SI been evolved in the flowering plants. Based on the morphology of flower, systems of self-incompatibility have been characterized into two types: heteromorphic and homomorphic. Plants exhibiting homomorphic SI produce morphologically indistinguishable flowers whereas heteromorphic SI is seen in those plants whose flowers are dissimilar in morphology considered by the relative positions of their reproductive organs (Kao and McCubbin, 1996). In heteromorphic SI systems, a mixture of genetically controlled alterations in floral structure (heterostylous polymorphism) and diallelic sporophytically controlled SI system to pose blockades for self-pollination and then self-fertilization is used (McCubbin, 2008). Whereas homomorphic systems have been widely studied and two forms

have been documented till now; sporophytic (SSI) and gametophytic self-incompatibility (GSI) (Ebert *et al*., 1989).

A single multi-allelic locus, known as the S-locus governs the SI response in many plant species. In the pollen and the pistil, a minimum of two highly polymorphic genes, known as the S-determinant genes are expressed which are comprised in this S-locus (McCormick, 1998). Yet, new studies by many researchers have shown that additional loci might also be participating in the SI response, with S-locus involved in playing the key role of identification of non-self and self-pollen (Gaude *et al*., 2006).

1.3.1 Heteromorphic Self-incompatibility.

The differences in flower morphology, like a prominent difference in style and stamen length of the flower can cause heteromorphic self-incompatibility. This system is called heterostylous SI and can be separated into two types: distylous and tristylous. Distylous SI has two flower forms (long anthers-short style and short anthers-long style). Style and anther arrangement in these types are genetically organized by dominance between alleles at the single S-locus (Lewis, 1947). There are three flower forms in Tristylous SI (middle and high stamen with short style, low and high stamen with middle style and low and middle stamen with long style) (de Nettancourt, 2001). In tristylous SI, stamen and style arrangements are genetically organized by dominance relationships between alleles at two different loci, S and M (Cruzan, 1993).

1.3.2 Homomorphic self-incompatibility systems

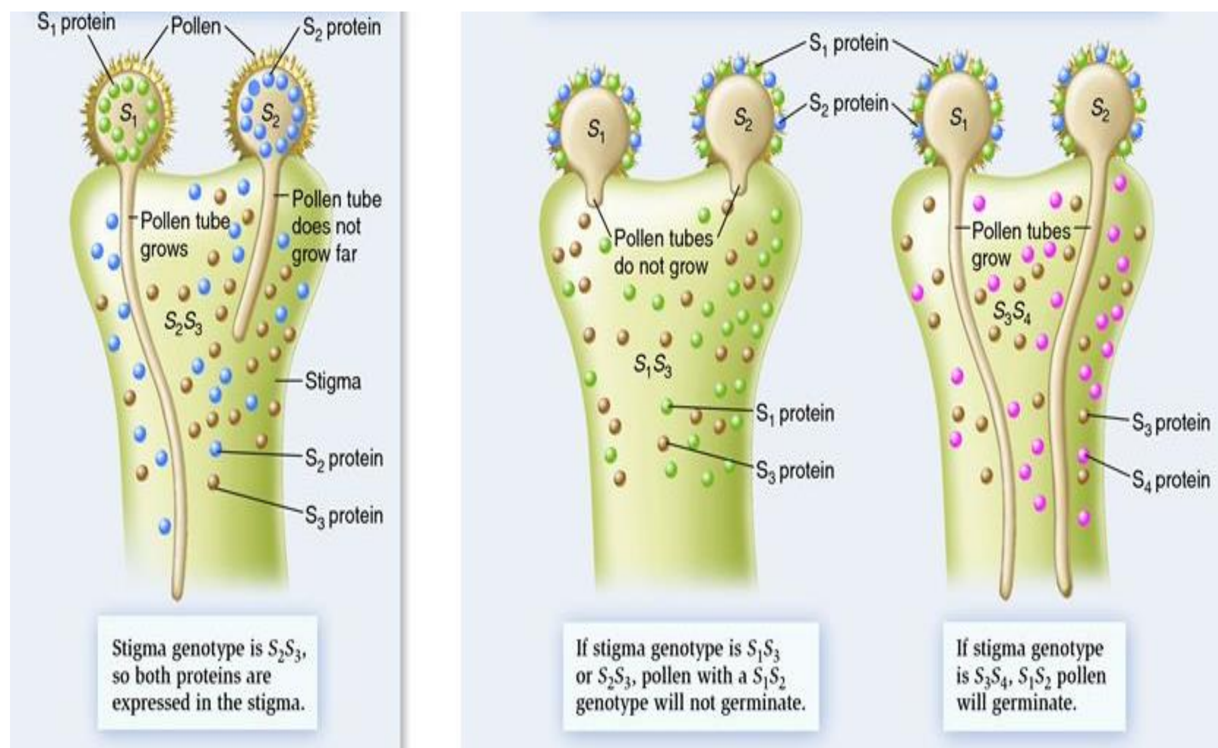
The foundation for classifying homomorphic self-incompatibility is based on the means of the genetic regulation of the pollen phenotype. The phenotype of pollen can be derived gametophytically or sporophytically. In gametophytic self-incompatibility system (GSI), the haploid genotype of pollen determines the phenotypic incompatibility whereas, in the sporophytic self- incompatibility system (SSI), determination of the phenotype of the pollen is due to the diploid genotype of the pollen parent (Figure 2).

1.3.2.1 Sporophytic self-incompatibility (SSI)

Initially, in two species from the Asteraceae family: *Crepis foetida* (Hughes & Babcock, 1950) and *Parthenium argentium* (Gerstel and Riner, 1950), the genetics of SSI was first clarified. It was studied later in the species from Brassicaceae (Bateman, 1955). A single polymorphic locus known as S-locus controls SSI. The main genetic dissimilarity between GSI and SSI is

that in SSI, the diploid parental genome determines the incompatibility phenotype of the pollen. Therefore, pollen grains comprise of the products of two SI (S) alleles, instead of only one (Figure 2b). Dominance interactions between S alleles are permitted by the diploid sporophytic expression of SI and such interactions can take place without restrictions for pollen and stigma. This frequently results in very complicated configurations of incompatibility and compatibility, with shared dissimilarities in incompatibility among individuals being a constant feature linked with contradictory dominance effects in pollen and stigma.

Figure 2: Homomorphic Self-incompatibility. **(A.) Gametophytic SI:** The pollen grain expressing either S_1 or S_2 allele falling on the stigma expressing S_2S_3 alleles results in the S_1 pollen, to be compatible on the S_2S_3 pistil. Full compatibility is only observed when the S alleles in pistil are totally different from the S_1S_2 alleles in the pollen grains, thus allowing the S_1 as well as S_2 pollen grains to germinate and pierce the style to penetrate the ovary thus permitting fertilization to take place. **(B) Sporophytic SI:** The matching of even a single of the S alleles in the pollen making parent with the pistil causes the arrest of pollen germination at the surface of the stigma. Pollen grains expressing the S_1S_2 are repressed on both the pistils expressing the S_1S_2 allele. Alternatively, pollen grains expressing the S_1S_2 allele are fully compatible with pistils expressing the S_3S_4, hence allowing the occurrence of fertilization (Hiscock, 2002).



A. B.

1.3.2.2 Gametophytic Self-incompatibility (GSI)

Most of the families of flowering plants including Solanaceae, Plantaginaceae, Rosaceae, Papavaraceae, Leguminoceae, Onagraceae, and Poaceae exhibit gametophytic self-incompatibility making it the most prevalent type of self-incompatibility (Kao and McCubbin, 1996). According to some detailed studies carried out at the molecular level, the GSI acts by two altered mechanisms to differentiate among self and non-self-pollen. One of which is the stylar ribonuclease (S-RNase) mechanism that was initially characterized in members of the Solanaceae, and then in the Plantaginaceae and Rosaceae. The other mechanism is seen in the Papavaraceae, specifically in poppy (*Papaver rhoeas*) (Franklin-Tong and Franklin, 2003). There are probably more distinctive GSI systems which are yet to be discovered (McClure and Franklin-Tong, 2003).

The S-allele products in the styles of flowers of Solanaceae family were recognized to be simple glycoproteins exhibiting ribonuclease activity accountable for hindering the development of the pollen tube in the style (Franklin-Tong and Franklin, 2003). These S- locus coded glycoproteins were found to be roughly 32kDa which were first recognized in the Solanaceae. These glycoproteins were identified to be ribonucleases (RNases) and later were confirmed to exhibit ribonuclease activity (McClure *et al*., 1989). It has been largely acknowledged that the S-RNase genes control the female specificity by messenger RNA (mRNA) or ribosomal RNA (rRNA) degradation in self-incompatible pollen tubes thus obstructing fertilization (McClure *et al*., 1989; Franklin-Tong and Franklin, 2003).

According to the molecular studies of S-RNases in the Solanaceae family, 5 conserved domains (C1, C2, C3, C4, and C5) and 2 hypervariable regions (HVa and HVb) situated between the C2 and C3 domains have been recognized (Ioerger *et al*., 1991). But the C4 domain is absent in the S-RNases of Rosaceae (Shouzheng *et al*, 2022). Also, it only has a single hypervariable region (RHV), unlike 2 such regions (HVa and HVb) seen in the Solanaceae (Takayama and Isogai, 2005). Initially, it was predicted that the conserved domains of S-RNase were accountable for the catalytic activity of the S-Rnases whereas the allelic-specificity determination was due to the hypervariable regions (Kaothien-Nakayama *et al*., 2010). With the help of X-ray crystallographic investigations, the 3D structure of single S-RNase in Solanaceae and Rosaceae was deduced which showed that the HV regions might be accountable for determination of specificity of S-RNase as they are exposed on the exterior of

S-RNases and hence could probably be interacting with the male determinant of the SI response (Ida *et al*., 2001).

In the gametophytic system the phenotype of pollen incompatibility is determined by its own haploid genotype (Figure 2a). Self-pollen is rejected during pollen tube growth in the style. This type of SI is by far the most widespread and it exists in more than 60 flowering plant families (Charlesworth and Awadalla, 1998). Only two forms have been described in detail at the molecular level revealing two different stylar *S-genes* so far, to achieve self-pollen recognition. The first form is the stylar ribonuclease or S-RNase system that was originally investigated and is broadly characterized in several members of the Solanaceae (Anderson *et al.,* 1986) and following this was described in the Rosaceae (Sassa *et al*., 1996) and Plantaginaceae (Xue *et al.,* 1996).

1.3.3 Gametophytic Self-incompatibility in Solanaceae.

The mechanism of self-incompatibility leading pollen-pistil interactions in the Solanaceae is characterized by the capability of incompatible (self) and compatible pollen grains to hydrate and go through normal germination on the surface of stigma of the pistil. As the pollen tube propagates through the transmitting tract of the style, callose plugs are deposited at regular intervals providing the tubes a 'ladder-like' form (Ebert *et al*., 1989). Initially, incompatible, and compatible tubes seem morphologically alike. Yet, incompatible tube development is arrested by manifestation of a sluggish growth rate of pollen tubes, uneven deposition of callose and tube cell wall thickening along with the enlargement and bursting of the tip in the upper one-third of the style (de Nettancourt, 2001). In this manner, incompatible pollen tubes are not capable to influence fertilization.

According to genetic studies, in the family of Solanaceae, the self-incompatibility trait is governed genetically by a single polymorphic locus known as the S locus (de Nettancourt, 1997). As the pollen phenotype is determined gametophytically, fertilization is stopped when the haploid pollen grain expressed S allele matches one of the S alleles expressed in the pistil (de Nettancourt, 1997). For instance, a heterozygous plant with two different S alleles, like S_1 and S_2 alleles, would yield pollen having one or the other of S_1 or S_2 allele. If these pollen grains fell on the stigma of the same flower, they would germinate, but the growth of all the pollen tubes will eventually be arrested in the style because of the same S alleles in the pistil. But, if S_1 and S_2 pollen grains fell on the pistil of a plant with S_1S_3genotype, the outcome of pollination outcome would not be the same. As anticipated, the S_1 pollen would be prohibited

owing to the same S_1 allele; but, the S_2 pollen would be accepted as non-self, and be permitted to germinate, grow through the style towards the ovary and result in fertilization (Kao *et al.*, 1996). Hence, when single S allele in the pollen parent is common with those in the pistil, only then there is rejection of half of the pollen (semi-compatible) (Thompson and Taylor., 1966).

1.3.3.1 Female Determinant

The female determinants in *Nicotiana alata* were initially identified as style glycoproteins (~30 kDa) that co-segregated with the S-haplotype in genetic crosses. As a result, several associated proteins from members of other Solanaceae families were identified and cloned. The style proteins contained a region that is homologous to the catalytic domain of the fungal T2-type ribonucleases, according to sequence data. Additional research demonstrated the presence of ribonuclease activity in these proteins, resulting in their designation as S-RNases (McClure *et al.*, 1989). S-RNases are only made in the pistil, where the protein is mostly restricted to the style's upper region where the self-pollen tubes are blocked. Also, all S-RNase genes have a signal sequence consistent with secretion into the stylar matrix. Otherwise, they would be cytotoxic to the stylar cells responsible for S-RNase synthesis. Gain- and loss-of-function experiments were used to determine the role of the S-RNases in self-incompatibility (Lee *et al.*, 1994). According to these investigations, the S-RNase is the sole female determinant responsible for the S-haplotype specificity of the pistil. The glycoproteins known as S-RNases typically have one or more N-linked glycan chains. The ability to reject S_3 pollen was retained by an experimentally made S_3-RNase of *Petunia inflata* that had only the N-glycosylation site knocked out, demonstrating that the protein backbone rather than the glycan chains are what determine the S-haplotype specificity of S-RNases.

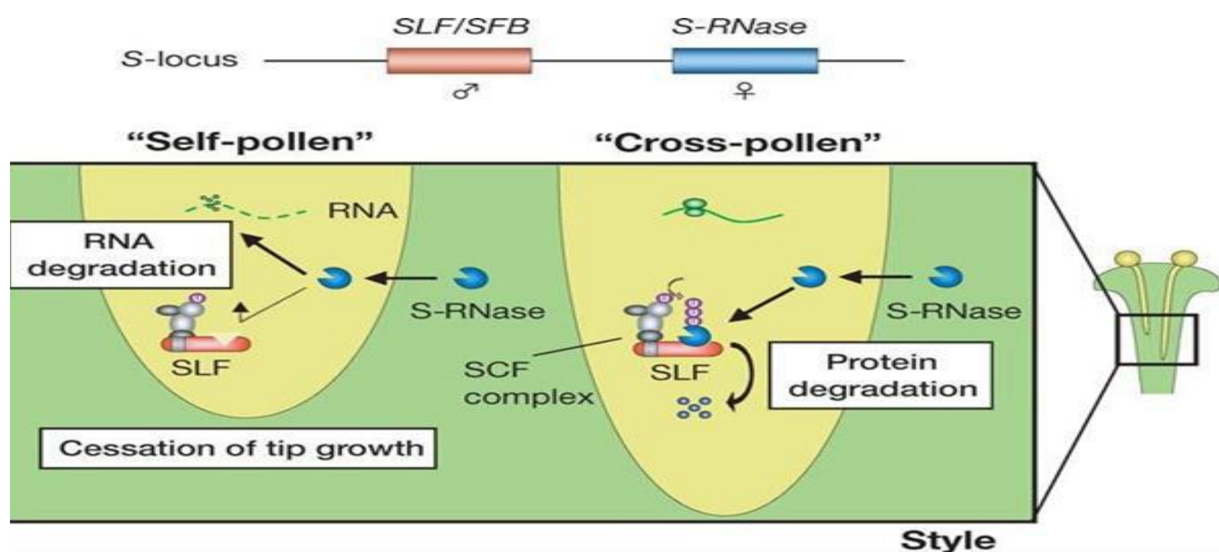
S-RNases' amino acid identity varies greatly among species of the Solanaceae family, from 38% to 98%. (McCubbin & Kao, 2000). Despite having a high degree of sequence diversity, the S-RNases contain several conserved regions. There are five highly conserved regions in Solanaceae S-RNases, numbered C1 to C5, and they share structural similarities with Rosaceae S-RNases except for the absence of the C4 region. The regions C2 and C3, which are comparable to fungal RNase T2, each contain a conserved histidine residue that is essential for catalysis. Additionally, the S-RNases in Solanaceae species have two hypervariable regions called HVa and HVb, whereas the S-RNases in the Rosaceae family only have one hypervariable region, which is equivalent to HVa of the solanaceous S-RNases. The 3D protein structure for two S-RNases, Sf11-RNase from *Nicotiana alata* (*Solanum* family) and S3-RNase

from *Pyrus pyrifolia* (Rosaceae family) have been determined by X-ray to have crystallography (Matsuura *et al.*, 2001). Both S-RNases have very similar structures made up of 8 helices and 7 beta-strands, which is typical of the RNase T2 enzyme family. These S-RNases' substrate-binding sites can be geometrically superimposed over those of the RNase T2 enzymes by residues of amino acids. The HV regions of the two S-RNases show the most notable differences. An extended, positively charged loop is followed, in that order, by a small, negatively charged α helix and a fragment of α helix in the solanaceous HVa and HVb regions of S-RNase. These HV regions are thought to form a domain that interacts with the male determinant because they are geometrically close to one another and exposed to the molecular surface. S-RNase experiments with domain-exchanging in transgenic *Solanum chacoense* support this model (Matton *et al.*, 1999).

Rejection of S-haplotype-specific pollen needs the expression of S-RNase at great levels. The S-RNase concentration in the extracellular matrix is measured at 10–50 mg/ml, and only the transformants with a comparable amount of expressed S-RNase can attain new S-haplotype specificities. The ribonuclease activity of S-RNases is essential for pollen rejection (Huang *et al.*, 1994). Moreover, radioactive tracer experimentations indicated that pollen RNA is destroyed specifically after incompatible pollination (McClure *et al.*, 1990). Therefore, S-RNases function as highly specific cytotoxins that prevent the growth of incompatible pollen (Figure 3).

Even though S-RNase is the one and only female factor influencing the S-haplotype specificity of the pistil, a necessity of other stylar factors for the complete function of S-RNase has been proposed (Cruz-Garcia *et al.*, 2003). One of such factors is HT-B, a protein rich in asparagine that was initially recognised during a differential screen implemented to recognise stylar genes expressed in the self-incompatible *Nicotiana alata* but not in the self-compatible *Nicotiana plumbaginifolia* (McClure *et al.*, 1999). Homologs of HT-B were also recognised in two other Solanaceae genera, Lycopersicon and *Solanum* (Kondo *et al.*, 2002a, 2002b & O'Brien *et al.*, 2002). In a comparative study of self-incompatible and self-compatible taxa of Lycopersicon, the expression of HT-B gene was not detected in all self-compatible taxa (Kondo *et al.*, 2002a, 2002b). A need for HT-B protein was verified by an RNAi suppression test in self-incompatible *Solanum chacoense*. Two HT-B-suppressed transformants expressed S-RNase ordinarily but did not display S-haplotype-specific pollen rejection. These results propose that the HT-B protein is associated in the SI response, even though its precise function remains uncertain (O'Brien *et al.*, 2002).

Figure 3: Molecular model of the SI reaction in the Solanaceae. The S-locus comprises of two genes, S-RNase, and SLF/SFB. S-RNase is produced in bulk quantities into the extracellular matrix of the style and is the female determinant. In a pollinated style, S-RNase is assimilated into the pollen tubes and acts as a cytotoxin that destroys RNA of the pollen. Though the S-RNase goes into the pollen tubes irrespective of their S-haplotypes, degradation of RNA takes place only in self-pollen tubes. SLF/SFB is a member of the F-box family of proteins, which usually acts as an element of an E3-ubiquitin ligase complex. Therefore, SLF/SFB is likely to be playing a part in ubiquitin facilitated protein damage of nonself-S-RNases. It is the male determinant (Takayama and Isogai, 2005).



1.3.3.2 Male Determinant.

The molecular character of the male determinant and the molecular processes of how S-RNases destroy pollen RNA in S-haplotype- specific means were long-standing ambiguities of S-RNase driven SI. The "inhibitor model" was one possible model where the pollen S-determinant was assumed to be an inhibitor that could obstruct all S-RNases except for the self S-RNase (McClure *et al*., 1999). The immuno-cytochemical interpretations that all S-RNases could cross the pollen tube irrespective of S-haplotype supported this model. Some advanced "inhibitor models" were also suggested (Luu *et al*., 2001).

Genomic analyses of the S-locus region allowed for the identification of the male determinant. Initially, Solanaceous species like *Lycopersicon peruvianum, Nicotiana alata,* and *Petunia inflata* were the focus of these studies. However, these species' S-loci are located in the sub-centromeric region and are surrounded by numerous repetitive sequences that have made chromosomal walking difficult (Entani *et al*., 1999). Sequence analysis of *Antirrhinum*

hispanicum, a member of the Plantaginaceae S-locus's region revealed the first indication of the male determinant. *A. hispanicum* S-locus F-box of S_2-haplotype, which is expressed in pollen grains and S_2-haplotype anthers, is confined to a region of the S_2-haplotype (Lai *et al.*, 2002). A gene with a very high sequence similarity (97.9%) has been found in other lines with various S-haplotypes, but no gene allelic to *AhSLF*-S_2 has been identified in other S-haplotypes. As a result, it was impossible to tell if *AhSLF*-S_2 encoded the pollen S-determinant. Four F-box genes are found in the roughly 60-kb genomic region nearby the S-RNase gene, according to genomic analysis of the S-locus of the Rosaceae plant *Prunus mume* (Entani *et al.*, 2003). Only one F-box gene, known as PmSLF, meets all the criteria for a pollen S-determinant gene, including: (a) its location within the highly divergent genomic region of the S-locus; (b) its S-haplotype-specific diversity (79% to 82% amino acid identity); and (c) its precise expression in pollen (Entani *et al.*, 2003). The S-locus region of *Prunus cerasus, Prunus dulcis,* and *Prunus avium* also contained polymorphic F-box genes that were independently referred to as SFB (S-haplotype-specific F-box) at around the same time (Ushijima *et al.*, 2003). *Prunus* species SLF/SFB met all requirements for the pollen S-determinant. These *Prunus* species' SLF/SFBs inferred amino acid sequence alignment revealed the presence of two hypervariable regions, HVa and HVb, at the C terminus (Ushijima *et al.*, 2003). Two *P. mume* and *P. avium* self-compatible haplotypes that lack the HVa and HVb regions in SLF/SFB encoded partial loss-of-function mutations (Ushijima *et al.*, 2004). This argument provides additional evidence that the SLF/SFB is the pollen S-determinant.

Through transformation experiments in *Petunia inflata*, proof of SLF/SFB encoding the pollen S-determinant was attained (Sijacic *et al.*, 2004). An extensive search for the pollen S-determinant in a large S-locus region revealed the polymorphic *PiSLF* F-box gene, which is located 161 kb downstream of the S-RNase gene. The *PiSLF* displayed the highest degree of sequence diversity, even though the genomic region outside of this contig contained two additional polymorphic F-box genes that were genetically linked to the S-locus. It was determined whether *PiSLF* encoded the pollen S-determinant by using a well-known phenomenon "competitive interaction." In tetraploid plants, competitive interaction is frequently observed. Even though the molecular cause of the breakdown is unknown, among the diploid pollen grains, those with hetero-allelic (two distinct S-haplotypes) but not homo-allelic (two identical S-haplotypes) characteristics perform poorly in SI tests (Entani *et al.*, 1999). Additionally, genotypic analyses of the offspring of S_2S_3/PiSLF$_2$ and S_1S_2/PiSLF$_2$ self-pollinated plants revealed that only S_1 and S_3 pollen carrying the *PiSLF*$_2$ transgene, which is

analogous to hetero-allelic pollen, turned self-compatible, but not S_2 pollen carrying the $PiSLF_2$ transgene, which is analogous to homo-allelic pollen. These results provide strong evidence that the long-sought pollen S-determinant is SLF/SFB.

1.3.3.3 Mechanisms of S-haplotype-Specific

Even though both male and female determinants have been recognised, the molecular mechanisms dictating how these molecules work together and explicitly stop self-pollen growth remain uncertain. The fact that RNase activity is needed for the function of S-RNases, and that S-RNases are taken up by both self- and non-self-pollen tubes, suggest that S-RNases function in pollen tubes as cytotoxins destroying the RNA of self-pollen. In contrast, SLF/SFB encloses a motif, called the F-box, which is well-known for facilitating interactions with other proteins that make up an enzyme complex referred to as the E3 ubiquitin ligase complex (Gagne *et al.*, 2002). E3 ubiquitin ligases act together with the E2 enzymes to ubiquitinate target proteins, which in many cases are destroyed by the 26S proteasome. Biochemical investigations propose the participation of AhSLF-S2 in this protein degradation pathway, though it remains to be elucidated whether AhSLF-S2 from *Antirrhinum* is an ortholog of PiSLF (Qiao *et al.*, 2004). AhSLF-S2 act together with ASK1- and CULLIN1-like proteins, which are the probable constituents of the SCF complex. AhSLF-S2 interacts with both self- and non-self S-RNases but seems to facilitate destruction of only non-self-S-RNases.

To explain the molecular mechanisms for this specificity, some hypothetical models that are compatible with the "inhibitor models" have been presented (Qiao *et al.*, 2004). One model hypothesises that SLF/SFBs comprise of two distinct interaction domains, like the conventional "inhibitor model." One domain would confine the hypervariable domain of its cognate S-RNase in an S-haplotype-specific means, and the other domain would confine to a domain mutual to all S-RNases. The specific interaction on S-haplotype is anticipated to somehow alleviate, or at least not change, the activity of S-RNase, and the overall interaction would lead to the poly-ubiquitination and destruction of S-RNases. A different process proposes the association of an additional molecule, such as a common inhibitor in a modified "inhibitor model." To back this up, a pollen-expressed RING-finger protein, PhSBP1 (*P. hybrida* S-RNase binding protein), binds explicitly with S-RNases in an S-haplotype-non-specific fashion (Sims and Ordanic, 2001). Since many RING-finger domain proteins also act as E3 ubiquitin ligases, PhSBP1 is presumed to have part in the overall degradation of S-RNases. In such

instances, SLF/SFB is estimated to confine to its cognate S-RNase as a pseudo-substrate and shield it from ubiquitination and following destruction (Franklin-Tong and Franklin, 2003).

Not one of these models, however, can adequately describe the phenomenon of "competitive interaction." These models assume that the S-haplotype-specific binding between S-RNase and its equivalent SLF/SFB is thermodynamically preferred over the common tie between S-RNase and non-self-SLF/SFB (or PhSBP1), and that the S-haplotype-specific binding prevents the ubiquitination of S-RNases, thereby allowing RNase activity. In "competitive interaction," two SLF/SFBs in hetero-allelic pollen bind to their respective S-RNases in an S-haplotype-specific manner, leaving the RNases active. Therefore, contrary to experimental observations, these models assume hetero-allelic pollen is incompatible. To clarify "competitive interaction," an advanced version of the "inhibitor model" has been proposed in which male S-determinants are anticipated to form a multimer prior to interacting with the S-haplotype-specific binding site of S-RNases (Luu *et al.*, 2001). However, it is not anticipated that SLF/SFBs will form a multimer during the interaction process. So, having identified both male and female determinants, it has been determined how S-haplotype-specific pollen selection is achieved (Kubo *et al.*, 2015).

1.4 Genome editing

Genetic modifications in plants was first seen 10,000 years ago in Southwest Asia, when people produced plants via artificial selection and selective breeding. Advances in agricultural research and technology have resulted in the present GM crop revolution. With established case studies in Indian cotton and Australian canola, GM crops are promising to reduce existing and future difficulties in commercial agriculture. However, current issues related to insect resistance and potential health risks, have harmed its reputation among the public and policymakers, leading to full and partial bans in some countries. Nonetheless, with a current compounded annual growth rate of 9.83-10% and potential research paths in biofortification, precise DNA integration, and stress tolerance, the GM seed industry is expected to bring productivity and wealth to commercial agriculture (Ruchir, 2017).

Genetic engineering has come a long way ever since the emergence of recombinant DNA technology at Paul Berg's research laboratory in 1972. A lot of genetic and molecular mechanisms and wonders have been explored and studied in detail. Due to all these advances, effective creation of not only transgenic lower microorganisms but also genetically modified higher organisms including various animals and plants species is possible.

Genome editing with engineered nuclease (GEEN) also known as new/novel genome editing tools, lets researchers cleave and re-join DNA molecules in specific sites to effectively alter the genetic makeup of cells. For this, distinct enzymes such as restriction endonucleases (REs) and ligase can be deployed for cutting and re-joining of DNA molecules in bacterial and viral genomes which are relatively short.

1.4.1 Zinc Finger Nucleases (ZFNs)

Zinc Finger Nucleases were the first era of genome altering tools which used chimerically designed nucleases that were recognized after the discovery of the working ideas of the functional Cys2-His2 zinc finger (ZF) domain. Every Cys2-His2 zinc finger domain comprises of 30 amino acid residues, which are folded up to ββαconfiguration. The development and application of these nucleases include modular design, assemblage, and optimization of zinc fingers alongside particular target DNA sequences trailed by joining of separate ZFs closer to bigger sequences (Durai *et al.*, 2005).

1.4.1 Transcription Activator-Like Effector Nucleases (TALENs)

The hunt for an effective and specific target genomic DNA altering technique resulted in the recognition of exclusive transcription activator-like effector (TALE) proteins that detect and initiate activation of plant promoters via a set of tandem repeats that formed the foundation for the establishment of a new genome-editing system comprising of nucleases called TALE nucleases (TALENs) which were chimeric in nature. TALE proteins involve a central domain accountable for binding of DNA, a signal for nuclear localization, and a domain that works as activator the target gene transcription. For the very first time, the capability of DNA-binding of these proteins was reported in 2007, and a year after that, two scientific groups deciphered the recognition code of the target DNA by transcription activator-like effector proteins (Nemudryi *et al.*, 2014).

1.4.1 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

In recent years, a new genome-editing system that has been developed and turned out to be extensively used is the clustered regularly interspaced short palindromic repeats (CRISPR) associated (Cas) protein system with CRISPR/Cas9 system being the most established one. This tool uses the adaptive archaeal and bacterial immune system, the procedure of which depends on the existence of distinct sites in the genome of the bacteria known as CRISPR loci. These loci are made of operons coding the Cas9 protein and a recurrent array of repeat-spacer

sequences. These spacers are short fragments that are derivatives of unfamiliar DNA in contrast to the TALEN proteins which are chimeric. In CRISPR/Cas9 system, the complementary sequence driven interaction between the guide RNA (non-coding) and the target site DNA completes the target site recognition. Nuclease activity is provided by the Cas protein complex for cutting the double-stranded DNA using Cas9 endonuclease (Jinek *et al*., 2012).

The most well-known system is the type II-A CRISPR/Cas system found in Streptococcus pyogenes and comprises of 3 genes coding CRISPR RNA (crRNA), trans-activating crRNA (tracrRNA), and Cas9 protein. General genetic constructs coding synthetic components of CRISPR/Cas "genome editor" have been generated using this system (Doudna and Charpentier, 2014). In addition, a simple kind of system, functioning as a complex of single guide RNA and Cas9 protein, consisting of CRISPR tracrRNA and short, mature crRNA was made. Based on complementarity, the DNA target site is recognized and bound by the guide sequence which is then cleaved by the Cas9 protein (Graham and Root, 2015).

CRISPR system can be employed for creating the cells which are genetically modified in living organisms and in cultures (Cho *et al*., 2013). Viral vectors or plasmids that offer a steady synthesis of CRISPR/Cas9 system components are introduced into cells in the first situation whereas, in the second situation, cultured protoplasts and a plasmid encoding CRISPR/Cas components are used to obtain GM plants (Shan *et al*., 2013). Thus, owing to its straightforwardness, competence, and wide proficiencies, CRISPR/Cas9 system has already established in many areas of applied and fundamental biology, biotechnology, and genetic engineering in a very short period.

1.5 Creating a Knockout via CRISPR

CRISPR can be used to create knockout cells or organisms by expressing an endonuclease like Cas9 or Cas12a and a target-specific gRNA together (Figure 4). The genomic target can be ~20 nucleotide DNA sequence if it fulfils two requirements:

1. The sequence is unique compared to remainder of the genome.

2. The target is positioned immediately next to a Protospacer Adjacent Motif (PAM).

The PAM sequence serves as a linking signal for Cas9; however, the precise sequence relies on which Cas protein is used. *S. pyogenes* Cas9 (SpCas9) is the most common one. Various other Cas nucleases used in CRISPR experiments are listed in (Table 1). When expressed, the gRNA and the Cas9 protein form a complex of ribonucleoprotein via interactions amongst the

gRNA scaffolding and surface-exposed positively charged channels on Cas9. Upon gRNA binding, Cas9 goes through a conformational change which changes it into an active DNA-binding state from an inactive state. Significantly, the spacer section of the gRNA remains open to fuse with the DNA target.

A specified region in the target DNA is cleaved by Cas9 only if the spacers sequence in gRNA has enough similarity with the target DNA. As soon as the Cas9-gRNA complex binds a known DNA target, the seed sequence (7-11 bases at the 3′ end of the sgRNA targeting sequence) will start to anneal to the target DNA. If the target DNA sequence and seed sequence match, the gRNA will carry on binding to the target DNA in a 3′ to 5′ direction. Hence, mismatches amid the target sequence in the 3′ seed sequence eliminate target cleavage, but mismatches near the 5′ end distal to the PAM frequently may allow cleavage of the target.

Table 1: Cas and other nuclease alternates used in CRISPR research with their PAM sequences.

CRISPR Nucleases	Organism Isolated From	PAM Sequence (5' to 3')
SpCas9	*Streptococcus pyogenes*	NGG
SaCas9	*Staphylococcus aureus*	NGRRT or NGRRN
NmeCas9	*Neisseria meningitidis*	NNNNGATT
CjCas9	*Campylobacter jejuni*	NNNNRYAC
StCas9	*Streptococcus thermophilus*	NNAGAAW
LbCpf1	*Lachnospiraceae bacterium*	TTTV
AsCpf1	*Acidaminococcus sp.*	TTTV

Cas9 goes through an additional conformational modification upon target binding that positions the domains of nuclease, called RuvC and HNH, to cut opposite strands of the targeted DNA. The final product of Cas9 facilitated DNA cleavage is a double-strand break (DSB) in the target DNA (~2-5 nucleotides upstream of the PAM).

The resultant DSB is then restored by one of either two common repair pathways:

1. An effective but a bit inaccurate non-homologous end joining (NHEJ) pathway

2. A less proficient but highly accurate homology-directed repair (HDR) pathway

The NHEJ repairing pathway is the most vigorous and dynamic repair mechanism, which often causes small nucleotide additions or deletions at the site of a double-stranded break. The uncertainty of NHEJ facilitated double-stranded break repair has significant practical effects as a population of cells expressing Cas9 and a gRNA will result in a miscellaneous range of mutations. In normal circumstances, NHEJ gives rise to small insertions or deletions in the target DNA resulting in amino acid frameshift mutations, amino acid deletions or insertions producing early stop codons inside the open reading frame (ORF) of the target gene. The ideal final result is a loss-of-function mutation for the targeted gene. Nevertheless, to check the strength of the phenotype of the knockout for a specific mutant cell, screening experiments must be carried out (Peters *et al.*, 2019).

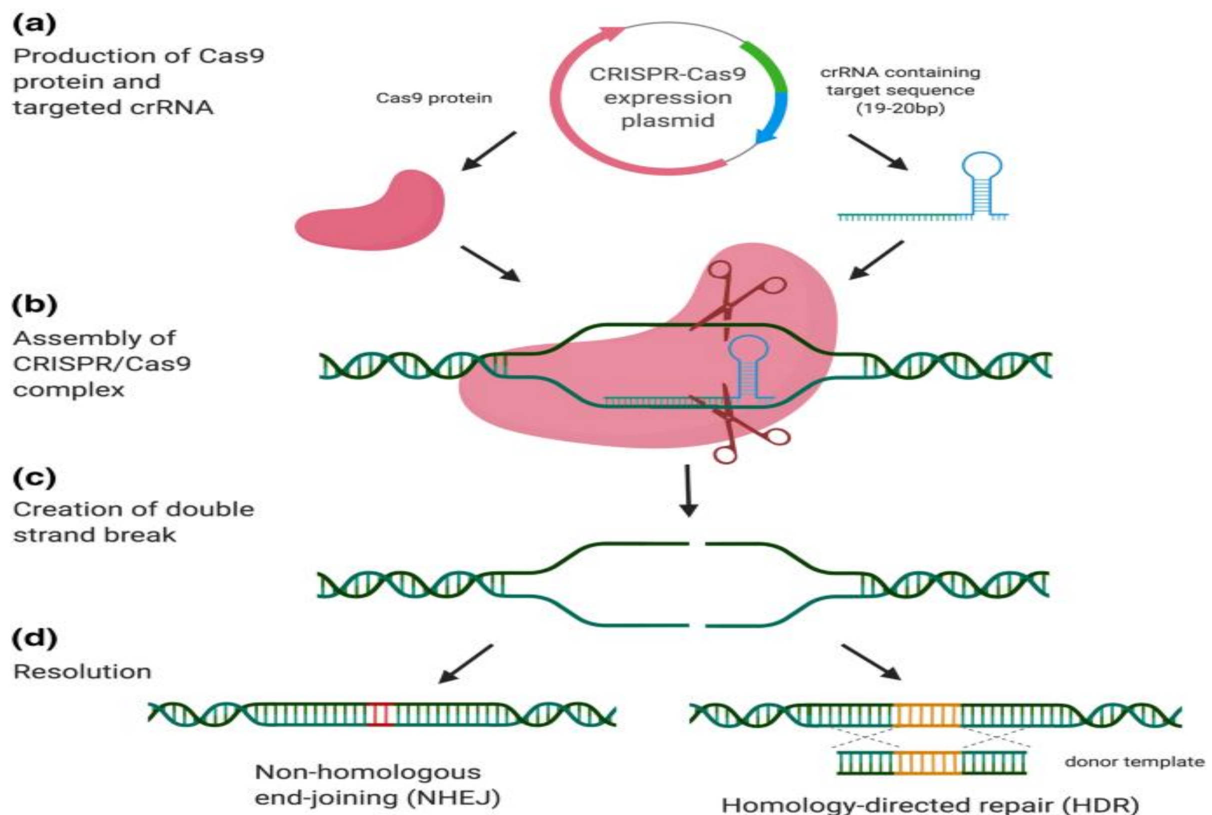
1.5.1. Crop Improvement using CRISPR

Currently, the most serious challenge faced by humankind is to deliver food security for a growing population. By 2050, the human population will reach 10 billion and to feed the world, global food production needs to rise by 60–100% (FAOSTAT, 2016). In addition to the growing population rate, reduced agricultural land availability, extreme weather conditions, increasing abiotic and biotic stresses are substantial limitations for farming and food production. Techniques involving genetic manipulation via chemical, physical, and biological mutagenesis have majorly contributed in identifying the role of genes and finding the biological mechanisms for the enhancement of crop species in the past decades (Ma *et al.*, 2015). So far, nearly 20 crop species have been genetically modified using CRISPR/Cas9 (Ricroch *et al.*, 2017) for different traits including abiotic and biotic stress management, yield improvement. Several published articles are regarded as proof-of-concept studies as they define the application of CRISPR/Cas9 system by knocking out specifically reported genes playing a vital role in biotic or abiotic stress tolerant mechanisms.

Potato is an essential food crop for world food security and, with the change in climate it is vital that potato varieties be adapted and that breeding materials be established which can be used to expand the region in which it is grown. The quality of potato starch is important for many of its food applications and is a significant research area. In tetraploid potato, using CRISPR/Cas9 mediated genome editing (CMGE), the waxy genotype was formed by mutating the granule-bound starch synthase (GBSS) gene and characterization of starch in genome-edited lines showed the presence of amylopectin, with a complete absence of amylose, signifying that all four GBSS alleles were knocked out (Andersson *et al.*, 2017). Genome

editing via CRISPR/Cas9 can be used to enhance horticultural crops such as vegetable and fruit crops to increase yield, resistance to disease and shelf life. Increasing the availability of data through sequencing of whole genome and transcriptome of chief horticultural crops would enhance the use of CMGE to improve crops (Karkute *et al*., 2017). Positive knockout of carotenoid cleavage dioxygenase4 (InCCD4) in the white-flowered *Ipomoea nil, cv. AK77* resulted in the white petals turning light yellow, with a 20-fold rise in the overall carotenoid content in petals of ccd4 mutant plants. It proposed that in the petals of *Ipomea nil*, along with a low carotenogenic gene expression, carotenoid degradation adds to low carotenoid content (Watanabe *et al*., 2018). In *Lotus japonicas,* an effective inactivation of a symbiotic nitrogen fixation related gene, SYMRK (symbiosis receptor-like kinase) was described by Wang L. *et al*. (2016).

Figure 4: Mechanisms of CRISPR/Cas9 gene editing. (a.) The target cell is given a construct that expresses a crRNA segment and the Cas9 protein, **(b.)** The crRNA and Cas9 complex binds to the target DNA, **(c.)** The Cas9 nuclease causes a double-strand break to occur at the target site, **(d.)** The DNA ends are then repaired by the cell's DNA repair mechanisms, either by error-prone non-homologous end joining (NHEJ) or, with the addition of a homology template, by homology-directed repair (HDR) (Binnie *et al*., 2021).



1.6 Salinity stress in Potato

After rice, wheat, and maize, potatoes are the fourth most important staple food consumed worldwide (Lutaladio & Castaldi, 2009). It is a rich source of minerals, dietary fibre, ascorbic acid, proteins, and other nutrients (Raigond *et al.*, 2020). A growing population in developing countries will benefit from the potato's contribution to food and nutritional security (Devaux, 2014). Abiotic stresses like heat, drought, and salinity, which are occurring more frequently because of climate change, could, however, reduce potato production and productivity (Hijmans, 2003). Therefore, creating new strategies to deal with potential effects of climate change and ensuring food and nutritional security in the coming decades are the two most important emerging challenges for policymakers and the government (Tiwari *et al.*, 2020). One of the main abiotic stresses that prevents potatoes from being produced and productive is salinity stress (Levy & Veilleux, 2007).

In addition to climate change, soil salinization has emerged as a global issue. Approximately 20% of cultivable land and 33% of irrigated land are affected by salinity, resulting in a significant decrease in crop yield and quality (Qadir *et al.*, 2014). The Green Revolution of the 1950s and 1960s encouraged the worldwide adoption of fertilizer-responsive, high-yielding wheat and rice varieties (Eliazer Nelson *et al.*, 2019). The soil quality has deteriorated over time due to the extensive use of fertilisers, chemicals, and altered rainfall patterns. As a result of soil salinity and sodicity, approximately 1.12 billion hectares (Mha) are impacted globally (Sharma & Singh, 2015). The Middle East has the largest salt-affected area (189 Mha), followed by Australia (169 Mha), North Africa (144 Mha), and South Asia (52 Mha). The area of saline soil is increasing over time, and it is anticipated that by 2050, the area of South Asia under salinity stress will nearly triple (Sharma & Singh, 2015). This scenario illustrates the overlap between salt-affected and the world's major potato-growing regions. Therefore, soil salinization is becoming a significant issue, especially in arid regions.

Saline soils have electrical conductivity of the saturated extract (ECe) values greater than 4 dSm1 at 25 °C and exchangeable sodium less than 15% (Shao *et al.*, 2019). Potato is very adaptable and can be grown in a variety of soils (Ma *et al.*, 2020). Salinity stress, however, negatively impacts its growth and yield. Until a specific salinity threshold (ECt) is exceeded in soil, salt stress has no effect on relative crop yield. Being salt sensitive, most vegetables, including beans, carrots, eggplants, potatoes, muskmelon, onions, peas, lettuce, okra, and tomato, have very low values of this threshold, which ranges from 1-2.5 dSm^{-1} (Machado &

Serralheiro, 2017). Since irrigation water salinity (ECw) is 1.1 dSm^{-1} and saturated soil extract (ECe) is 1.7 dSm^{-1}, the potato crop is categorised as a moderately salt-sensitive crop (Machado & Serralheiro, 2017).

For tolerant potato species, the yield reduction is 50% when irrigation water has an electrical conductivity (EC) of 5 dSm^{-1}, while it is 25% for sensitive species (Blom-Zandstra *et al.*, 2014). When plants are under salt stress, sodium (Na^+), chloride (Cl^-), and boron (B^{3+}) ions accumulate in the tissues of the shoots and leaves. High Na^+ and Cl^- concentrations are a limiting factor for growth when exposed to salt stress. Na^+ interferes with K^+ and Ca^{2+} transport, and it also lowers photosynthetic efficiency because chlorophyll is degraded (Tavakkoli *et al.*, 2010). Additionally, these ions hinder biochemical processes like enzyme inactivation and protein synthesis. Extreme salinity causes harm to organelles, including chloroplasts (Hirasawa *et al.*, 2017). Additionally, high salt accumulation in tuber cells, which results in altered osmotic potential and nutrient uptake capacity, prevents tuber formation and bulking in soil salinization (Dahal *et al.*, 2019).

Plants have evolved a variety of defence mechanisms to withstand salinity stress. Three categories—osmotic tolerance, ion exclusion, and tissue tolerance—have been broadly used to group these mechanisms (Chakraborty *et al.*, 2020). The main regulatory mechanisms for plant cell adaptation to salinity are osmotic adjustment and toxic ion compartmentalization. According to the evidence, most vegetables' cells experience oxidative stress when exposed to high salinity (Machado & Serralheiro, 2017). By adjusting and accumulating large amounts of osmolytes, such as proline, sugars, other metabolites, and various inorganic ions (Na^+, K^+, Ca^{2+}, and Cl^-) in the high salinity condition, osmotic balance can be achieved (Sarker & Oba, 2018). Plant species have been divided into two categories based on how they respond to salinity: salt-tolerant (4 dsm^{-1} or 40 mM NaCl) and salt-sensitive (2 dsm^{-1} or up to 20 mM NaCl) (Ödemiş & Çalişkan, 2014) under salt stress, salt-tolerant plant species maintain their typical metabolic processes like water use efficiency (WUE). In contrast, later species lack native metabolic mechanisms to adjust to high salt concentrations (Jha *et al.*, 2017).

1.6.1 Effects of Salt Stress on Potato Development and Growth

Given that abiotic stress is a complex trait, breeding for it is never easy. There are numerous instances where efforts are being made to create potato varieties that are salt-resistant (Londhe, 2016). There have been few concentrated efforts to create potato cultivars that can tolerate salt, though. Due to the complexity of both genetic and physiological traits, the success rate is

generally low (Ashraf & Foolad, 2013). The tolerance mechanism in potatoes exhibits all the features of a quantitative trait, leading to the development of various tolerance levels under various environmental circumstances (Velásquez *et al.*, 2005). The results show that, potato salinity stress reduces both yield and tuber quality (Ödemiş & Çalişkan, 2014). A yield reduction of about 60% was observed under salinity stress as a result of tuberization inhibition. However, there was a yield loss of only 21 to 59% when the potato plant was first exposed to salinity during the early stages of sprouting, emergence, and plant development (Levy, 1992). Due to salinity stress, the micro-tuberization process is also negatively impacted (Zhang *et al.*, 2005). It is a well-known fact that high concentrations of sucrose act as a signal for the emergence of microtuber. According to Dobránszki *et al.* (2008), the development of microtubers was constrained under salinity stress because sugar translocation was inhibited (Kumar & Ezekiel, 2006). According to another report, the higher salt concentrations in the growing medium may inhibit potato microtuber formation by reducing plant growth and rooting ability (Dobránszki *et al.*, 2008).

The roots, and in particular the root meristem, are the main receptors of salinity stress (Richardson *et al.*, 2001). Root number, root diameter, and root length decrease as salt concentration rises (especially that of Na^{+}, Ca^{2+}, Mg^{2+}, Cl^{-}, SO^{2-}, and HCO^{3-} ions) (Faried *et al.*, 2016). The root system, where the reduction in root growth was severely hampered by salinity stress, exhibits the first signs of salt toxicity (Jbir-Koubaa *et al.*, 2015). Compared to susceptible genotypes, tolerant genotypes experience a less significant reduction in root length, diameter, and number. In potato breeding programmes, this parameter can be successfully used as a selection parameter for genotypes that are salt-tolerant (Jbir-Koubaa *et al.*, 2015). The main location for gas exchange and photosynthesis is the leaf. According to one study, a reduction in leaf area lowers potato yield (Acosta-Motos *et al.*, 2017). Potato growth is severely hampered by salinity levels over 0.6%, which also cause shoot tip necrosis and the development of compound leaves (Naik, 1993). The plant's built-in tolerance mechanism causes it to typically try to deal with salinity by excluding and transferring extra salts to older leaves (Acosta-Motos *et al.*, 2017). Toxicity, early senescence, disturbed osmotic potential, disturbed pigment compositions, and disturbed relative leaf water content are all results of this. Moreover, the disruption of Mg^{2+} ions concentration in leaves also results in stomatal closure and a decrease in chlorophyll content. In potatoes, salt accumulation reduces the leaf water potential by 0.24 to 0.54 MPa (Chakraborty *et al.*, 2020). Stomata closure and a decrease in chlorophyll content are primarily caused by toxic Na^{+} and Cl^{-} accumulation (Tiwari *et al.*,

2020). These ions' homeostasis can be actively maintained by halophytes. While salt-sensitive plants, like potatoes, are unable to control the influx of ions, which lowers the concentration of K^+ in the stems and roots (Murshed *et al.*, 2015). Reduced photosynthesis overall, transpiration rate, leaf stomatal conductance, stem number, dry matter, and ultimately growth are the results of such disruption to plant tolerance mechanisms (Murshed *et al.*, 2015). When two potato cultivars, *May Queen* and *Dejima*, were stressed by high salinity, Ghosh and colleagues (2001) found that leaf nitrogen reductase (NR) activity was reduced.

Potato has a very low seed multiplication ratio (1:4), and many farmers use a significant portion of the produce, or tubers, as seeds in the following season (Chindi *et al.*, 2013). So, for profitable potato farming, good plant stand, emergence, and germination are essential factors. Studies on potatoes indicate that salinity stress is very likely to affect plant emergence and germination (Ghosh *et al.*, 2001). Tubers exposed to salinity directly after planting experienced sparse emergence and severe yield losses (Levy, 1992). Seed tubers that didn't fully emerge under salt stress due to soil electrical conductivity greater than 1.95 dSm^{-1} rotted (Ghosh *et al.*, 2001). Processing potatoes lowers post-harvest losses and overproduction glut, boosting farm income and ensuring the safety of the nation's food supply (Lal *et al.*, 2020). Typically processing industries can use potato cultivars with a dry matter content of over 21-23%. Numerous studies show that the accumulation of dry matter in the economically valuable plant parts decreases as the salt level rises (Zaman *et al.*, 2015). Compared to other potato plant parts, the dry matter loss in tubers is comparatively higher. Consequently, it is evident that increased abiotic stress, specifically salinity, will influence potato processing industries.

1.6.2 Improving Potato Salt Tolerance

Plants can withstand high salinity stress through tolerance and avoidance mechanisms. The development of salt tolerance involves limiting root salt uptake, excluding ions from leaf tissues, controlling ion concentration and distribution, compartmentalization, hormonal interactions, and various sensory mechanisms (Chen *et al.*, 2018). We now have a better understanding of the various mechanisms underlying salt stress and how it affects crop productivity as a result of extensive research conducted in recent years. Though not extensive, the research, particularly in potatoes, may point the way in the future to a better understanding of salinity stress. The most effective and cost-effective method for reducing the impact of salinity stress on the crop is to use genetic strategies and approaches, which include utilising

wild relatives and species, breeding for salt-tolerant cultivars, and genetically altering the suitable agronomical cultivars (Ashraf & Foolad, 2013).

1.6.2.1 Wild Potato Relatives and Primitive Species

The evolution of wild relatives has always been a significant source of trait utilisation in the potato industry, especially for biotic resistance. By providing a gene pool to be used in potato breeding for salinity tolerance, wild species of potatoes offer tremendous potential in addressing the issue of salinity (Heřmanová *et al*., 2007). In this regard, the International Potato Center (CIP), Peru, has assessed the salinity tolerance of its accessions. Seven accessions in total—six hybrids and one landrace species, *Solanum juzepczukii*—are resistant to salinity. Nine accessions, including one subspecies from Andigena, are moderately resistant. When it comes to salt tolerance, Andean potatoes are quite variable (Chourasia *et al*., 2021). CIP, Peru (https://genebank.cipotato.org/gringlobal/search.aspx, accessed on 01 December 2022) has reported 21 potato accessions that are salt tolerant, 14 accessions that are moderately salt resistant and 8 accessions that completely salt resistant.

1.6.2.2 Breeding Methods to Improve Salt Tolerance

The breeding strategies for enhancing salt tolerance include: (i) pre-breeding, which involves utilising natural genetic variations, trait selection, hybridization, and subsequently developing lines of salinity tolerance; (ii) marker-assisted breeding, which involves population development, mapping quantitative trait loci, identification of markers, and subsequently marker-assisted selection; and (iii) genomic approaches, which include genome editing with CRISPR/Cas9 systems and (iv) creating transgenic plants to add a gene for salinity resistance or to overexpress and modify the levels of genes already present (Chourasia *et al*., 2021).

1.6.2.3 Using Genetic Engineering to Increase Potato Salt Tolerance

The moderate sensitivity of potato plants to salinity is well known, but they are vulnerable during the tuber bud initiation phase. Many attempts have been made worldwide to create salt-tolerant potato varieties by introducing salt-tolerant genes for their functional proteins, including Glyceralde-hyde-3-phosphate dehydrogenase (GPD), osmotin-like protein, proline synthesis protein, and trehalose synthesis protein, as well as regulatory proteins, including StRD22 (responsive to desiccation 22), StEREBP, and CBF (Byun *et al*., 2007). In their comprehensive review about salt stress in potato, Chourasia *et al*., (2021) have mentioned various salt tolerant genes that have been successfully incorporated in potato.

1.6.2.4 Use of Biostimulants

Plant biostimulants, also known as agricultural biostimulants, are a broad category of compounds that can be added to the environment around a plant and have beneficial effects on plant growth and nutrition as well as on the ability of the plant to withstand biotic and abiotic stress. Although most of the plant biostimulants are added to the rhizosphere to promote nutrient uptake, many of these also have protective effects against environmental stress, including exposure to suboptimal growth temperatures, soil salinization, and water deficit (du Jardin, 2015). Biostimulants don't act as nutrients; rather, they help with nutrient absorption, or they help with stress resistance or growth promotion (Brown & Saa, 2015). A recently developed paradigm emphasises that plants are not independent living things in their environments, but rather are hosts and partners to a variety of bacterial and fungal microorganisms. Because of these associations, both externally and internally in their tissues, plants are able to respond to and adapt to biotic and abiotic stress (Vandenkoornhuyse *et al.*, 2015). We could strengthen these associations' function in plant stress defence if we functionally optimise these associations.

1.7 Melatonin as a Biostimulant

Melatonin (MT), also known as N-acetyl-5-methoxytryptamine, is an indoleamine with a variety of roles in both humans and animals. In 1995, melatonin was then found in plants, where it serves a variety of regulatory roles (Arnao & Hernández, 2019). Mammalian MT regulates seasonal changes at various neuroendocrine and physiological levels, affecting circadian rhythms and having hypnotic effects as well (Zhao *et al.*, 2019). Due to its small size and high solubility in both water and lipids, melatonin is an eco-friendly biomolecule capable of penetrating cell compartments. MT is regarded as an alternative and cost-effective strategy for enhancing plant tolerance to abiotic stressors such as salinity, pH, and heavy metals. Phytomelatonin is produced from tryptophan through the activation of multiple enzymes (Dhole & Shelat, 2018).

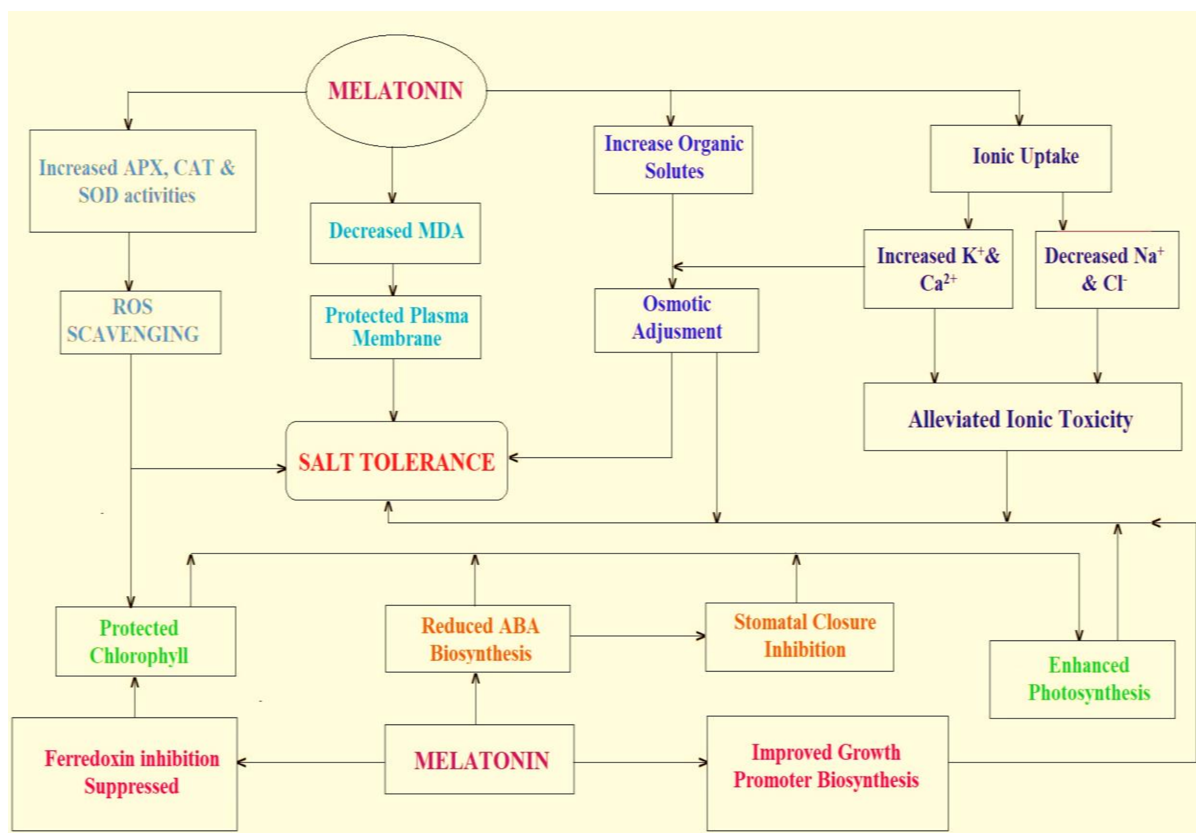
Reactive oxygen species (ROS) and reactive nitrogen species are both effectively combated by melatonin's antioxidant properties (RNS). Melatonin is also a protective substance against various abiotic stresses (Arnao & Hernández, 2019). Although each stressor induces specific physiological responses, MT generally promotes physiological processes such as stomatal absorption, growth, roots, germination, photosynthesis, osmoregulation, anti-senescence, and primary and secondary metabolism. (Arnao & Hernández, 2019).

1.7.1 Melatonin and Salinity Stress

Melatonin is well known for its antioxidant properties, and it has been shown that MT plays a regulatory role in enhancing plant tolerance to various types of abiotic stress, including salinity (Li *et al.*, 2012) (Figure 6). Exogenous applications of MT have been observed to enhance under saline conditions and improve the antioxidant system in tomato (Zhou *et al.*, 2016, Martinez *et al.*, 2018). In response to NaCl and $ZnSO_4$, the content of MT in barley roots increased over control. This increase is crucial for stress tolerance (Arnao & Hernández, 2019). Additionally, MT reduces the closure of stomata (Li *et al.*, 2017), safeguards chlorophyll (Wang *et al.*,2016) and enhances photosynthesis by increasing light absorption and CO_2 fixation. Application of melatonin promotes the accumulation of organic osmolytes, such as proline, soluble sugars, and water-soluble proteins, preventing cells from becoming dehydrated when exposed to salt stress (Zeng *et al.*, 2018). In addition, it has been observed that MT improves ion homeostasis in *Malus hupehensis* under high salinity conditions (Li *et al.*, 2012) and lessens ion toxicity by reducing Na^+ and Cl^- uptake. Additionally, it controls energy production, which improves germination and increases uniformity in cucumber seeds under salt stress (Zhang *et al.*, 2017). It has been demonstrated that MT not only increases the content of gibberellins and indole-3-acetic acid, two plant hormones that are important in numerous biological processes under saline conditions, but also decreases the content of abscisic acid (ABA) (Dawood *et al.*, 2015).

Melatonin inhibits oxidative stress in several ways to protect plants from salt stress (Szafrańska *et al.*, 2016). Through an increase in the TaSNAT transcript, which encodes essential enzymes in the MT biosynthesis pathway, exogenous MT application causes the accumulation of endogenous MT under salinity stress in wheat (Ke *et al.*, 2018). MT increases the expression of genes associated with antioxidants under salinity stress. For instance, MT was found to upregulate the transcripts for the enzymes ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD) in salt-stressed Arabidopsis (Chen *et al.*, 2017). Additionally, under salt-stress conditions, it upregulated genes involved in ascorbate metabolism, such as VTC4 and APX4. This could explain how MT helps plants produce more antioxidants (Wei *et al.*, 2015).

Figure 6: An illustration of how melatonin affects the plant's ability to tolerate salt stress (according to existing research findings) (Moustafa-Farag *et al.*, 2020).



Melatonin shields the photosynthetic apparatus from oxidative damage brought on by salt (Rangani *et al*., 2016). By increasing permeability and lowering lipid peroxidation, melatonin may help maintain the integrity of biological membranes, which reduces toxicity and promotes plant growth (Jiang *et al*., 2016). A healthy energy status is required for the maintenance of proton pump activity across the tonoplast and plasma membrane, and MT aids plants in increasing the amount of energy produced from lipids stored in sweet potato cells (Yu *et al*., 2018). Melatonin may improve salinity tolerance by increasing the expression of ion-channel genes in leaves, which helps to maintain ion homeostasis (Wang *et al*., 2012). According to some reports, MT does not act alone to alleviate salinity stress. It increases the accumulation of endogenous bioactive molecules known for their salt-stress mitigation role. Melatonin has been shown to increase polyamine biosynthesis from precursor amino acids and decrease salt-induced polyamine degradation (Ke *et al*., 2018). Although many aspects of melatonin biochemistry and physiology in plants are already known, more technical data pertaining to specific crops is required. The ideal dosage of melatonin to be used in root or foliar treatments, for example, must be determined.

THESIS AIMS & OBJECTIVES

The primary aim of this thesis was to investigate the unrealised potential of *Solanum okadae*, an Andean wild diploid potato species, *Solanum okadae*, by screening for its nutritional content and salt tolerance, which can potentially be used to improve cultivated potato. Due to *Solanum okadae's* self-incompatible nature, which would impede the production of inbred lines, this thesis also aimed to create self-compatible lines of *Solanum okadae* via CRISPR-Cas9 mediated knockout. To achieve these aims, this project had six specific objectives:

Objective 1: Optimisation of a micropropagation system for *Solanum okadae* to produce sterile genetic stock.

Objective 2: Biochemical analysis of tubers to assess their nutritional content via sugar, mineral, vitamin, and amino acid profiling.

Objective 3: Screening *Solanum okadae in vitro* for salt tolerance using NaCl at different concentrations.

Objective 4: Designing two different CRISPR/Cas9 constructs to knockout S-RNase gene and delivering these CRISPR/Cas9 constructs in *Solanum okadae* via *Agrobacterium*-mediated transformation to obtain self-compatible lines.

Objective 5: Sequencing 3 putative S-Locus F-Box (SLF) gene sequences in *Solanum okadae* from S_2 haplotype via polymerase chain reaction and using *Petunia integrifolia subsp. inflata* SLFs as reference.

Objective 6: Assessing the effect of exogenously supplied melatonin on photosynthetic and antioxidant system of severely salt stressed *Solanum okadae.*

To the best of knowledge and after a comprehensive assessment of the literature, there is no data available on any of the above-mentioned topics linked to *Solanum okadae*, and thus this thesis proves to be useful in bridging the gap in lack of information related to the specific diploid potato species.

CHAPTER 2: Nutritional evaluation of *Solanum okadae*

1. Introduction

Cultivated in almost 80% of the world's countries, potato (*Solanum tuberosum*) ranks fourth in terms of production and harvested area after maize, wheat, and rice as a staple crop for human diet, with more than 368 million tonnes produced (Sulli *et al*, 2017). It is a versatile, carbohydrate-rich food that is eaten by many people and prepared in several different ways all over the world. This tuber is typically consumed as a complementary vegetable or as a garnish for other important meal components and is frequently believed to make a negligible difference to the nutritional benefit of a meal, considered primarily for their caloric value, with little thought given to their nutrient density (Navarre *et al*., 2009).

Potato contains a wide range of biochemical and nutritional components, including reducing, non-reducing and total sugars, starch, phenolic content, ascorbic acid, carotenoids, polyamines, and flavonoids, all of which are highly desirable in the diet due to their health benefits (Mishra *et al*., 2020). Due to the presence of essential amino acids especially lysine, along with high levels of starch and dietary fibre and a low concentration of fats, the potato tubers have high nutritional value (Keutgen *et al*., 2019). Potato tubers with no or low-fat additionally contain a high concentration of bioactive compounds and antioxidants, like ascorbic acid. Potato tuber consumption may increase blood and tissue antioxidant levels and protect against oxidative stress, which is responsible for lipid, protein, and enzyme damage. Increased nutrient availability to a large portion of the world's population is one of the global health goals. A reasonable strategy for achieving this goal would be to increase the nutritional content of commonly consumed crops such as potato. Additionally, potatoes are amenable to development through breeding and biotechnology methods and have superior biochemical and nutritional qualities. The utilisation of the crop and its future economic significance, as well as increased knowledge of its biochemical and nutritional qualities, have significant effects on domestic and international human food systems (Nzaramba *et al*., 2007).

1.1 Nutritional composition of cultivated potato

Researchers have discovered that potatoes are nutrient-rich even though just 20% of newly harvested tubers is dry matter and nearly 80% is water. Although starch makes up the bulk of a potato dry matter, it also contains protein and alkaline salts. Potato is an excellent source of many vitamins, including beta-carotene, vitamins C, A, B1, B2, B6, and folic acid. It also has

some amino acids and nicotinic acid in very low concentrations (Navarre *et al.*, 2009). Many of potato's beneficial nutrients are in the skin, so consuming them unpeeled may provide greater health benefits (Brar *et al.*, 2017). Potatoes are highly desirable crops for commercial use, and they are also vital to ensuring food security (Sabeena *et al.*, 2012).

Carbohydrates

Starch, sugars, polysaccharides, and non-starch are the four different categories of carbohydrates found in potato. Granules of starch are present and consist of a constant 3:1 ratio of amylopectin to amylose. Amylopectin has about 105 glucose residues and is a large, ramified molecule. The amylopectin fraction, which is chemically linked to starch, contains traces of phosphorus. Potato starch production is economically viable in developed nations due to its high starch content and is utilised in the creation of food, textiles, adhesives, and derived products like alcohol and glucose. These starch gels, as opposed to cereal starches, have a high pot-paste viscosity, and set quickly. Only a small portion of the dry matter in tubers is made up of non-starch polysaccharides. Non-starch polysaccharides, which serve as dietary fibre, enhance the nutritional value of potato. With traces of other smaller sugars, sucrose, fructose, and glucose are the three main sugars present in potatoes (Navarre *et al.*, 2009).

Crude protein

Potato has a protein content of 2 to 3 percent based on its fresh weight (Mishra *et al.*, 2020), which is comparable to most other root and tuber staples except for cassava, which has half this amount. It is comparable to cereals when dry, and it is comparable to boiled rice when cooked (Navarre *et al.*, 2009). Their high lysine content is a benefit of potato over cereal grains. It contains fewer sulphur-containing amino acids (such as methionine and cysteine) than cereals. Potato can supplement low-lysine diets when combined with other foods, such as rice, which provides a higher-quality protein. In some developing countries, meals are frequently comprised of boiled potatoes and rice or pasta. However, consumers in developed nations frequently erroneously believe that such mixtures provide only a high carbohydrate energy content (AnjumSahair *et al.*, 2018). It has been suggested that potato has a nutritional advantage in the tropics. According to the most recent data on protein and energy needs, 100 g of potato can provide 7 %, 6 %, and 5 % of the daily energy needs, and 12 %, 11 %, and 10 % of the daily protein needs, respectively, of children aged 1-2, 2-3, and 3-5 years. 100 g of tuber can provide between 3 and 6 % of an adult's daily protein requirements, depending on body weight and gender (Bereje & Nwankwo, 2021).

Nitrogen

Rarely consumed on its own, potato is more often consumed in combination with other foods, which can have additive or synergistic effects. Potato is not a high-energy food, providing only about 80 kilocalories per 100 g, but it does provide high-quality protein. This is especially crucial in underdeveloped regions where access to energy is more common than access to protein (AnjumSahair *et al.*, 2018). Abderhalden (1912) reported that adult dogs fed a proline-free diet could maintain a positive nitrogen balance, while those fed a tryptophan-free diet exhibited a negative nitrogen balance. They classified amino acids (AA) as either nutritionally essential AA (EAA) or non-essential AA based on this discovery (NEAA). Potato tubers have a high nitrogenous content, making them a particularly valuable vegetable crop. Nitrogen is not evenly distributed throughout the tuber, with the highest concentration found in the skin, followed by the cortex, and then rising again toward the pith. Total nitrogen found in potatoes is split up into the following components: soluble, coagulable (true) protein; insoluble protein; and soluble non-protein nitrogen, which is made up of free amino acids, the amides asparagine and glutamine, as well as trace amounts of nitrate nitrogen and basic nitrogen compounds like nucleic acids and alkaloids. Peel is where the bulk of the insoluble protein fraction is present and is responsible for the total nitrogen content of only about 4% (FAO, 2008).

Minerals

Minerals are a crucial component of a balanced diet. Potato, a significant staple food crop, has a relatively high concentration of several macro and trace minerals and may be useful in the fight against mineral deficiency. While boiling whole or cut-up potatoes can raise mineral levels, boiling thinly sliced potatoes will result in a significant decrease (Bethke & Jansky, 2008).

Potato is an excellent source of several essential minerals and is notable for its iron, potassium, zinc, and magnesium content. Potassium (320 mg/100 g raw) is the most abundant mineral, with a higher concentration in the skin and, as a result, a lower concentration in peeled potato products. 100 g of raw potato contains about 18% of the recommended daily allowance for potassium, 6% of the RDA (Recommended Dietary Allowance) for iron, phosphorus, and magnesium, and 2% of the RDA for calcium and zinc. Most minerals are effectively preserved in boiled tubers with the skin. Keeping the skin on when baking potatoes is a good way to retain minerals. There are significant differences between *Solanum tuberosum* cultivars in major and trace mineral content (Bereje & Nwankwo, 2021).

Ascorbic acid

Ascorbic acid is a potent reducing agent in plant metabolism; as such, it enhances the absorption and transport of iron and zinc from other plant sources. It has been reported that potato tubers contain up to 46 mg of ascorbic acid per 100 g of fresh tuber weight, and their availability is dependent on the variety, maturity level, and environmental conditions under which the crop is grown. Ascorbic acid levels decreased with storage time in tubers of all varieties and ranged from 6.5 to 36.9 mg/100 g in fresh weight and 22.2 to 121.4 mg/100 g in dry weight for newly harvested, peeled, raw tubers. A British study examined the levels of vitamin C in 33 varieties raised in three different parts of Europe. Between 13 and 30.8 mg of vitamin C were present per 100 g of fresh weight. The amount of vitamin C in potatoes drops significantly during cold storage, with losses approaching 60%, according to numerous studies (Brar *et al*., 2017, Finlay *et al*., 2003).

Numerous primitive forms of the potato exist in the wild, and their vast genetic diversity makes them a rich source of genes that can be used to improve nutrition. It has been estimated that wild species contain more than twice as many alleles per locus as commercial cultivars, making them a rich source of genes not present in current cultivars (Bethke *et al.,* 2017). The International Potato Centre (CIP) estimates that less than 1% of the genetic diversity available has been incorporated into modern cultivars, making this germplasm a largely untapped resource (Jansky, 2000). Therefore, the aim of this study was to evaluate the content of various nutritional components like carbohydrates, proteins, minerals, amino acids, proteins, fats, sugars and ascorbic acid in wild diploid potato species, *Solanum okadae*.

2. Materials & Methods

2.1 Plant material

In vitro raised potato (*Solanum okadae*) plantlets with well-developed roots were carefully rinsed with sterile water to remove excess agar and planted in sterile CNSC compost. Before being transferred to the glasshouse, potted plants were kept in the controlled growth chamber for four weeks at 22± 2 ºC and 70% relative humidity. After 8 weeks, the potato tubers were harvested, rinsed with distilled water, air dried (Figure 1) and were stored at 4 ºC for dormancy for further analysis. Commercially available tubers of *Solanum tuberosum* 'Celandine' were used as control samples due to their close resemblance (size and shape) with *Solanum okadae* tubers.

Figure 1: Tubers harvested from 8 weeks old *Solanum okadae* plant raised in glasshouse.

2.2 Elemental profiling using ICP-MS

The ionomics analysis of potato powder was carried out as described (Danku *et al*., 2013). Briefly, 10 g of potato tubers (unpeeled) were washed with deionised water and thinly sliced samples were transferred into Pyrex test tubes (16 × 100 mm) and dried at 80 °C for 24 h. After the appropriate number of samples had been weighed, the tubes were filled with trace metal grade HNO_3 (Fisher Chemicals) mixed with indium internal standard (1 mL per tube). The samples were then digested for 4 hours at 115 °C in a dry block heater (DigiPREP MS, SCP Science; QMX Laboratories, Essex, UK). Using 18.2Mcm Milli-Q water, the digested samples were diluted to 10mL. Elemental analysis was carried out using inductively coupled plasma mass spectrometry (ICP-MS) (PerkinElmer NexION 2000 equipped with an Elemental Scientific Inc. auto sampler and the Syngistix software in collision mode (He)). Fourteen elements (Na, K, P, S, Mg, Ca, Fe, Mn, Co, Cu, Ni, Zn, Mo, and Se) were monitored. Before the sample run started, a liquid reference material made up of pooled samples was prepared, and it was used for the duration of the sample run. In order to account for variation within an ICP-MS analysis run, it was performed after every ninth sample. The calibration standards (with blanks and indium internal standard) were made from solutions of single element standards sold by Inorganic Ventures and Essex Scientific Laboratory Supplies Ltd, respectively. In the instrument's software, sample concentrations were calculated using an external calibration method. Using Microsoft Excel spreadsheet, additional data processing was performed. The concentrations of minerals in tubers were expressed in ppm on a dry weight basis.

2.3 Nutritional analysis

Tubers of *Solanum okadae* and *Solanum tuberosum* 'Celandine' were weighed (500g) and sent to Eurofins food testing labs in Wolverhampton, UK for nutritional profiling . The nutrients analysed along with test code and method used for detection are shown in Table 1 below.

Table 1: Nutrients and their respective detection methods provided by Eurofins Basic Group 2 Nutrition Profile service.

Test code	**Analyte**	**Method**
UD08D	Moisture	H/091 (Gravimetry)
UD001	Crude Protein (Nx6.25) (Dumas)	Z/001 (Dumas (TCD))
UD007	Ash	Q/001 (Gravimetry)
UD017	Carbohydrates (available)	Q/035
UD08W	Fructose	CHROM/344 (IC-PAD)
UD08W	Galactose	CHROM/344 (IC-PAD)
UD08W	Glucose	CHROM/344 (IC-PAD)
UD08W	Lactose	CHROM/344 (IC-PAD)
UD08W	Maltose	CHROM/344 (IC-PAD)
UD08W	Sucrose	CHROM/344 (IC-PAD)
UD08W	Total sugars	CHROM/344 (IC-PAD)
UD08C	Total Fat	H/090 (NMR)
B7039	Total dietary fibre (AOAC 991.43)	H/085 (Enzymatic-gravimetry)
UD771	Energy (Kcal)	Q/035
UD771	Energy (KJ)	Q/035
UD815	Salt	ICP/003 (ICP-OES)
Fatty acids		
UDFA1	Trans fatty acids	CHROM/215 (GC-FID)
UDFB1	Monounsaturated fatty acids	CHROM/215 (GC-FID)
UDFB1	Polyunsaturated fatty acids	CHROM/215 (GC-FID)
UDFB1	Saturated fatty acids	CHROM/215 (GC-FID)
Vitamins		
A7291	Ascorbic acid (Vitamin C)	LC-UV/DAD
DJCDE	Cyanocobalamin (Vitamin B12)	LC-UV/DAD

2.4 Amino acid profiling

2.4.1 Preparation of working solution & standards:

i. **Formic acid/phenol solution:** 735 ml of formic acid (Fisher. Cat. No. F/1900) and 111 ml of deionized water were mixed with 4.73 g of phenol (Fisher. Cat. No. P/2320). Phenol in the oxidation solution is a group 3 carcinogen, therefore the mixing was done inside a fume hood.

ii. **Oxidation solution:** 10 ml of 30% hydrogen peroxide ((Fisher. Cat. No. H/1800) was dispensed in a 100ml volumetric flask. The volume was made up to 100ml with formic acid/phenol solution. This solution was incubated at 20-30°C for 1 hour till the solution turned yellow in colour and stored in fridge.

iii. **Hydrolysis reagent:** Inside a fume hood, 492ml of conc. HCl (Fisher. Cat. No. H/1200) was carefully diluted with 508ml of deionised water to make up the volume to 1L.

iv. **Following standards were used directly for the analysis:**
   - Amino acid standard, 2.5µmol/ml (Sigma. Cat. No. AA-S-18).
   - Cysteic acid (Sigma. Cat. No. C7630).
   - Methionine sulfone (Sigma. Cat. No. M0751).
   - Alanine 15N (Sigma 332127)
   - Phenylalanine 15N (Sigma 490105-500MG)
   - Phenylalanine 13C6 (Kenneth Smith)
   - Cell Free Amino Acid Mixture - 13C,15N (Sigma-Aldrich: Cat No 767964).

v. **Concentrated solution of Cysteic acid and Methionine sulfone (1.25 mM):** 0.2340 g of cysteic acid and 0.2265 g of methionine sulfone were dissolved in 200ml of tri-Sodium citrate buffer (or Loading Buffer) (pH: 2.20). The volume was made up to 1L with tri-sodium citrate buffer using a volumetric flask. This solution was stored in a freezer.

vi. **Concentrated solution of tryptophan, 10µmol/ml (10 mM):** 0.1021 g of tryptophan was dissolved in 100 ml of tri-Sodium citrate buffer (pH: 2.20). The volume was made up to 50 ml with tri-sodium citrate buffer using a volumetric flask. This solution was aliquoted and stored in a freezer.

2.4.2 Amino acid analysis

i. 10 g of unpeeled potato tubers (*Solanum okadae* and *Solanum tuberosum* 'Celandine) were thinly sliced and lyophilised using freeze dryer (Alpha 1-4 LDplus, Germany) for 48h before being powdered using an agate mortar and pestle.

ii. Approximately 30mg of samples (potato powder) were weighed and transferred into a 20ml crimp top tubes making sure that the samples were deposited to the bottom of the tubes. A static eliminator was used to prevent static and the weight (W) of each tube was recorded to 4 decimal places before being placed in the fridge for 5-6 hours.

iii. The tubes were taken out of the fridge using a dispenser and 2.5ml of chilled oxidation solution was added to each tube making sure the solid samples were rinsed down to the bottom. The samples were kept in the fridge overnight.

iv. After oxidation, samples were taken out of the fridge and 0.42g of sodium metabisulphite was added to each tube to decompose any excess of oxidation reagent.

v. 2.5ml of 12M HCl was then added to each sample followed by 0.5ml of hydrolysis reagent (6M). The samples were left to cool at room temperature.

vi. The sample tubes were sealed tightly and incubated at 110°C for 24hrs using an oven.

vii. Post hydrolysis, the tubes were taken out of the oven and left to cool for 10mins.

viii. The samples were quantitatively transferred into 50ml falcon tubes. Ammonium formate buffer (pH: 2.8, 20mM) was used to rinse the contents of the sample tubes into 50ml falcon tubes.

ix. The samples were partly neutralised by addition of 16ml of 4M ammonium formate solution before adjusting the pH of each sample to 2.8 using 4M, 100mM ammonium formate and formic acid. The total volume of each sample was made up to 50ml with ammonium formate buffer (pH2.8, 20mM).

x. The samples were centrifuged at 3000 RPM for 10mins, and the supernatant was passed through a 0.22µm filter into a HPLC vial before being passed through the amino acid analyser.

xi. The concentration of amino acid is expressed as gram per kilogram sample (g/kg or mg/g). It is calculated as following:

$(A*MW*F/W)*10^{-3}$ = g amino acid per kilogram sample (g/kg, or mg/g)

A = concentration of hydrolysate obtained by the instrument (uM), MW = molecular weight, W = g sample (corrected to original weight if dried or defatted), F = ml total hydrolysate, in this case it is 50ml.

All trials were conducted in a completely randomised fashion and each test was performed in triplicate. Data were presented using the mean±standard deviation (SD). Using SPSS software (version 21.0, SPSS Inc., Chicago, USA), an analysis of variance (ANOVA) was used to determine differences between samples. The significant difference between the variables was evaluated using the Duncan's multiple range test ($P<0.05$).

3. Results

3.1. Proximate Composition Analysis (PCA)

The proximate composition of *Solanum okadae* and *Solanum tuberosum* 'Celandine' is summarised in Table 2 below. The results of Table 2 show that *Solanum okadae* presented significantly higher values for carbohydrates (18.47 g/100g) and energy (368 KJ) in *Solanum okadae* when compared to *Solanum tuberosum* (10.61 g/100g and 216 KJ respectively). Overall, it was observed that the values for ash, total proteins and total dietary fibre were only slightly higher (0.16%, 0.9% & 0.04% respectively) in *Solanum okadae* when compared to those of *Solanum tuberosum*, expect for the moisture content. The moisture content in *Solanum tuberosum* (85.1 g/100g) was almost 11% more than that of *Solanum okadae* (75.9 g/100g).

Table 2: Proximate Composition Analysis (g/100g) of *Solanum okadae* and *Solanum tuberosum* 'Celandine'

Analyte	***Solanum okadae***	***Solanum tuberosum***
Moisture	75.9	85.1
Ash	1.2	1
Protein	2.1	1.1
Protein (% DM)	15.74	10.71
Carbohydrates	18.47	10.61
Total dietary fibre	2.3	2.2
Energy (Kcal)	87	51
Energy (KJ)	368	216

Figure 2: Graphical representation of proximate composition analysis (g/100g) of *Solanum okadae and Solanum tuberosum* 'Celandine'

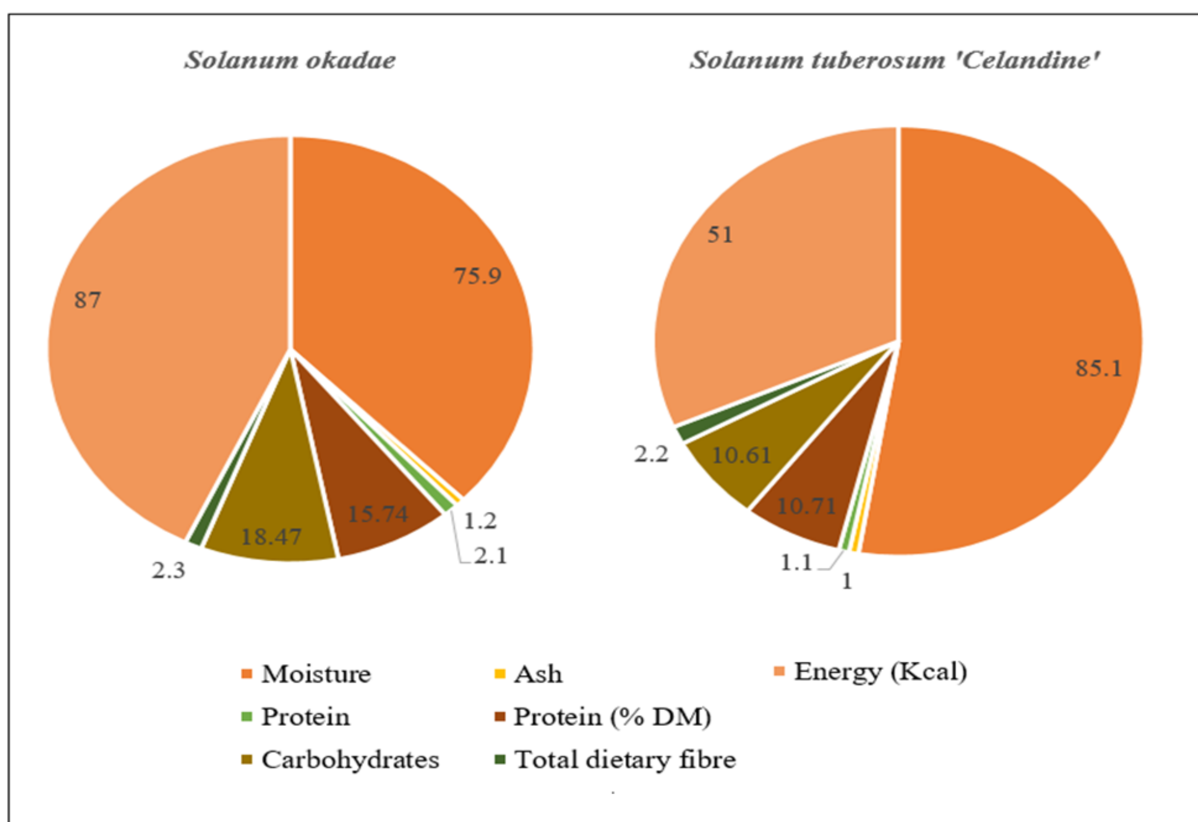


Table 3: Sugar content (g/100g) in tubers of *Solanum okadae* and *Solanum tuberosum* 'Celandine'

	Solanum okadae	***Solanum tuberosum***
Total sugars	2.6	1.5
Fructose	0.9	0.8
Galactose	0.5	<0.1
Glucose	1.3	0.6
Lactose	<0.1	<0.1
Maltose	<0.1	<0.1
Sucrose	0.2	<0.1

The total sugar content along with individual sugars in both potato species is shown in Table 3 above. The total sugar content in *Solanum okadae* was estimated to be 2.6 g/100g whereas that in *Solanum tuberosum* 'Celandine' was 1.5 g/100g. There was no significant difference in the amount of individual sugars between both the species except for glucose. The glucose content of *Solanum okadae* was 1.3 g/100g whereas that of *Solanum tuberosum* 'Celandine' was 0.6 g/100g. This study also indicated that *Solanum okadae* had lower amounts of trans, monosaturated and polysaturated fatty acids compared to Celandine (Table 4).

Table 4: Fatty acid profile of *Solanum okadae* and *Solanum tuberosum* 'Celandine' (g/100g)

	Solanum okadae	***Solanum tuberosum***
Trans fatty acids	<0.02	0.23
Monounsaturated fatty acids	<0.1	0.2
Polyunsaturated fatty acids	<0.1	0.2
Saturated fatty acids	<0.1	<0.1

3.2 Mineral concentration

In this study, whole tubers were cleaned and powdered for the analysis to assess overall concentrations more accurately. The concentrations of 6 macrominerals (Na, K, Ca, Mg, P and S) for the potato samples are shown in Table 5. There was a significant difference between the concentrations of macrominerals among the two potato species except for phosphorous (P), where *Solanum okadae* had a P concentration of 2.91±0.81 g/kg and that of *Solanum tuberosum* was of 2.76 ± 0.31 g/kg. Amongst all the macrominerals quantified, the potassium (K) content was highest in both the species. *Solanum okadae* had a K content of 30.10 ± 1.46 g/kg whereas the K concentration of *Solanum tuberosum* was found to be 21.50 ± 1.9 g/kg. The Calcium (Ca) content in *Solanum okadae* was 2.92 ± 0.23 g/kg whereas that in *Solanum tuberosum* 'Celandine' was 0.62 ± 0.23 g/kg. Also, the magnesium content in this study was estimated to be 1.47 ± 0.22 g/kg and 0.83 ± 0.85 g/kg in *Solanum okadae Solanum tuberosum* 'Celandine' respectively.

Table 5: Macrominerals (g/kg) in *Solanum okadae* and *Solanum tuberosum* 'Celandine'

Macromineral	*Solanum okadae*	*Solanum tuberosum*
Sodium (Na)	2.02 ± 0.3^{c}	1.64 ± 0.74^{bc}
Potassium (K)	30.10 ± 1.46^{a}	21.50 ± 1.9^{a}
Calcium (Ca)	2.92 ± 0.23^{b}	0.62 ± 0.23^{c}
Magnesium (Mg)	1.47 ± 0.22^{d}	0.83 ± 0.85^{c}
Phosphorous (P)	2.91 ± 0.81^{b}	2.76 ± 0.31^{b}
Sulphur (S)	1.48 ± 0.15^{d}	1.14 ± 0.13^{c}

Table 6: Microminerals (ppm) in *Solanum okadae* and *Solanum tuberosum* 'Celandine'

Micromineral	*Solanum okadae*	*Solanum tuberosum*
Iron (Fe)	28.422 ± 2.69^{a}	25.20 ± 1.96^{a}
Zinc(Zn)	21.31 ± 0.65^{b}	12.98 ± 1.64^{b}
Copper (Cu)	4.17 ± 1.58^{c}	4.10 ± 1.58^{c}
Manganese (Mn)	4.52 ± 0.03^{c}	1.32 ± 0.03^{d}
Cobalt (Co)	0.34 ± 0.01^{d}	0.14 ± 0.0^{d}
Molybdenum (Mo)	0.56 ± 0.01^{d}	0.29 ± 0.03^{d}
Selenium (Se)	1.09 ± 0.27^{d}	1.00 ± 0.09^{d}

All values in the tables represent the means ±S.E. Different letters within a column indicate significant differences at $P< 0.05$ by Duncan's multiple range test

Table 6 shows the micromineral compositions of *Solanum okadae* and *Solanum tuberosum* 'Celandine'. 7 microminerals measured were Fe, Zn, Cu, Co, Mo, Mn, and Se. Iron (Fe) was found in higher concentrations in both potato species amongst all the tested elements. *Solanum okadae* had a Fe, Zn, and Mn content of 28.422 ± 2.69 ppm, 21.31± 0.65 ppm and 4.52 ± 0.03 respectively whereas the Fe, Zn, and Mn content of *Solanum tuberosum* was found to be 25.20 ± 1.96 ppm, 12.98± 1.64 ppm and 1.32 ± 0.03 ppm respectively. There was no significant difference between the copper (Cu) content in of *Solanum okadae* (4.17± 1.58 ppm) and *Solanum tuberosum* 'Celandine' (4.10 ± 1.58 ppm). Other trace minerals like cobalt (Co), molybdenum (Mo) and selenium (Se) did not show a significant difference amongst the two potato species but the content of these three trace minerals was higher in concentration in *Solanum okadae* compared to *Solanum tuberosum*.

3.3 Vitamins C and B12

In current investigation, LC-UV/DAD (Liquid Chromatography Ultraviolet Diode Array Detection) technique was used to quantify the vitamin C and B12 concentration in the potato tubers (Appendix II). *Solanum okadae* had a vitamin C concentration of 10.69 mg/100g whereas that of *Solanum tuberosum* was 7.34 mg/100g showing that *Solanum okadae* had 31.33% more vitamin C compared to *Solanum tuberosum.* Vitamin B12 was estimated to be 0.28 mg/100 g in *Solanum okadae* whereas that in *Solanum tuberosum* was less than 0.25µg/100 g (limit of quantitation) (Table 7).

Table 7: Vitamin C and B12 content of *Solanum Okadae* and *Solanum tuberosum* 'Celandine'

	RDA for Adults and Children ≥ 4 years (mg)	***Solanum okadae*** (mg/**100g)**	***Solanum tuberosum***
Vitamin C	75-90	10.69	7.34 mg/100g
Vitamin B12	0.09	0.28	<0.25 (LOQ) µg/100 g

LOQ: Limit of Quantitation

3.4 Essential (EAA) and Non-essential (NEAA) amino acids

In the present study, all nine different types of EAA and eight different types of NEAA were quantified in both potato species (Table 8 and 9). The concentration of all the EAAs in *Solanum okadae* was higher compared to that of *Solanum tuberosum* (Figure 3). There was a significant difference in the amount of each EAA in both potato species, with leucine (5.62 ± 0.40 g/kg)

and lysine (5.46 ± 0.08 g/kg) having the highest values in *Solanum okadae*, which were 66.19% and 47.61% more that those detected in *Solanum tuberosum* 'Celandine' respectively. Traditional classifications of NEAAs in mammals include Ala, Asn, Asp, Cys, Glu, Gln, Gly, Pro, Ser, and Tyr. All the analysed NEAA had higher values in *Solanum okadae* when compared to *Solanum tuberosum* (Figure 4). There was a significant difference in the amount of each NEAA in both potato species, with aspartic acid (Asp) (9.51 ± 0.46 g/kg) and glutamic acid (Glu) (8.13 ± 0.22 g/kg) having the highest values in *Solanum okadae*, which were 34.27% and 22.63% more that those detected in *Solanum tuberosum* 'Celandine' respectively.

Table 8: Essential amino acids (g/kg) in *Solanum okadae* and *Solanum tuberosum* 'Celandine'

Essential AA	***Solanum okadae***	***Solanum tuberosum***
Arg	4.05 ± 0.07^{c}	2.29 ± 0.04^{b}
His	1.76 ± 0.04^{e}	0.86 ± 0.06^{f}
Iso	4.23 ± 0.15^{c}	1.78 ± 0.02^{cd}
Leu	5.62 ± 0.40^{a}	1.90 ± 0.03^{c}
Lys	5.46 ± 0.08^{a}	2.86 ± 0.08^{a}
Met	2.38 ± 0.08^{d}	1.11 ± 0.10^{e}
Phe	4.90 ± 0.01^{b}	1.87 ± 0.13^{c}
Thr	4.37 ± 0.02^{c}	1.62 ± 0.09^{d}
Val	4.86 ± 0.15^{b}	2.38 ± 0.19^{b}

All values represent the means ±S.E. Different letters within a column indicate significant differences at $P < 0.05$ by Duncan's multiple range test

Figure 3: Concentration of essential amino acids in *Solanum okadae* and *Solanum tuberosum* 'Celandine

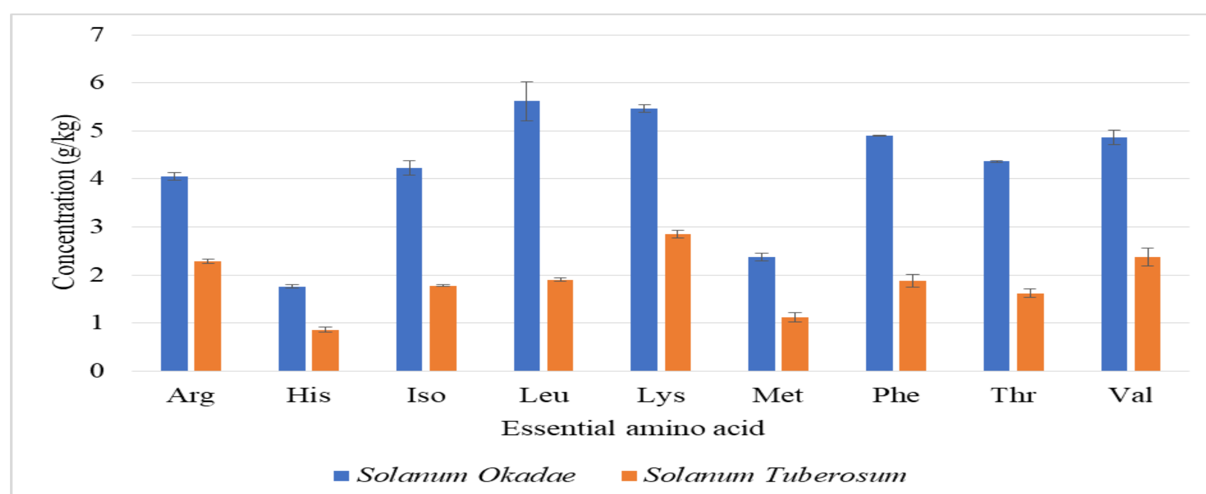
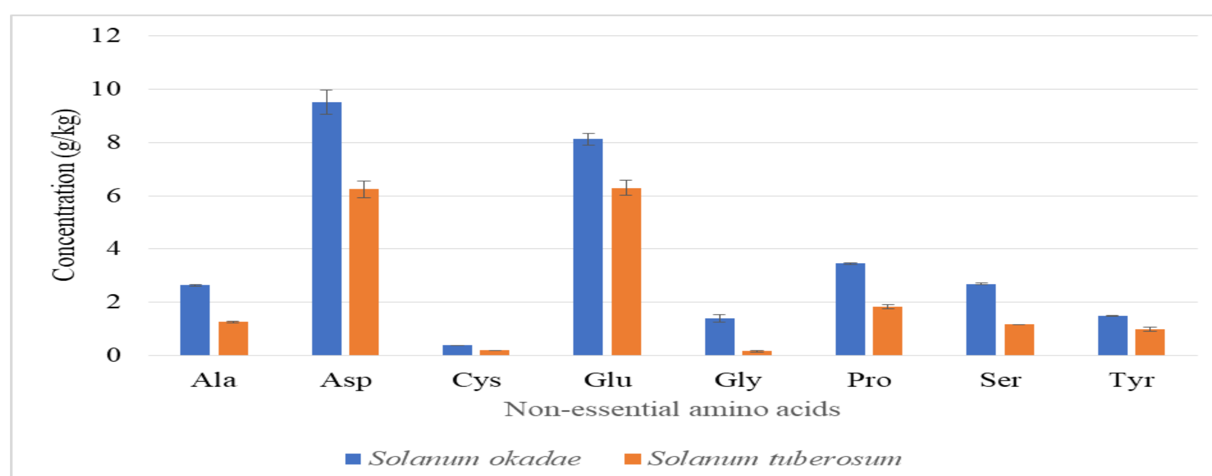


Table 9: Non-essential amino acids (g/kg) in *Solanum Okadae* and *Solanum tuberosum* 'Celandine'

Non-essential AA	*Solanum okadae*	*Solanum tuberosum*
Ala	2.64 ± 0.04^{d}	1.26 ± 0.03^{c}
Asp	9.51 ± 0.46^{a}	6.25 ± 0.31^{a}
Cys	0.40 ± 0.00^{f}	0.20 ± 0.00^{d}
Glu	8.13 ± 0.22^{b}	6.29 ± 0.28^{a}
Gly	1.39 ± 0.14^{e}	0.17 ± 0.02^{d}
Pro	3.45 ± 0.03^{c}	1.84 ± 0.08^{b}
Ser	2.70 ± 0.03^{d}	1.17 ± 0.01^{c}
Tyr	1.49 ± 0.03^{e}	0.99 ± 0.07^{c}

All values represent the means ±S.E. Different letters within a column indicate significant differences at $P < 0.05$ by Duncan's multiple range test

Figure 4: Concentration (g/kg) of non-essential amino acids in *Solanum okadae* and *Solanum tuberosum* 'Celandine'



4. Discussion

4.1 Proximate Composition Analysis

As a result of global population growth, there is an increase in the demand for food on a global scale, and especially in developing nations, large populations suffer from malnutrition, hunger, and famine. To meet these nutritional needs and population growth, it is necessary to investigate and develop new food resources (Embaby and Rayan, 2016). The moisture, ash, lipid, protein, and carbohydrate contents are all part of the food's proximate composition. These food ingredients may be useful to the food industry for developing new products, conducting quality control (QC), or meeting regulatory requirements. Therefore, this study examined the

proximate composition and nutritional value of the wild diploid potato species *Solanum okadae* for the first time.

Similar to findings in both the potato species in this study, the protein contents of the commercial potato cultivars ranged from 0.6 to 2.1 g/100 g FW in the experiments conducted by Eduardo *et al*., (2017), which were also comparable to values reported in raw unpeeled commercial Andean potato genotypes grown in Argentina (from 0.8 to 2.6 g/100 g FW) (Jiménez *et al*., 2009), raw unpeeled Andean cultivated and Spanish commercial potato genotypes (from 0.9 to 4.2 g/100 g FW) (Ritter *et al*., 2008), and raw unpeeled commercial potato genotypes grown in Spain (from 1.9 to 2.4 g/100 g FW) (Galdón *et al*., 2012). The value of protein (% DM) of *Solanum okadae* (15.47%) in this study was significantly higher than that of *Solanum phureja* (5.2%) and *Solanum stenotomum* (8.0%) as reported by Bártová *et al*., (2015). Among the 14 *Solanum tuberosum* cultivars tested by Zhou *et al*., (2019), the highest protein (%) was found in Neida 34 (14.10±0.06) which was almost 9% less than that of *Solanum okadae* in present study. However, the protein content in *Solanum tuberosum* 'Celandine' (1.1g/100g) in this study was similar to that of *cv. Violetta* (1.1g/100g) and *Salad Blue* (1.19g/100g) with cultivar *Highland Burgundy* having the highest value (1.65g/100g) as reported by Vaitkeviien (2019) which was less than the protein content in *Solanum okadae* (2.1g/100g) in this study.

The tuber's high moisture content indicates that it can be kept in storage after harvest for extended periods of time. The recorded moisture content value of 75% in *Solanum okadae* in current study was more than that of *Solanum tuberosum* (69%) recorded by Williams *et al*., (2020) falls within the range of 65% to 82% for *Dioscorea dumetorum* and *Ipomoea batatasa*, respectively (Alam *et al*.,2016). Like current study's findings, Zhou *et al*., (2019), found the moisture content in *Solanum tuberosum cv. Neida 35* to be 76.96%.

Table 10 below compares the PCA of *Solanum okadae* estimated in this study with those of some popular commercial *Solanum tuberosum* varieties; Swift, King Edward and Inova as reported by the Swedish National Food Administration during 2009-2010. It is clearly observed that, *Solanum okadae* has better carbohydrate and fibre content compared to other varieties. The protein content in Inova is same as that of *Solanum okadae*. The selection of high-calorie *Solanum okadae* will enable potato breeders to increase the competitiveness of future potato varieties in the modern food market.

Table 10: PCA of *Solanum okadae* and *Solanum tuberosum* varieties Swift, King Edward and Inova

Analyte	***S. okadae***	**Swift**	**King Edward**	**Inova**
Moisture	75.9	85.4	77.6	84.0
Protein	2.1	1.1	1.8	2.1
Carbohydrates	18.47	11	18.2	11.3
Dietary fibre	2.3	1.6	1.4	1.7
Energy (Kcal)	87	53	85	58

When compared to other dietary staples like rice and pasta, white potatoes are regarded as a high-calorie food. However, when compared on a gram-weight basis to most other staple foods, potatoes have less energy (94 kcal/100 g) while offering significant amounts of important nutrients (King and Slavin, 2013). The results of this study revealed that the amount of carbohydrates in *Solanum okadae* was 18.4%, falling within the 16.34–19.74% range reported by Ezekiel *et al*. (2019) in *Solanum tuberosum*. As reported by Narváez-Cuencaa *et al*., (2018), the diploid commercial potato cultivars of genotype Criolla Colombia, Criolla Galera, Criolla Paisa and Criolla Guaneña had respective energy values (321.9 kcal, 326.2 kcal, 322.2 kcal and 319.2 kcal) which were significantly higher than *Solanum okadae* in current study.

The tuber is the primary site of demand for photoassimilates during its growth and development. These substances are transported from the synthesis sites (leaves), primarily in the form of sucrose (Fernie *et al*., 2002). An essential quality criterion for potatoes is their sugar content. It has been demonstrated that, even at temperatures below 10 °C, the tuber sugar content can vary by genotype but is largely influenced by storage and treatment (Kumar *et al*., 2004). So that no environmental factor could act as a variable, all the analysed samples in this study were kept at a constant temperature of 4ºC. The highest content of glucose was 0.239 g/100g in *Solanum tuberosum cv Vivaldi* as reported by Morales-Fernández *et al*., (2018) which is approximately 18.46% less than the glucose content of *Solanum okadae* in current study. This research also indicates that *Solanum okadae* could be recommended as a dietary supplement for patients with cardiac disease or those at risk for lipid-induced disorders due to its low-fat content.

4.2 Minerals

Mineral elements are necessary for the structural and functional integrity of the human body; they are also an integral part of many enzymes and play a crucial role in the regulation of metabolism (White & Broadley, 2009). As a result, the mineral composition of potato is a significant factor to consider when assessing its role in the human diet (White *et al*., 2009). Iron and Zinc deficiencies are estimated to affect approximately 60% -30% of the current global population, respectively. Minerals such as Cu, Ca, and Mg are frequently lacking in human diets. Potato cultivars, tuber size and shape, as well as the geographical conditions in which they are grown, may all affect the mineral content of the crop (Vaitkevičienė, 2019).The distribution of different mineral elements throughout the potato tubers is uneven because some elements are concentrated more in the skins than in the flesh, and some studies also reveal a heterogeneous distribution between the stem and distal end of the tubers (Andre *et al*., 2007).

The potassium (K) level was the highest among all the macrominerals measured in both species in current investigation. *Solanum okadae* had a K content of 30.10±1.46 g/kg, whereas *Solanum tuberosum* had a K concentration of 21.50±1.9 g/kg. Potassium is an essential nutrient for acid–base balance, as well as heart, liver, nerve, and muscle function and potato tubers are well-known for their high potassium content. Also, *Solanum tubersoum* K content in this study is comparable to levels of inorganic K (22.83 g/kg) found in *Solanum tuberosum* tubers reported by Wszelaki *et al*., (2005). In the studies conducted by Vaitkevičienė (2019), on different *Solanum tuberosum* cultivars, the K content in Red Emmalie was the highest (24.0 g/kg) while Rosalinde and Highland Burgundy cultivars had the lowest values (19.5g/kg each). The Salad Blue cultivar had similar K concentration (21.3 g/kg) as that of *Solanum tuberosum* in this study. In comparison to foods like bananas, oranges, and broccoli, which are frequently known to be high in potassium, potatoes offer one of the most concentrated sources of potassium, and evidence suggests that they are also one of the least expensive vegetables in the National School Lunch Program (Drewnowski, 2013). Consuming approximately 60g of *Solanum okadae* tubers would fulfil almost 50% of potassium RDA.

Calcium (Ca) is essential for skeletal and neural health, as well as metabolic processes. In the current study, the Ca content in *Solanum okadae* was almost 5 times more than *Solanum tuberosum* 'Celandine'. The Ca content ranged from 0.66 to 1.10 g/kg in various cultivars of *Solanum tuberosum* analysed by Vaitkevičienė (2019), with cv. Valfi having 0.66 g/kg Ca which is similar to *Solanum tuberosum* in present study. Saar-Reismaa *et al*., (2020) also

reported a Ca concentration of 0.72 g/kg in *Solanum tuberosum*. Magnesium (Mg) performs a variety of functions within the body, such as enhancing the nervous system's signalling and communication, assisting cell division processes, and regulating other minerals such as calcium. On a more fundamental level, Mg also reduces feelings of tiredness and fatigue, increases energy levels, and improves heart health, especially for those with high blood pressure. Potatoes contain a significant amount of magnesium in their skin and flesh and are the most widely consumed form of the nutrient. In this study, the concentration of Mg in *Solanum okadae* was found to be 1.47 ± 0.22 g/kg which was similar to the Mg concentration in *Solanum tuberosum* cv. Neida 28 and Neida 42 (1.45 & 1.44 g/kg respectively) as reported by Zhou *et al*., (2019). Gugala *et al*., (2019), reported a Mg content of 1.13 g/kg and 0.89 g/kg in Balbina and Igra cultivars of *Solanum tuberosum* which is similar to the Mg content (0.83 ± 0.85 g/kg) of *Solanum tuberosum* 'Celandine' in this study.

In terms of microminerals, similar to current investigation, iron (Fe) contents of *Solanum tuberosum* cultivars ranged from 23.96 ppm (Neida 26) to 30.99 ppm (Neida 29) in the studies conducted Zhou *et al*., (2019). Also, the Fe content of *Solanum tuberosum* cv. Vulfi was 23.2 ppm as reported by Vaitkevičienė (2019) and Amoros *et al*., (2020), reported a mean Fe content of 21.75 ± 0.73 ppm in potato groups *Stenotomum* and *Phureja*. The human body needs iron to make oxygen-carrying proteins like haemoglobin and myoglobin, as well as heme enzymes and other iron-containing enzymes that help with electron transfer and oxidation-reduction reactions (Hurrell, 1997). Almost two-thirds of the iron in the body is in the haemoglobin in the circulating erythrocytes, 25% is in an easily accessible iron store, and the last 15% is bound to myoglobin in muscle tissue and enzymes that help with oxidative metabolism and many other cell functions (McDowell, 2003). Consuming 100 g of *Solanum okadae* tuber would fulfil 32.18% of RDA for men over 18 and women over 50 and 18.91% of RDA for women aged 19 to 50.

Zinc (Zn) is crucial for human survival. As a component of the chromatin structure, it is essential for important metabolic processes, including enzyme activation, protein and carbohydrate synthesis, DNA replication, and RNA transcription, despite being required in small amounts (Broadley *et al*., 2007). However, approximately 30% of the world's population suffers from Zn deficiency, which increases the likelihood that individuals will develop infectious diseases, DNA damage, stunted growth, and immunodeficiency (Gibson, 2012). In present study, the Zn concentration of *Solanum okadae* was found to be 21.31± 0.65 ppm. Similar Zn concentrations (20.58 ppm, 19.22 ppm and 19.84 ppm) were reported by Zarzecka

et al., (2016) in 3 different cultivars Cekin, Satina and Tajfun of *Solanum tuberosum* respectively. Potato groups *Stenotomum* and *Phureja* grown by Amoros *et al*., (2020) showed a Zn content of 17.8±0.98 ppm and 19.2±1.0 ppm when grown in Acco and Huancayo regions of Peru respectively. Vaitkevičienė, (2019) reported the highest Zn content of 18.3 ppm in *Solanum tuberosum* cv. Red Emmalie and the lowest (12.2 ppm & 12.7 ppm) in *Solanum tuberosum* cv. Highland Burgundy & Rosalinde, which is similar to the Zn content (12.98 ppm) of *Solanum tuberosum* 'Celandine' in present study. Potato has an excellent potential for agronomic biofortification due to its high productivity and presence in the diets of the majority of the world's population, as well as its good digestibility (Subramanian, 2011). However, increasing Zn content in tubers is difficult due to the low mobility of this micronutrient in the phloem and the strong adsorption of Zn to soil constituents, making post-harvest fortification an option to consider (Dimkpa & Bindraban, 2016).

Copper (Cu), a vital micromineral, occurs naturally in some foods and is also available as a nutritional supplement. It is a cofactor for several enzymes (called "cuproenzymes") involved in energy production, iron metabolism, activation of neuropeptides, connective tissue synthesis, and neurotransmitter synthesis. Cu is also involved in numerous physiological processes, including angiogenesis, neurohormone homeostasis, gene expression regulation, brain development, pigmentation, and immune system function (Collins, 2020). In addition, the defence against oxidative damage is largely dependent on superoxide dismutases containing copper (Allen & Klevay, 1994). In present study, there was no significant difference between the Cu content in *Solanum okadae* (4.17± 1.58 ppm) and *Solanum tuberosum* 'Celandine' (4.10 ± 1.58 ppm). Similar Cu contents were reported by Vaitkevičienė, (2019) in *Solanum tuberosum* cv. Highland Burgundy (4.55 ppm), Violetta (4.15) and Red Emmalie (4.01 ppm). According to Zhou *et al*., (2019), the Cu concentrations varied within a narrow range, from 3.70 to 5.45 ppm for *Solanum tuberosum* cv. Neida 26 and Neida 3, with a mean of 4.62 ppm. Saar-Reismaa *et al*., (2020), reported a Cu concentration of 4.11 ppm in *Solanum tuberosum* (crossbreed Blue Congo and Desiree) which agrees with the findings of this study. Contrary to the findings of this investigation, Zarzecka *et al*. (2016) reported Cu concentrations (6.189 ppm, 6.22 ppm, and 6.19 ppm) in *Solanum tuberosum* cv. Cekin, Satina, and Tajfun respectively.

Manganese (Mn) is a crucial trace element that can be found as a dietary supplement and naturally in many foods. Many enzymes, such as manganese superoxide dismutase, arginase, and pyruvate carboxylase, require manganese as a cofactor (Li & Yang, 2018). Manganese is

involved in bone formation, reproduction, immune response, and the metabolism of amino acids, cholesterol, glucose, and carbohydrates through the action of these enzymes (Chen *et al.,* 2018). In current investigation, *Solanum okadae* had a Mn concentration of 4.52 ± 0.03 ppm whereas that of *Solanum tuberosum* was 1.32 ± 0.03 ppm. Zhou *et al.*, (2019) found that, *Solanum tuberosum cv Neida 3*, had 14.95 ppm of Mn which is 30.23% higher than the Mn content of *Solanum okadae* in this study. As per Saar-Reismaa *et al*., (2020), Mn concentrations in Blue Congo and Granola crossbreed tubers ranged from 5.3 ppm to 12.0 ppm which is also high compared to results of this study. Also, Vaitkevičienė, (2019), reported a range of Mn concentrations from 4.93 ppm to 10.1 ppm in various cultivars of *Solanum tuberosum*. All these findings suggest *Solanum okadae* might not be a rich source of Mn.

Molybdenum is an important trace element that is found in many foods and is also sold as a supplement. Molybdenum is part of molybdopterin, which is a cofactor made by the body and needed by four enzymes to work: sulphite oxidase, xanthine oxidase, aldehyde oxidase, and mitochondrial amidoxime reducing component (mARC). These enzymes break down amino acids that have sulphur in them and heterocyclic compounds, like purines and pyrimidines (Klein *et al*., 2006). Drugs and toxins are also broken down by Xanthine oxidase, Aldehyde oxidase, and mARC (Terao *et al*., 2016). Chaparro *et al*., 2018, found that their advance line of *Solanum tuberosum* yellow market class (variety: CO07131-1W/Y) had the highest Mo concentration (0.435 ppm) similar to *Solanum okadae* Mo content in this study.

Selenium is a component of over two dozen selenoproteins that play crucial roles in reproduction, thyroid hormone metabolism, DNA synthesis, and protection against oxidative damage and infection (Alexander, 2015). Whereas cobalt, which is a part of vitamin B12, is reserved in the liver. It is crucial to the healthy growth of red blood cells. Additionally, it contributes to the formation of myelin nerve coverings and is involved in a number of enzyme reactions (Winter., 2000). The Co and Se concentration of *Solanum okadae* was 0.34 ± 0.01 ppm and 1.09 ± 0.27 ppm respectively, whereas that of *Solanum tuberosum* was 0.14 ± 0.0 ppm and 1.00 ± 0.09 ppm respectively. Chaparro *et al*., 2018, found that their advance line of *Solanum tuberosum* yellow market class (variety: CO99045-1W/Y) had a Co concentration of 0.328 ppm, which is similar to this study. Also, their variety CO05228-4R variety of red market class had a Se content of 0.36 ppm.

4.3 Vitamins

Most of the vitamin C (L-ascorbic acid) we consume comes from plants. The molecule is widely recognised as one of the most important antioxidants in living cells, as it helps to detoxify a broader range of reactive oxygen species (ROS), including hydrogen peroxide, hydroxy radicals, superoxide anion radicals, singlet oxygen, and peroxy radicals (Kazmierczak-Baranska *et al*., 2020).The USDA's SR21 database says that a medium red potato (173 g) has about 36% of the RDA and based on the cost to provide 10% of the RDA, potatoes were the least expensive source of vitamin C (Drewnowski and Rehm, 2013). In addition to absorbing vitamin C from leaves and stems, tubers can also synthesise vitamin C (Tedone *et al*. 2004). The high vitamin C content of potatoes may also improve the body's ability to absorb iron, a mineral for which there is a widespread problem (Cook and Reddy 2001).

Freshly harvested potato tubers contain between 10-25 mg of vitamin C per100 g of fresh material (FM), according to Brown (2005) which was in agreement with the vitamin C content in *Solanum okadae*. Significantly lower figures were reported by Tudela *et al*. (2002), who determined the vitamin C content to be 6.2 mg/100g potatoes which is similar to the vitamin C concentration in *Solanum tuberosum* in this study. Hrabovská *et al*., 2013, reported the highest vitamin C content (8.10 mg/100g) in Sipnela variety of potato tubers whereas Hamouz *et al*., 2018 and Love *et al*., (2004) found 12.6 mg/100g and 11.9 mg/100g of vitamin C in *Solanum tuberosum cv. Herbie* and A8792-11 respectively. The amount of vitamin C in potato cultivars varies greatly, with measurements from 21.77–68.95 mg/100 g and ~22 mg to up to 122 mg vitamin C /g in various studies (Andre *et al*., 2007, Wu *et al*., 2020). This highlights the genetic potential for breeding high vitamin C varieties and illustrates the marketing potential for specific potato varieties with high vitamin C contents and their nutritional value.

Vitamin C functions as an antioxidant in the body (FAO, 2008), which plays a crucial role in protecting against oxidative stress (Bates, 1997). It is also necessary for several biosynthetic processes, including the synthesis of collagen and carnitine, the amidation of some peptide hormones, and the metabolism of tyrosine (Kumar *et al*., 2016). Potatoes are one of the most significant sources of vitamin C, but its importance in human nutrition is frequently undervalued (Finley *et al*., 2003). Potato can provide adequate vitamin C to consumers daily. Given the significance of this vitamin to the human metabolism and health, the crop is a

valuable source of this vitamin. However, there is also a good possibility of biofortifying this essential phytonutrient in the tuber through breeding efforts or genetic engineering.

Vitamin B12, a water-soluble vitamin, can be found in some foods naturally, added to others, and consumed in the form of a supplement or prescribed medication. Compounds with vitamin B12 activity are collectively known as "cobalamins" due to vitamin B12's mineral cobalt content (Watanabe *et al*, 2014). It is the only vitamin known to be lacking in plant-based meals, placing vegetarians and vegans at danger of megaloblastic anaemia due to vitamin B12 deficiency. (Ghifari, 2021). The main dietary sources of vitamin B12 are foods made from animals (meat, milk, eggs, fish, and shellfish). Essential functions of the nervous system, including myelination and nerve conduction, red blood cell formation, and DNA synthesis, are all reliant on vitamin B12. Methionine synthase and L-methylmalonyl-CoA mutase are two enzymes that rely on vitamin B12 as a cofactor (Frey, 2010). To produce the essential amino acid methionine, homocysteine must be converted by the enzyme methionine synthase. Methionine is used to make S-adenosylmethionine, a universal methyl donor for nearly 100 different substrates (Marriott, 2020). These substrates include DNA, RNA, proteins, and lipids. In the metabolism of the short-chain fatty acid propionate, the enzyme L-methylmalonyl-CoA mutase converts L-methylmalonyl-CoA to succinyl-Co (Takahashi-Iñiguez *et al*., 2012).

The high vitamin B12 content in *Solanum okadae* in present investigation could be attributed to its high cobalt content (0.34 ± 0.01 ppm), as one of the elements that makes up vitamin B12 is cobalt (González-Montaña *et al*., 2020). The Swedish National Food Administration examined the nutritional value of six potato varieties during the 2009–2010 period, including the Swift, Solist, Asterix, Inova, King Edward, and Almond varieties. Neither variety contained any Vitamin B12 which was in accordance with the United States Department of Agriculture (USDA) and official Diet and Fitness Today website [Accessed 08 July 2022: http://www.dietandfitnesstoday.com/vitamin-b12-in-potatoes.php], which contradicts the findings of this study. Adults (aged 19 to 64) require approximately 1.5µg of vitamin B12 per day (NHS, UK), therefore consuming only 0.5g of *Solanum okadae* would fulfil 100% vitamin B12 RDA.

In the current investigation, for the first time we found a significantly higher quantity of vitamin B12 in *Solanum okadae* compared to any other cultivated potato variety. Commercial potato varieties are known for their rich vitamin C and B6, and poor vitamin B12 content. In contrast,

Solanum okadae has a higher vitamin B12 content along with significant amount of vitamin C making it an ideal choice for vegans and vegetarians to derive their vitamin B12 from.

4.4 Amino acids

An essential amino acid (EAA) is one that cannot be synthesised by the organism quickly enough to meet its demand and must therefore be obtained from the diet. The nine amino acids that humans cannot synthesise are phenylalanine, valine, threonine, arginine, methionine, leucine, isoleucine, lysine, and histidine, which are found in all living things (USDA, 2014). The oxidation of other AAs increases gradually with increasing dietary intake of AAs or protein when an EAA is deficient in a diet. This is because the limited availability of this EAA prevents other AAs from being used for protein synthesis, leading to the tissue-specific degradation of all excess AAs. Low appetite and vomiting are symptoms of EAA deficiency, as well as problems with nutrient absorption, transport, and storage, decreased neurotransmitter synthesis, emotional disorders (such as irritability, severe depression, and anxiety), irritability and insomnia, anaemia, and impaired oxygen transport (Wu, 2017).

The leucine content of *Solanum okadae* (10.67g/16g N) in this study was more than that of *Solanum phureja* (4.95 g/16 g N) and *Solanum stenotomum* (4.79 g/16g N) as reported by Bartova´ *et al*., (2015). Also, like the content of lysine content in *Solanum okadae* (10.38 g/16g N) in this study, they discovered a comparable lysine content in *Solanum phureja* (10.49 g/16g N) and in contrast, a lower lysine content in *Solanum stenotomum* (8.55 g/16g N) was reported by them. The lysine and leucine contents of *Solanum tuberosum cv. Desiree* quantified by Bartova´ *et al*., were almost 1.4 and 3.28 times lower compared to *Solanum okadae* in present investigation. Bartova´ *et al*., also reported significantly lower concentrations of other EAAs like valine, threonine, histidine, isoleucine, methionine, and phenylalanine in *Solanum stenotomum*, *Solanum phureja* and *Solanum tuberosum cv. Desiree* when compared to *Solanum okadae* in present study. Ježek *et al*., (2011), found the leucine and lysine concentration to be 3.67 g/kg and 3.14 g/kg respectively in *Solanum tuberosum* variety *Karin* which were lower to those in *Solanum okadae* in current investigation. Mushinskiy *et al*., (2021) reported the highest lysine (4.5 g/16g N) and leucine (9.8 g/ 16g N) content in *Solanum tuberosum* '*Nevsky*' contradicting the findings of this study. Amongst various British and Irish grown *Solanum tuberosum* varieties tested by Davies, (1977) for amino acid content, the highest content of leucine was found in variety Duke of York (0.76 g/kg) and that of lysine was found in variety Desiree (1.12 g/kg). Kowalczewski *et al*, 2019, reported the leucine and lysine content in

Solanum tuberosum to be 9.06 g/16g N and 8.33 g/16g N respectively, which was almost 3.35 and 4.38 times more than the leucine and lysine content found in *Solanum tuberosum* 'Celandine', but approximately 1.18 and 2 times less than the leucine and lysine content found in *Solanum okadae* in this study.

It had long been assumed that NEAA are synthesised in sufficient quantities in animals and humans to meet the requirements for maximum growth and health (National Research Council, 2012). This assumption, however, is not supported by experimental data. While it is known that all organisms have metabolic requirements for all proteinogenic and other physiologically important AA, animal production and human health have largely ignored the requirements for dietary NEAA. Mammals, birds, and fish have dietary needs for all NEAA for optimal growth, development, lactation, reproduction, and health, based on recent advances in AA biochemistry and nutrition and this new nutritional paradigm shift has led to the recognition of the dietary importance of "nutritionally non-essential AA" for both humans and animals (Hou *et al*., 2015).

Ohara-Takada *et al*., (2005), reported a very low concentration of aspartic acid (0.14 g/kg) and glutamic (0.57 g/kg) in *Solanum tuberosum* when compared to that of *Solanum okadae* in this study. The highest concentration of aspartic acid (0.12 g/kg) and glutamic acid (0.137 g/kg) was reported in *Solanum tuberosum cv. Neida 42* and *Neida 11* respectively by Zhou *et al*., (2019). Also, Kowalczewski *et al*., (2019), reported the aspartic acid and glutamic acid content in *Solanum tuberosum* to be 12.74 ±0.52 g/16g N and 11.22 ±0.55 g/16g N respectively which was approximately 30% and 27% less than aspartic and glutamic acid content estimated in *Solanum okadae* but 42% and 33% higher than the aspartic and glutamic acid content estimated in *Solanum tuberosum* 'Celandine' in this study. Similarly, in *Solanum tuberosum* variety Epicure, Borsook *et al*., (1958), had reported the lower aspartic and glutamic acid values (11.3 and 13.0 g/16g N respectively) when compared to those of *Solanum okadae* (18.07 and 15.44 g/16g N respectively) in present study. On the other hand, diploid potato species like *Solanum phureja* and *Solanum stenotomum* had similar aspartic acid (18.05 and 17.04 g/16g N) and glutamic acid (13.63 and 12.93 g/16g N) content when compared to *Solanum okadae* in this study (Bartova´ *et al*., 2019). Interestingly, in the studies conducted by Bartova´ *et al*., (2019), the alanine, glutamic acid, serine, and tyrosine contents were less in *Solanum tuberosum 'Desiree'* compared to *Solanum okadae* in present studies. Interestingly, Ježek *et al*., (2011), found the aspartic and glutamic acid concentration to be 9.01 g/kg and 7.74 g/kg in *Solanum tuberosum* variety *Karin* which were similar to those in *Solanum okadae* in present investigation.

Leucine and lysine are the two main free amino acids studied in potato research when it comes to essential amino acids, while glutamic acid and aspartic acid are primarily studied when it comes to non-essential amino acids (Wichrowska & Szczepanek, 2020). In terms of tuber protein quantity and quality, non-traditional potato species grown in some parts of South America has promising potential for breeding programmes as well as for human nutrition. Present study validated the importance of *Solanum okadae* in terms of its essential amino acid as well as non-essential amino acid content. This indicates that this species may be useful for potato breeding programmes and human nutrition. This species also exhibited a significant difference in EAA and NEAA content in tubers compared not only to other diploid potato species tested, but also to the widely cultivated *S. tuberosum* cultivar Desiree.

5. Conclusion

The metabolic complexity of tubers and how it relates to their varied phytonutrient content will be known to a plant biochemist. The perceived nutritional value of a food may be becoming more significant in societies where there is an abundance of food and consumers are becoming more health conscious. According to a consumer survey conducted by the Oregon Potato Commission, appearance, flavour, and nutritional value were the three main factors influencing consumers' decisions to buy fresh-market potatoes.

While potatoes already have high levels of vitamins, minerals, and phytonutrients, future cultivars can be improved in many ways, including nutritional content, flavour, and appearance. In the current study, among the two species tested for nutritional value (*Solanum okadae* and *Solanum tuberosum* 'Celandine'), and when compared to other cultivated potato varieties according to various studies, *Solanum okadae* can be considered promising as parental resources to breed new cultivars for nutritional traits such as higher carbohydrate and vitamin B12 content. Thus, providing health benefits for a variety of consumers in the form of essential microelements, amino acids and overall immune system and health improvement.

CHAPTER 3: *In vitro* screening for salt tolerance in *Solanum okadae* and the impact of melatonin in alleviating severe salt stress.

1. Introduction

Salinity is a significant environmental stress factor that frequently poses a serious obstacle to the production of crops (Peethambaran *et al*. 2018). Additionally, it is predicted that increasing salinization of cultivatable land will have disastrous global effects, leading to a 30 percent loss of land within the next 25 years and up to a 50 percent loss by the middle of the 21st century (Wang *et al*. 2003). According to estimates from the United Nations Environment Program, 50 percent of cropland and 20 percent of agricultural land worldwide are both under salt stress (Yokoi *et al*. 2002). The most common salt that cause toxicity and are connected to saline soils is NaCl. Salinity is a serious issue that affects the productivity and growth of many crops by altering the metabolic processing, causing decreased stomata conductance and respiration, decreased water potential, ion imbalances, and toxicity of specific ions (Wahid *et al*., 2007). Salinity negatively impacts plant growth via two physiological challenges: osmotic stress and ion toxicity (Munns &Tester, 2008). Osmotic stress reduces plant growth due to decreased water potential, while sodium ions (Na^+) congest to a toxic concentration that decreases potassium ion absorption. These obstacles disrupt a variety of biological processes, including physiological characteristics and enzyme activity, in plants (Fricke *et al*., 2004). The maintenance of a low Na^+ concentration and a normal range of K^+/Na^+ ratio in the cytoplasm of higher plants is required to increase plant salt tolerance (Zhang *et al*., 2014).

In comparison to other crops, potatoes are considered to be moderately salt sensitive because their tuber yield may suffer at field salinity EC levels of 2.0–3.0 dSm-1 (roughly 22–33 mM NaCl) (Maas and Hoffman, 1977). During the reproductive phase, salinity stress can disrupt the normal K^+/Na^+ ratio in the leaves, have a negative impact on the ultrastructure of the leaves, and reduce photosynthesis in the leaves (Levy & Veilleux, 2007). Also, the movement of carbon from the vegetative tissues to the tubers is slowed down, along with the number and size of tubers, when the K^+/Na^+ ratio in potato leaves is abnormal (Li *et al*., 2019). Salinity has a significant impact on potato tuber growth and may significantly lower tuber quality (Jha *et al*., 2017). Reactive oxygen species (ROS), which are toxic to plant cells at high concentrations, are produced by salinity, which increases ionic stress, osmotic stress, and salt stress in plant cells. One of the main mechanisms that results in tolerance against salt stress is osmotic

adjustment (Bündig *et al.*, 2017). Under *in vitro* and in vivo conditions, the potato is one of the moderately sensitive crops to salinity, and the salinity stress has significant and disruptive effects on potato tuber production.

Abiotic stresses like salinity are a complex traits, making breeding for them challenging. There are many instances where attempts are made to create potato varieties that can withstand salt (Londhe, 2016). However, there hasn't been much effort put into creating potato cultivars that can withstand salt. The complexity of both genetic and physiological traits accounts for the relatively low success rate (Chourasia *et al.*, 2021). The mechanism of tolerance in potatoes exhibits all the traits of a quantitative trait, leading to the development of various tolerance levels under various environmental circumstances (Velásquez *et al.*, 2005). There have been several observations of differences in salt sensitivity between cultivated and wild species, but no comprehensive comparison of cultivated and wild species has been reported (Zaki & Radwan, 2022).

Higher osmotic pressures in saline soils inhibit plant development and cause plant death (Tuteja, 2007). Numerous researchers are diligently investigating biological responses to salt stress to prevent salt injury. Exogenous use of cellularly recognised molecules, such as betaines, proline, and antioxidants, to resist stressors is one strategy (Lopez & Satti, 1996). Melatonin (MT) improves salt tolerance in a variety of plants, including maize, wheat, cucumber, tomato, cotton, and rice (Liang *et al.*, 2015; Zhou *et al.*, 2016; Ke *et al.*, 2018; Zhang Y. *et al.*, 2021). Salt stress has been shown to increase endogenous melatonin levels and alter the expression patterns of crucial melatonin biosynthetic enzymes (Arnao and Hernández-Ruiz, 2019).

Exogenous melatonin application controls antioxidant enzyme expression and activity, polyamine metabolism, and NO signalling to protect plants from salt stress (Zhan *et al.*, 2019). Major antioxidant enzymes are activated, which lowers ROS and H_2O_2 levels brought on by salinity thus enhancing photosynthetic efficiency, protecting the photosynthetic system, and enabling growth under salt stress. Melatonin improves plant growth under salt stress by increasing photosynthetic efficiency, K^+ influx, and Na^+ efflux, decreasing ROS production, enhancing antioxidant activities, and accumulating compatible solutes. Therefore, the application of MT exogenously can reduce salt stress in plants.

It is possible to use genetic variation in salt tolerance within and between plant species to screen and choose breeding candidates (Epstein and Rains, 1987). Since the whole plant response to salinity stress is complex and depends on environmental conditions (such as season, light, climate, and soil type), conventional field trials are not only time- and labour-intensive but also challenging to replicate (Siddique *et al*. 2014). Choosing and utilising salt-tolerant crop plants is the most efficient way to address soil salinity issues (Ashraf and Wu, 1994). To identify varieties that can be grown in highly saline soil conditions or to utilise salt-tolerant genotypes in breeding programmes, it is crucial to screen cultivated and wild species for their salt tolerance. *In vitro* conditions can provide quicker and more accurate assessments of plant growth under saline stress considering the environmental variations in conventional field trials (Banu *et al*., 2014). A diploid potato *Solanum chacoense Bitt*, known as the most prevalent, aggressive, and adaptable wild potato in South America with resistance to more than 20 pests and diseases, recently underwent an *in vitro* evaluation to determine the extent of genetic variability for salinity tolerance and its clones 'A-6', 'C-8', and 'D-2' had the highest salt tolerance overall (Zaki & Radwan, 2022). They also determined whether exogenous applications of the antioxidants and osmoregulators could reduce salt stress injury in the genotypes of potatoes under study. There are limited researches about effect of salinity on potatoes *in vitro* (Ahmed *et al*, 2020), and no known studies regarding the effect of melatonin on salt stressed potato plants. Therefore, this study aimed to evaluate the salt tolerance of another important wild diploid potato species, *Solanum okadae in vitro* and observe the effects of exogenously applied melatonin on salt stressed plantlets.

2. Materials & Methods

2.1 Plant material and culture conditions

Sterile genetic stocks of *Solanum okadae* (discussed in chapter 4) growing in the magenta boxes kept in controlled growth rooms at 22±2 ºC, and a 16-h photoperiod and 2500 lux light intensity were used in this experiment.

For screening, five salt stress treatments were used. Treatment zero (T_0) was a control (without NaCl), while treatments T_1, T_2, T_3 and T_4 represented stress conditions induced by the addition of 50mM, 100mM, 150mM and 200mM NaCl, respectively, to MS medium containing 30 g/L sucrose and 0.8% agar. To examine melatonin’s effect on potato plantlets grown under severe salt-stressed conditions (200mM NaCl), only one treatment was used; T_{SM} (with 200mM NaCl and 100µM melatonin) (Ahmed *et al*., 2020).

Before all the media were autoclaved for 20 minutes at 120°C, the pH was adjusted to 5.8. To evaluate salt tolerance and effect of melatonin on salt stressed plantlets, 1.5-2.0 cm long stem cuttings with one or two axillary buds were prepared by excluding the basal and apical portions of the plantlets and used as explants. Five explants were inoculated in the magenta box, each with about 30 ml of the growing medium. For evaluation, a total of 30 explants in 6 boxes were considered for each treatment. Before measuring root and shoot related morphological traits, the explants were cultured for 30 days at a constant temperature of 22±2ºC, and a 16-h photoperiod with a photosynthetically active photon flux density of approximately 2500 lux.

2.2 Measurement of Morphological Traits

When the plantlets were 30 days old and fully grown with robust stems and broad leaves in the control treatment (T_0), all the plantlets were carefully removed from the magenta boxes and rinsed gently with sterile distilled water to remove excess agar and blotted dry on a tissue paper.

The following data were recorded for various morphological characteristics:

I. Shoot height (cm): This was determined by measuring the length of the stem from the base to the tip of the shoot.
II. Number of shoots
III. Root length (cm): This was determined by measuring the length of the primary root from the point of root emergence to the tip of the root.
IV. Number of roots
V. Number of leaves
VI. Fresh weight (FW, g)
VII. Dry weight (DW, g): This was measured after fresh samples were dried for 72 hours in a drying cabinet (oven) set at 70°C (Schafleitner *et al.*, 2007).
VIII. Plant water content (PWC %): The percent PWC was calculated using the equation: PWC, % = (FW – DW) / FW × 100 (Rashid *et al*, 2019).

2.3 Analysis of Leaf Photosynthetic Pigments

Estimation of photosynthetic pigments was done according to the method described by Arnon, (1949). 100mg of leaf sample was crushed in 10ml of 80% acetone using mortar and pestle. This mixture was transferred to a 15ml falcon tube and centrifuged at 5000rpm for 5 mins in a centrifuge (Pico 21, Thermo Scientific). The supernatant was carefully transferred to a new

15ml falcon tube following the absorbance of the solution at 480nm, 645nm and 663nm against the blank (acetone) using a spectrometer (CE 2041 UV/VIS, Buck Scientific, Inc.).

The concentrations of chlorophyll a, chlorophyll b and total chlorophyll and carotenoid were calculated using the following equations:

- Total Chlorophyll: $20.2(A_{645}) + 8.02(A_{663})$
- Chlorophyll a: $12.7(A_{663}) – 2.69(A_{645})$
- Chlorophyll b: $22.9(A_{645}) – 4.68(A_{663})$
- Carotenoid: $[A_{480} +(0.114(A_{663}) - (0.638-A_{645})] \times V/1000\times W$

2.4 Proline determination

Proline was colorimetrically determined according to the studies carried out by Marín *et al.*, (2009) based on proline's reaction with ninhydrin reagent. 500mg of leaf tissues were homogenised in 2 ml of 3% sulfosalicylic acid. This solution was centrifuged at 1500g for 10 mins in a centrifuge (Pico 21, Thermo Scientific). The supernatant was mixed with 8% acidic ninhydrin and glacial acetic acid (1:1:1 v/v/v) and heated to 100°C for one hour in a water bath. The reaction was stopped using an ice bath, and the chromophore was extracted with 4 ml of toluene. After the solution cooled to room temperature, a spectrophotometer (CE 2041 UV/VIS, Buck Scientific, Inc.) was used to measure the chromophore's absorbance at 520 nm. The method was calibrated for each determination with standard L-proline solutions within the detection range of the method (0-100 µg/ml).

2.5 Antioxidant enzyme assay

2.5.1 Preparation of crude protein/enzyme extract

200mg of leaf tissue were powdered in a mortar & pestle using liquid nitrogen. This leaf powder was homogenised by addition of 3 ml of 100mM PBS buffer (pH 7.8). The homogenate was the transferred to 1.5ml centrifuge tubes and centrifuged at 10,000x g for 20 min at 4 °C. The supernatant was transferred to new centrifuge tubes and kept on ice. The concentration of crude protein (mg/ml) in the supernatant was measured using Nano-Drop Spectrophotometer (ND-1000 Spectrophotometer, NanoDrop Technologies, Inc., and Wilmington, USA) and the following equation:

Protein concentration (mg/ml) = $1.55 \times A_{280} - 0.76 \times A_{260}$ (Simonian and Smith, 2006)

In this protocol, all solutions—aside from NaH_2PO_4 and Na_2HPO_4 stock solutions—were freshly made on the day of the assay and kept at room temperature. All samples were kept on ice or at 4 °C during crude protein/enzyme extraction.

2.5.2 Catalase (CAT) activity assay

Reaction solution needed for this assay was prepared by mixing 39µl of 30% H_2O_2 in 25ml of 100mM PBS buffer (pH 7.8). A spectrophotometric cuvette was filled with 50µl of crude enzyme before being placed inside the holder. WPA S2000 Lightwave UV/VIS spectrophotometer was then used to record the absorbance at 240 nm immediately after adding 1 ml of the reaction solution to the cuvette. For one minute, the absorbance was measured every 15 seconds to look for a steady average change. Reaction solution with 50µl 100mM PBS (pH 7.8) was used as blank. The CAT activity was calculated using following formula (Chen & Zhang, 2016).

CAT activity (unit: u/mg protein) = $\triangle A_{240}$ x $(V/V_t)/(0.1 \times t)/C_p$

- $\triangle A_{240}$: the change of absorbance at 240 nm during every 15 sec
- V: total volume of crude enzyme solution
- V_t: volume of crude enzyme used in the testing tube
- t: reaction time (min)
- C_p: crude protein concentration (mg/ml)
- 0.1: One unit of CAT is defined as the amount of enzyme that decreases 0.1 of absorbance at 240 nm per minute.

2.6 Detection of Hydrogen Peroxide by DAB Staining

Using an adaptation of earlier techniques staining (Daudi *et al*., 2012), hydrogen peroxide (one of several reactive oxygen species) was detected *in situ* in leaves by 3,3′-diaminobenzidine (DAB) stain.

2.6.1 DAB staining solution preparation

50mg of DAB was added to 45ml sterile water in a 50ml beaker for a final 1 mg/ml DAB solution. 25µl Tween 20 (0.05% v/v) and 2.5 ml 200mM Na_2HPO_4 was added to the beaker and the solution was stirred till its pH was 3.0 with 0.2 M HCl (to dissolve DAB). The beaker was covered with aluminium foil due to the light sensitivity of DAB. The DAB solution

remains active only for the day, and therefore was made fresh every time the assay needed to be performed.

2.6.2 Staining leaves with DAB

Leaves were carefully picked using plastic forceps to avoid any damage to the tissue and transferred into a 1.5ml centrifuge tube. 2ml of DAB solution was added to this tube ensuring that the leaf samples were completely immersed in the staining solution. To ensure that the DAB solution was taken up by leaves, the tubes were subjected to a gentle vacuum infiltration for 30 mins. The tubes covered in aluminium foil and transferred to a shaker for 1 h at 80–100 rpm shaking speed. Following incubation on shaker, the DAB staining solution was pipetted out of the tubes and the leaf samples were immersed in bleaching solution (ethanol: acetic acid: glycerol, 3:1:1 v/v/v). The tubes were then carefully placed in a boiling water bath for 30 mins to bleach out chlorophyll and keep the brown precipitate formed by DAB stain reacting with H_2O_2. The bleaching solution in the tubes was replaced with fresh bleaching solution after boiling and allowed to stand for 10 mins. Leaves were then mounted on clean glass slides and covered with coverslip and visualized under microscope (LAS AF, Leica Microsystems) on a plain white background under uniform lighting.

2.7 Elemental profiling using ICP-MS

The ionomics analysis of samples was carried out as described (Danku *et al.*, 2013). Briefly, 1g of leaf sample was washed with deionised water and transferred into Pyrex test tubes (16 × 100 mm) and dried at 80 °C for 24 h. After the appropriate number of samples had been weighed, the tubes were filled with trace metal grade HNO_3 (Fisher Chemicals) mixed with indium internal standard (1 mL per tube). The samples were then digested for 4 hours at 115°C in a dry block heater (DigiPREP MS, SCP Science; QMX Laboratories, Essex, UK). Using 18.2Mcm Milli-Q Direct water, the digested samples were diluted to 10mL. Elemental analysis was carried out using an ICP-MS, a PerkinElmer NexION 2000 equipped with an Elemental Scientific Inc. auto-sampler and the Syngistix software in collision mode (He). Four elements (Na, K, Mg, & Ca) were monitored. Before the sample run started, a liquid reference material made up of pooled samples was prepared, and it was used for the duration of the sample run. The calibration standards (with blanks and indium internal standard) were made from solutions of single element standards sold by Inorganic Ventures and Essex Scientific Laboratory Supplies Ltd, respectively. In the instrument's software, sample concentrations were calculated using an external calibration method. Using Microsoft Excel spreadsheet, additional data

processing was performed. The concentrations of minerals in tubers were expressed in ppm on a dry weight basis.

2.8 Salt Tolerance Trait Index (STTI) & Salt Tolerance Index (STI)

To evaluate the limit of salt tolerance (i.e., maximum salt concentration in which plantlet could survive) and check the effect of melatonin on stressed plantlets, average of each trait (morphological and biochemical) as STI was estimated using the following equation (Heon Kim *et al*, 2018):

$$\mathrm{STTI} = \frac{\text{Value of trait under stressed condition}}{\text{Value of trait under no stress}} \times 100$$

$$\mathrm{STI} = \text{The mean of STTIs}$$

2.8 Statistical analysis

All trials were conducted in a randomised fashion and each test was performed in triplicate. Data were presented using the mean±standard deviation (SD). Using SPSS software (version 21.0, SPSS Inc., Chicago, USA), an analysis of variance (ANOVA) was used to determine differences between samples. The significant difference between the variables was evaluated using the Duncan's multiple range test ($P<0.05$).

3. Results

3.1 Morphological parameters

In this study, as the NaCl concentration in the MS medium increased, morphological parameters of the plantlets declined, resulting in a low water potential. At 50 and 100mM NaCl, plants produced stems and leaves with no evidence of leaf necrosis. Only at 150mM NaCl were morphological differences observed, whereas at 50mM NaCl plant responses were comparable to the control (Figure 2). Under extreme water stress conditions induced by 200mM NaCl, all plantlets produced fewer shoots and roots and in some cases none at all. Thus, the proposed *in vitro* system for evaluating the effect of melatonin against salt stress could only be used at a severe stress (200mM NaCl).

All NaCl concentrations tested had a statistically significant impact on the variations in plantlet length, number of shoots, number of leaflets, number of roots, root length, fresh plantlet weight, and dry plantlet weight (Table 1). With increasing NaCl concentration, the shoot lengths of all plantlets significantly decreased. The control plantlets had the longest shoot length (7.3±0.3 cm) and minimum shoot length (1.2 cm) was observed at 200mM NaCl. The shoot length of

plantlets at 50mM NaCl (7.2 cm) was unaffected by salt. Additionally, all plantlets shoot lengths were significantly impacted by a 100-200mM NaCl concentration.

Table 1: Effect of various salt stress treatments on different morphological parameters of *Solanum okadae*

NaCl (mM)	FW (g)	DW (g)	PWC (%)	Number of leaflets	Number of shoots	Shoot length (cm)	Number of roots	Root length (cm)
0	2.2±0.5[a]	0.1±0.06[a]	85.3±0.87[a]	9.5±4.3[ab]	11.1±4.9[a]	7.3±0.3[a]	3.5±0.7[b]	1.3±0.0[c]
50	1.5±0.9[b]	0.1±0.02[a]	85.8±1.54[a]	6.6±2.1[a]	4.6±4.0[b]	7.2±0.4[a]	3.4±1.5[a]	1.4±0.1[bc]
100	1.3±1.9[bc]	0.08±0.02[bc]	85.9±2.85[a]	5.8±0.9[ab]	3.3±3.7[b]	2.3±0.2[d]	2.0±1.0[b]	1.7±0.2[a]
150	1.1±0.7[bc]	0.06±0.02[bc]	86.2±2.28[a]	4.3±2.0[ab]	0.3±0.4[c]	1.9±0.7[d]	0.0[c]	0.0±0.0[d]
200	0.8±0.2[c]	0.03±0.01[ab]	86.4±1.97[a]	3.3±1.7[b]	0.00±0[c]	1.2±0.3[d]	0.0[c]	0.0±0[d]
200mM NaCl + 100µM MT	0.9±0.3[c]	0.10±0.01[bc]	85±1.86[a]	6.3±2.9[ab]	1.2±1.1[bc]	3.4±0.2[b]	1.1±0.2[c]	1.1±0.1[ab]

All values represent the means ±S.E. Different letters within a column indicate significant differences at $P< 0.05$ by Duncan's multiple range test

As salt concentration increased, leaflet formation decreased. When the means of the number of leaflets of the plantlets, excluding control plantlets, were evaluated at all salt concentrations, T_1 had the highest number of plant leaflets (6.67), while T_4 had the lowest number (3.33). Increased salt levels also led to a reduction in root length and number. The longest roots were measured in the control (3.50 cm) treatment, whereas the shortest roots were measured in the 200mM NaCl treatment. 50mM NaCl had no effect on the number of roots in when compared to control plantlets. The values of root length fluctuated between 0 and 1.80 when the mean root lengths of the plantlets, including control plantlets, was determined at each salt concentration. In control, the greatest number of root length was determined whereas T_3 and T_4 yielded the lowest root length (0 cm) compared to control. Table 1 also shows the effect of salinity stress on plantlet water content (PWC) after 4 weeks of growth. Apart from a slight increase in PWC values of plantlets in salt stress of 150 and 200mM, there was no significant change in PWC values of plantlets in any treatment. PWC % was 85.38% under control (no

stress); however, under stress conditions, PWC% varied from 85.83% (50mM) to 86.46% (200mM).

Figure 2: Effect of NaCl on the development of *in vitro Solanum okadae* shoot cultures. The *in vitro* shoots were cultured onto the MS medium different concentrations of NaCl (0, 50 100, 150 and 200mM).

The MS media with additional melatonin (100µM) was used to assess the effects of exogenous melatonin application on salt stress in the plantlets. Table 1 demonstrates a distinction between plantlets grown in 200mM salt stress with (T_{SM}) melatonin treatment. There was a significant increase in the values of shoot length (62.64%), root length (100%), shoot number (100%) and number of leaves (47.4%) in plantlets treated with melatonin when compared to salt stressed (200mM) plantlets (Figure 3). No significant difference was observed in the values for fresh weight, dry weight, plant water content and number of roots in plantlets treated with melatonin when compared to salt stressed (200mM) plantlets.

3.2 Photosynthetic Pigments

In current study, chlorophyll (Chl) and carotenoid (Car) content in the leaves was found to be significantly impacted by the rising salt stress level on potato plantlets (Table 2). The decrease in chlorophyll a, b and total chlorophyll in all salt stressed plantlets was drastic compared to control. On the other hand, the Car content increased with increase in salt concentration. The highest Car content (4.20±1.12 µg/g FW) was found in plantlets growing in 200mM NaCl. Interestingly, in this study, the chlorophyll a, b and total Chl content in plantlets grown in NaCl

concentration of 150mM was higher (4.86±1.5 µg/g FW, 7.22 µg/g FW, 11.81±3.6 µg/g FW, respectively) when compared to plantlets in other salt treatments.

Figure 3: Effect of 100µM Melatonin on *Solanum okadae* under 200mM (T4) salt stress after, **(A)** 7, **(B)** 15 and **(C)** 30 days.

Table 2: Effect of various salt stress treatments on photosynthetic pigments (µg/g FW) of *Solanum okadae*

NaCl (mM)	Total Chl	Chl a	Chl b	Car
0	37.74 ± 5.36^{a}	14.44 ± 3.2^{a}	23.31 ± 4.63^{a}	1.53 ± 1.12^{a}
50	6.42 ± 2.6^{c}	4.59 ± 1.1^{b}	1.56 ± 0.96^{e}	1.38 ± 0.36^{c}
100	4.60 ± 1.23^{d}	3.02 ± 1.6^{c}	1.58 ± 0.84^{e}	2.60 ± 1.36^{d}
150	11.81 ± 3.6^{b}	4.86 ± 1.5^{b}	7.22 ± 2.36^{b}	3.75 ± 0.63^{e}
200	4.48 ± 1.6^{d}	2.17 ± 1.03^{d}	2.31 ± 1.12^{d}	4.20 ± 1.12^{b}
200mM NaCl + 100µM MT	5.58 ± 1.9^{c}	2.08 ± 1.1^{d}	3.51 ± 0.36^{c}	2.69 ± 0.18^{d}

All values represent the means ±S.E. Different letters within a column indicate significant differences at $P < 0.05$ by Duncan's multiple range test

In terms of the effect of exogenous melatonin treatments in our study, the results in Table 2 show a significant increase in total chlorophyll (5.58±1.9µg/g FW) and chlorophyll b (3.51±0.36 µg/g FW) content under high salt stress levels.

3.3 Elemental concentration

In this study, as salt stress levels rose in the *Solanum okadae* plantlets, their Na^+ concentrations significantly increased and K^+ concentrations significantly decreased in comparison to the control (Table 3). The Na^+ content in control was lowest (55.75±12.5mmol/g) whereas it increased to 2797.20±93.6mmol/g in plants under 200mM NaCl stress. It is noteworthy, though, that despite the significant Na^+ accumulation in plantlets in various salt treatments, we found no discernible variations in their respective K^+ content. The calcium (Ca) ion concentration rose as the salt concentration increased with highest value (87.54±4.33mmol/g) being recorded at a NaCl concentration of 200mM. Also, the magnesium (Mg) content rose as the salt concentration increased with highest value (155.35±5.63mmol/g) being recorded at a NaCl concentration of 150mM, which was almost 53% more than control.

The K^+, Na^+, Ca^{2+}, and Mg^{2+} content in melatonin-treated NaCl-stressed (T_{SM}) plantlets is shown in Table 3. In 200mM NaCl-stressed plants treated with melatonin, melatonin application significantly increased the K^+ (6%), Ca^{2+} (13%), and Mg^{2+} (17%) ion concentrations while also significantly decreasing the Na^+ (44%) ion concentration when compared to plantlets in 200mM NaCl without exogenous melatonin application.

Table 3: Effect of various salt stress treatments on ionic content (mmol/g) of *Solanum okadae*

NaCl (mM)	Na^+	K^+	Ca^{2+}	Mg^{2+}
0	$55.75±12.5^{d}$	$783.13±43.0^{a}$	$55.11±1.89^{d}$	$73.13±2.35^{c}$
50	$616.73±30.12^{c}$	$700.11±79.75^{ab}$	$65.03±4.14^{c}$	$111.46±5.63^{abc}$
100	$733.89±19.41^{c}$	$610.15±21.53^{b}$	$60.95±2.66^{cd}$	$112.71±4.23^{abc}$
150	$1551.03±68.29^{b}$	$696.21±38.05^{ab}$	$82.90±5.05^{a}$	$155.35±5.63^{abc}$
200	$2797.20±93.6^{a}$	$580.16±15.44^{c}$	$87.54±4.33^{c}$	$91.69±4.36^{bc}$
200 mM NaCl + 100 µM MT	$1563.2±70.36^{b}$	$619.2±29.36^{b}$	$77±3.66^{b}$	$110.2±4.96^{ab}$

All values represent the means ±S.E. Different letters within a column indicate significant differences at $P< 0.05$ by Duncan's multiple range test

3.4 Proline and Total Protein concentration

In this study, salt exposure increased proline levels in comparison to the control (Table 4), but these levels significantly decreased in plants treated with 200mM NaCl. Plantlets grown in 150mM NaCl had the highest proline content (6.32 mg/g FW), nearly 27% higher than the control. The proline content of *Solanum okadae* plantlets increased under NaCl stress, as shown in Table 4. The total protein content, on the other hand, decreased as the salt stress increased. Melatonin significantly decreased the proline content of the plantlets in the NaCl+metatonin treatment compared to the NaCl treatment alone. In contrast, exogenous melatonin application increased the total protein content in severely stressed plantlets. It was observed that the use of exogenous melatonin helped the plantlets recover from the stress damage.

Table 4: Effect of various salt stress treatments on proline content and CAT activity of *Solanum okadae*

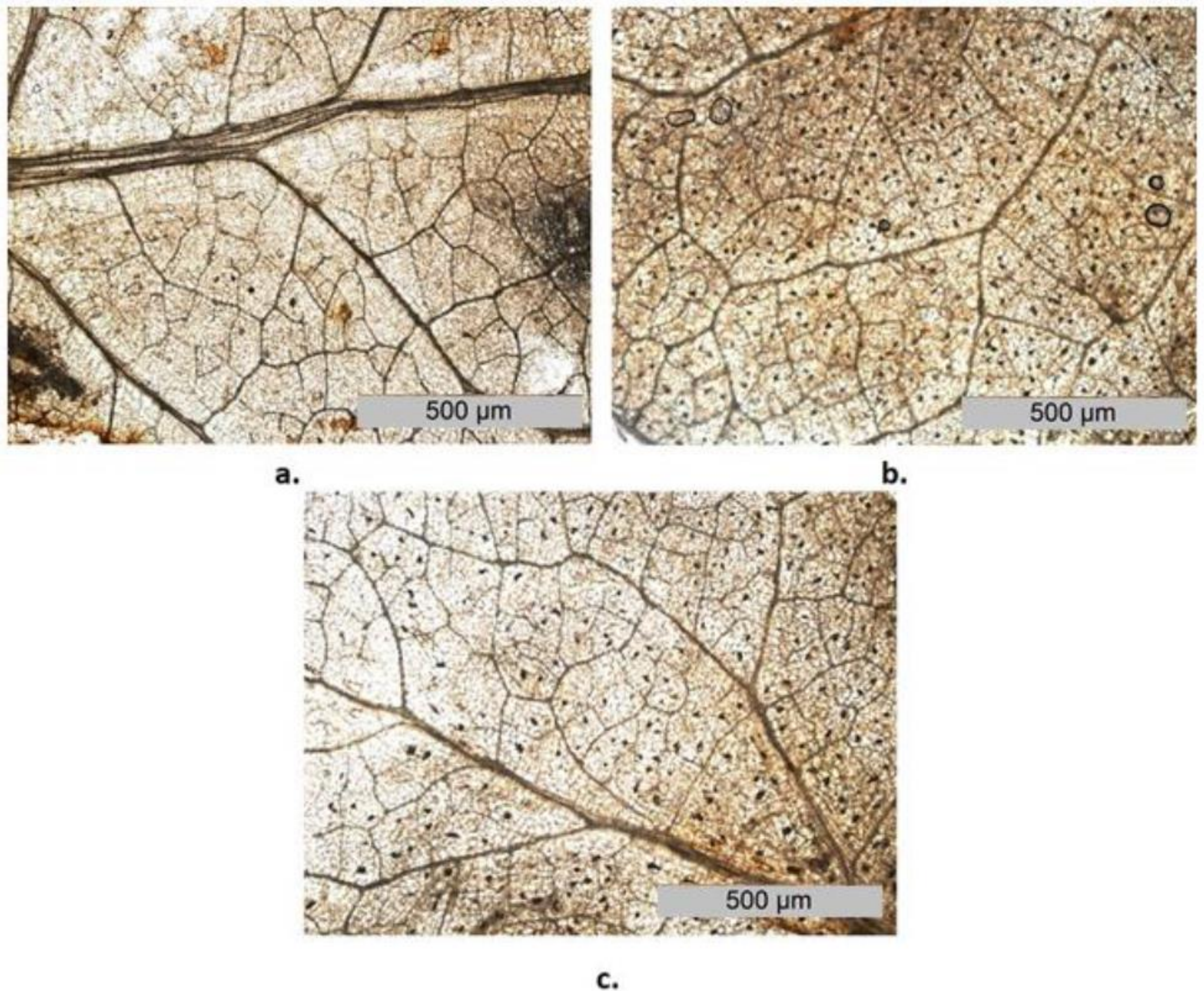
NaCl (mM)	Proline (mg/g FW)	Protein (mg/g FW)	CAT (U/mg protein)
0	4.63[c]	21.96[a]	759.4[c]
50	5.13[bc]	20.93[b]	766.55[bc]
100	5.39[b]	19.65[bc]	795.8[b]
150	6.32[a]	19.82[bc]	803.33[a]
200	5.94[b]	18.34[cd]	818.03[a]
200 mM NaCl + 100 µM MT	3.92[d]	20.66[b]	760[b]

Different letters within a column indicate significant differences at $P < 0.05$ by Duncan's multiple range test

3.5 CAT activity and H_2O_2 content

Catalase (CAT) is the most effective enzyme for preventing oxidative damage. In the leaves of *Solanum okadae* plantlets, increasing concentrations of salt stress led to a significant increase in total CAT activity (Table 4). High salt concentration (200mM) in the growth medium also significantly increased the contents of hydrogen peroxide (H_2O_2) (indicated by small dark spots as microbursts under microscope because of DAB staining) in the tissues of *Solanum okadae* (Fig 4). Supplying melatonin to the salt stressed plantlets helped in lowering the H_2O_2 content and ultimately lowered the CAT activity.

Figure 4: Visualization of H_2O_2 microbursts in response to salt stress using DAB (3, 3′ Diaminobenzidine) staining. (a.) Control, (b.) 200mM NaCl & (c.) 200mM NaCl with 100 µM melatonin.



3.6 Salt Tolerance Trait Index (STTI)

Fernandez (1993) defined the stress tolerance index (STI), which can be used to identify genotypes with high yield under both stressed and non-stressed conditions. In the current study, the salt tolerance trait index was used to identify the salt-tolerant traits and the highest concentration of NaCl that *Solanum okadae* could tolerate *in vitro* based on the ratio of non-stress to stress indices. The STTI of various studied parameters of *Solanum okadae* was determined using different concentrations of NaCl (Table 5). 50mM NaCl stress exhibited the highest STTI for morphological traits (89.76%) and photosynthetic pigments (31.29%) whereas 150mM NaCl stress exhibited the highest STTI for ionic content (145.78%) and for

CAT activity, proline and protein content (108.01%). Plantlets growing in 150mM NaCl had the overall highest salt tolerance index (81.84%).

Table 5: STTIs for various traits under different NaCl concentrations

Treatment	Morphological STTI (%)	STTI for Photosynthetic pigments (%)	STTI for Ionic content (%)	STTI for CAT activity, proline and Protein content (%)	STI (%)
50mM	89.76[a]	31.29[a]	101.17[c]	98.44[ab]	80.16[a]
100mM	78.44[b]	21.72[bc]	107.48[c]	99.87[ab]	76.88[b]
150mM	43.81[d]	29.75[b]	145.78[a]	108.01[a]	81.84[a]
200mM	38.58[d]	28.19[b]	103.42[c]	101.55[ab]	67.94[c]
200mM+100µM Melatonin	63.31[c]	23.22[bc]	118.70[b]	90.55[c]	73.94[b]

Different letters within a column indicate significant differences at P< 0.05 by Duncan's multiple range test

4. Discussion

Salt tolerance is a complex trait that typically involves three key mechanisms: osmotic tolerance, K^+ homeostasis, and tissue tolerance (Wu *et al.*, 2015). Potassium (K^+) is an essential element for plant resistance to salinity, drought, and fungal diseases and, prior research has demonstrated a strong positive correlation between the ability to retain K^+ and overall plant salt tolerance when challenged with NaCl in several species, including barley (Chen *et al.*, 2007) and wheat (Cuin *et al.*, 2008). Under salt stress, proline accumulates in plant cells, and exogenous proline mitigates the negative effects of salt stress (Anamul *et al.*, 2007). Reactive oxygen species (ROS) that are detrimental to plant development can be neutralized by antioxidants produced by plants in response to salt stress and therefore, these antioxidants and ROS can be used as a potential salt tolerance selection criterion (Ashraf & Harris, 2004).

It has been proposed to use *in vitro* testing of potato genotypes for water stress tolerance as an alternative to costly, time-consuming, and occasionally problematic field-based testing (Ali Somoro *et al.*, 2008). According to Albiski *et al.* (2012), it is challenging to test a large number of genotypes for salt tolerance in the field because of the spatial heterogeneity of soil chemical and physical properties and seasonal fluctuations. It has been reported that the impact of water

or salinity stress on potato growth *in vitro* is comparable to what is seen in the field (Khenifi *et al*. 2011, Rashid *et al*., 2019).

4.1 Morphological parameters

In the present investigation, all NaCl concentrations evaluated had a statistically significant effect on plantlet length, shoot number, leaflet number, root number, root length, fresh plantlet weight, and dry plantlet weight. The shoot lengths of all plantlets reduced significantly as NaCl concentration increased. Our results are in line with studies that claim that higher salt concentrations have a negative impact on potato plant length and development *in vitro* and *in vivo* (Sudhersan *et al*., 2012; Zaman *et al*., 2015). Potato at 75mM NaCl showed poor growth and development, according to Zhang and Donnelly (1997) and at 75mM and 100mM NaCl, Ali Somoro *et al.,* (2008) also noted a reduction in shoot length *in vitro*. The development of leaflets was inhibited by the presence of salt in the current study similar to that reported in salt stressed *Solanum tuberosum L.* cv. 'Challisha' by Biswa *et al*., (2017). Due to the high osmotic pressure in the centre of the multiplication, a small number of leaflets may form under high NaCl concentrations. This is because most of the available energy is used to resist high pressure and maintain osmotic equilibrium within the cell for the metabolic process. According to Smith *et al*. (2008), measurements of canopy and shoot responses to salinity provided an excellent indicator of the yield potential of tomato plants under stress. High levels of salt also inhibited the development of new roots. Above 100mM NaCl, Naik and Widholm (1993) observed poor root development in potato. In addition, (Evers *et al*., 1999) reported low profile rooting in potato under conditions of salt stress which agrees with our findings. The plant water content (PWC) of the plantlets in this study did not vary significantly in any of the treatment which contradicts the findings of Zaki & Radwan (2022), where the PWC% decreased with salt stress in tetraploid cultivars of *Solanum tuberosum* and diploid clones of *Solanum chacoense*. Salinity kills plants through progressive dehydration that leads to irreversible loss of turgor (Tyree *et al*. 2003), when living cells lose function. The exact mechanism by which living plant cells detect dehydration is unknown, but it involves changes in cell volume, turgor, and osmolyte concentration (Sack *et al*. 2018). Dehydration eventually leads to membrane dysfunction and death in most plants (Chaturvedi *et al*. 2014). Plants must retain a minimum pool of water to prevent permanent turgor loss to avoid death. Plant water pools are dependent on both (i) the ability of the xylem to maintain a supply of water and (ii) the ability of living cells to retain such water by preventing water loss to the xylem and the atmosphere during stress. Water retention in living cells is dependent on their ability to reduce cell water potential

(WP) to match that of adjacent xylem, which is accomplished by concentrating solutes. The regulation of water loss by the opening and closing of stomata. If the stomata are continuously open, there is a constant water loss via transpiration without replenishment from the soil; as a result, the potato plant loses turgidity and experiences water stress (Aliche *et al.*, 2020). The salinity-tolerant potato plant can maintain water uptake through osmotic adjustment, cell turgor, and allowing physiological metabolism to proceed at a moderate rate, with minimal interference to plant growth, development, and yield (Chourasia *et al.*, 2021). Our findings suggest that *Solanum okadae* might be able to withstand high salinity conditions by retaining internal water content and prevent turgor loss.

To evaluate the effects of exogenous melatonin treatment on salt stress in the plantlets, the MS media was supplemented with extra melatonin (100 M). When compared to salt-stressed plantlets, plantlets treated with melatonin had significantly higher values for shoot length, root length, shoot number, and leaf count. Comparing melatonin-treated plantlets to salt-stressed plantlets, no discernible difference was seen in the fresh weight, dry weight, plant water content, or number of roots. These findings concur with research done on melatonin treated drought stressed tomato seedlings grown in greenhouse by Altaf *et al* (2022).

4.2 Photosynthetic apparatus

Due to the disruption of the photosynthetic pigment system caused by salinity stress, photosynthesis is significantly reduced (Sarker *et al.*, 2019). The uptake of nitrogen from the soil, which is a crucial component needed by the plant for the synthesis of chlorophyll, is inhibited by the higher concentration of NaCl (Kaya *et al.*, 2020). Magnesium ion (Mg^{2+}), which functions as an enzyme cofactor and a component of chlorophyll, is required for the activation of numerous enzymes during the synthesis of chlorophyll. However, under high salinity stress conditions, Mg uptake is also hampered (Abdel Latef & Chaoxing, 2011). Under salinity stress, the stomata close more frequently, decreasing CO_2 uptake through the stomatal pore and, consequently, photosynthesis. A decrease in the number of stomata in potatoes resulted in a decrease in the rate of photosynthesis per unit area (Charfeddine *et al.*, 2019).

The current study demonstrated that escalating salt stress levels on potato plantlets had a substantial impact on chlorophyll (Chl) and carotenoid (Car) content in the leaves. All salt stressed plantlets had a significant drop in chlorophyll a, b, and total chlorophyll compared to controls. Three wild potato species, *S. stoloniferum, S. bulbocastanum,* and *S. acaule*, produced similar results to our research (Daneshmand *et al.*, 2010). Several crop species, including

tomato (Juan *et al.* 2005), soybean cultivars (Abd El Samad and Shaddad 1997), *Kochia prostrata* (Karimi *et al.* 2005), and apple rootstock, have reported a reduction in the amount of photosynthetic pigments in response to salinity (Molassiotis *et al.* 2006). The decrease in chlorophylls in salinized plants may be attributable to an increase in chlorophyll-degrading enzyme activity and ion accumulation in the leaves (Sultana *et al.* 1999).

In this study, it was interesting to observe that the amount of chlorophyll a, b, and total Chl in plantlets grown in 150mM NaCl was higher than in plantlets grown in other salt treatments. The results regarding the "increase" in chlorophyll content with increasing salt concentrations are consistent with the findings of Misra *et al.*, (1997). They found that stressing rice seedlings *Oryza sativa L.* with sodium chloride significantly increased the chlorophyll content of 15-day-old seedlings. Even though salt stress lowers the Chl content, the degree of the reduction depends on the plant species' tolerance to salt. For instance, it is well known that under saline regimes, Chl content increases in salt-tolerant species while it decreases in salt-sensitive species (Akram and Ashraf, 2011). In light of this, an accumulation of Chl has been suggested as one of the potential biochemical indicators of salt tolerance in various crops, such as wheat (Arfan *et al.*, 2007), pea (Hernandez *et al.*, 2000), sunflower (Ashraf and Sultana, 2000), alfalfa (Sabir *et al.*, 2009). Since all the crops on this list are either dicots or monocots, Chl accumulation cannot be used to determine a plant's susceptibility to salt. Although the aforesaid studies indicate that Chl accumulation may be used as a biochemical marker for salt tolerance in various crops, other studies have found that salt tolerance is not always linked to Chl accumulation under saline stress. For instance, Juan *et al.* (2005) found that in tomato cultivars with different salinity tolerance levels, there was only a weak correlation between leaf Na^+ and photosynthetic pigments. They concluded that Chl a and Chl b are poor predictors of tomato salt tolerance. Therefore, the nature of the plant species or cultivar must be considered when using Chl accumulation as a measure of salt tolerance.

Chlorophyll b is primarily found in the light-harvesting antennae surrounding the photosystem II in land plants. As a result, the ratio of chlorophyll b to chlorophyll a is higher in shade-adapted plants, which have a higher ratio of photosystem II to photosystem I. This is adaptive because rising chlorophyll b raises the spectrum of wavelengths that the shade chloroplasts can absorb (Kitajima & Hogan, 2003). Out of both pigment systems, photosystem II (PS II) is indispensable for photosynthesis. PS II is the more sensitive of the two photosystems to salinity stress, and its efficiency was reported to be reduced in potato leaves (Kolomeichuk *et al.*, 2020). In contrast to our study, under high salinity stress, the magnitude of photosynthetic activity

reduction was found to be greater in the control potato reported by Wang *et al*., (2019). This might be due to high amount of Chl b in *Solanum okadae* indicating its high salt tolerance.

Carotenoids (Car) are important as signalling precursors during plant development under abiotic/biotic stress and are required for photo protection of photosynthesis. They have a great potential to increase plant yield and nutritional quality. Increased Car contents in plants are receiving a lot of attention for plant breeding and genetic engineering (Li *et al*., 2008). Researchers working with sugar cane discovered that applying salt stress (7-8 dS m-1) at different plant growth stages resulted in a noticeable decrease in Chl and Car contents, but salt-tolerant varieties showed higher membrane stability and pigment contents (Gomathi & Rakkiyapan, 2011). Ziaf *et al*. (2009) discovered significantly higher Chl and Car contents at 60mM NaCl and suggested that relative water (RWC) and Car contents could be utilised as reliable selection criteria for salt tolerance in hot pepper.

In current investigation, Car content increased as salt concentration increased. Similar results were recorded by Wang and Nii (2015), Koyro (2006) and Daneshmand *et al*., (2010) in *Amaranthus tricolour*, halophype *Plantago coronopus* and wild potato, *Solanum acaule* in response to salt stress. Given that carotenoids oversee quenching singlet oxygen and preventing lipid peroxidation and subsequent oxidative damage, the increase in carotenoids in tolerant plants may protect plants from salinity stress (Juan *et al*., 2005). Additionally, carotenoids can stabilise the membranes in chloroplasts and release excess energy from photosystems I and II as heat or in non-harmful chemical reactions (Koyro 2006).

Regarding the effect of exogenous melatonin treatments in this investigation, the results indicate a considerable increase in total chlorophyll and chlorophyll b concentration under high salt stress. These results are similar to those recorded by Altaf *et al*., (2021) in tomato seedlings udder salt stress. They also recorded an increase in chlorophyll a and carotenoid content in seedling under salt stress treated with melatonin which does not agree with findings of current study as the values for chlorophyll a and carotenoid content were not affected by melatonin application when compared to those in plantlets growing in high salt concentration in our study. In contrast to the findings of this study, melatonin (100 µM) at 200mM NaCl (Wang *et al*., 2015) reduced chlorophyll contents in cucumber plants. But there was an insignificant decrease in carotenoid content in plantlets treated with when melatonin (100 µM) at 200mM NaCl in present study.

It is well known that endosymbiotic relationships between cyanobacteria and plants led to the evolution of chloroplasts and cyanobacteria have been found to contain melatonin and the enzymes that produce it. Therefore, it is conceivable that chloroplasts may have kept the ability to synthesise melatonin that was present in cyanobacteria (Tan, D. *et al.*, 2013). It was also discovered that the enzyme N-serotonin acetyltransferase (SNAT), necessary for the synthesis of N-acetylserotonin, the primary precursor of melatonin, is localised in the chloroplasts of rice plants (Back, K. *et al*, 2016). Furthermore, there is evidence to support the idea that under typical circumstances, plants prefer to perform melatonin biosynthesis in chloroplasts. Melatonin is made in the mitochondria when the chloroplast pathway is blocked (Tan and Reiter, 2020). It's significant to note that additional stress increases melatonin synthesis in chloroplasts. Apple leaf lipid peroxidation was significantly increased by salt stress, and melatonin production was upregulated to reduce the oxidative stress (Zheng *et al.*, 2017). It implies that the melatonin biosynthetic pathway has evolved into being stress-inducible in terrestrial plants. The purpose of melatonin production in chloroplasts appears to be to shield this organelle from oxidative stress, such as that caused by high temperatures, high salinity, and excessive light exposure (Murch & Erland, 2021).

4.3 Mineral composition

Salt stress is also referred to as hyper-ionic stress. During the growth stages of the potato plant, the shoot tissues begin to accumulate salt (Rahnama *et al.*, 2011). The uptake of K^+ ions, one of the essential minerals for growth and development, is inhibited by the higher Na^+ concentration in the plant (Ishikawa & Shabala, 2019). It experiences salinity stress in response to the unbalanced ionic concentration within the cell, which causes the production of ROS like singlet oxygen, hydrogen peroxide, and superoxide radicals, which further impair the potato plant's essential cellular processes (Jaarsma & de Boer, 2018). Under salt stress, K^+ and Na^+ ions are modified by their acquisition and distribution in plants, maintaining the ionic homeostasis (Basu *et al.*, 2020).

In the present investigation, Na^+ concentrations considerably increased and K^+ concentrations significantly dropped when salt stress levels increased in the media. Similar results were reported by Zaki & Radwan (2022) and Altaf *et al.*, (2021) in salt stressed tetraploid cultivars of *Solanum tuberosum*, diploid clones of *Solanum chacoense* and tomato seedlings respectively and by Assaha *et al.*, (2015) in *Solanum melongena.* Maintaining cellular metabolism necessitates a high cytosolic K^+/Na^+ ratio. In the current study, Na^+ levels

gradually rose as salt concentration increased, whereas K^+ levels appeared to decline as salinity stress increased though higher at 50mM NaCl similar to the studies reported by Gumi *et al.*, (2013) in *Solanum lycospersicum L. (Cv. Dan eka)*. High levels of Na^+ inside the cell prevent K^+ from being taken up, increasing the Na^+/K^+ ratio, which then has an impact on plant metabolism (Maathuis & Amtmann, 1999). The ability of Na^+ to outcompete K^+ for binding sites necessary for cellular function is a major contributor to its metabolic toxicity. K^+ is known to activate more than fifty (50) enzymes, and Na^+ is unable to fill this role (Bhandal & Malik, 1988). Furthermore, the binding of tRNA to the ribosome and likely other functions of the ribosome require high concentrations of K^+ during protein synthesis (Blaha *et al.*, 2000). By improving osmotic stress-induced reactive oxygen species (ROS), boosting the activity of antioxidant enzymes, and improving nitrogen use efficiency (NUE) in plants, K+ may be able to lessen the effects of salinity on plants. This could help maintain crop yield under stressful conditions (Tittal *et al.*, 2021). An essential component of salinity tolerance is the maintenance of low cytosolic Na^+ concentration and Na^+/K^+ homeostasis, and salt-tolerant lines exhibit lower Na^+/K^+ levels (Chattopadhyay *et al.*, 2002).

The ability to maintain K^+ homeostasis during salt stress is regarded as a trait of plants with greater salt tolerance (Hauser & Horie, 2010). When potato plants were moderately salt stressed (50–75mM NaCl), as in the comparison of the salt tolerant (Kennebec) and salt sensitive (Concord) cultivars (Aghaei *et al.*,2009) and four other potato cultivars (Rahnama & Ebrahimzadeh, 2005), K^+ levels decreased. K^+ levels decreased in the salt stressed *Solanum okadae* plantlets examined in this study, which is consistent with these studies. It is noteworthy, though, that despite the significant Na^+ accumulation in plantlets in different salt treatments, there was no discernible variations in their respective K^+ content.

Ca^{2+} functions in plant cells as a second messenger, linking a variety of extracellular stimuli with a range of intracellular reactions (Conde *et al.*, 2011). According to Pottosin *et al.* (2009), salt stress causes a quick and brief increase in free cytosolic Ca^{2+} that is likely released from the vacuole and is recognised by Ca^{2+} sensors like calmodulin (CaM), calcineurin B-like proteins (CBLs), and CBL-interacting protein kinases (CIPKs). These Ca^+ sensors are frequently built as calcium-dependent protein kinases (CDPKs), which act as a CBL-CIPK complex (Conde *et al.*, 2011). Cytosolic Ca^{2+} sensors in turn activate numerous signal transduction pathways that control ion channel activity (for example, NSCCs are strongly blocked by external Ca^{+2}), enzymatic activity, and gene transcription, leading to ion homeostasis (Adams and Shin, 2014).

The calcium (Ca) ion concentration increased as the salt concentration increased in the current investigation. This was similar to the data recorded by Kashyap *et al.*, (2020) in wild tomato *Solanum chilense.* Numerous genes associated with Ca^{2+} signalling was upregulated in their study. In addition, calcium ion-binding proteins encoded by calmodulin genes and Ca^{+}/H^{+} exchangers have been demonstrated to play important roles in salt tolerance in *S. chilense*. In addition to the role of salt-responsive phytohormones, their results indicated that Ca^{2+} signalling was essential for salt tolerance in *S. chilense*. Ca^{2+}/H^{+} exchangers (CAXs), cyclic nucleotide-gated channels (CNGCs), and calcium-transporting ATPases (ACAs) were also significantly up-regulated in *S. Chilense*, which may be the primary factor in Ca^{2+} fluxes during salinity. Sodium/hydrogen exchanger or NHXs are known to be involved in the compartmentalization of K^{+}, $Na^{+,}$ and pH homeostasis. Cyclic nucleotide-gated channels (CNGCs) are responsible for the uptake of Ca^{2+}, Na^{+}, and K^{+}. All of these functions are controlled by the pH gradient generated by V-ATPases (Reguera *et al.*, 2014). K^{+} transporters also play a crucial role in the ionic balance process, which is impeded by salinity stress (Dreyer, & Uozumi, 2011). Transport of Na^{+}, osmolytes, heavy metals, auxin, and fatty acids are among the multiple functions of ABC transporters (Kim *et al.*, 2010). During salt stresses, the primary function of cyclic nucleotide-gated channels is to influx the Ca^{2+} ion across the plasma membrane, whereas the Ca^{2+}/H^{+} exchangers and calcium-transporting ATPases perform the Ca^{2+} efflux process across the plasma membrane and tonoplast, respectively.

Understanding the fundamental physiology and genetics of cells under salt stress is crucial for developing any transgenic strategy because salt stress poses a serious environmental threat to agriculture. Calcium is a crucial factor in determining a plant's ability to tolerate salt, and it is especially important for maintaining sodium and potassium homeostasis. Due to its ability to increase K^{+} and Ca^{2+} uptake while decreasing Na+ uptake in plants exposed to NaCl salinity, calcium is a particularly crucial nutrient for plant growth (Munns, 2002). High Ca^{2+} concentrations can alter the cell wall's properties and decrease the plasma membrane's permeability to Na^{+}, which can reduce the accumulation of Na^{+} by passive influx (Vitart *et al.*, 2001). According to available data, the SOS pathway is essential for coordinating the operations of several transport systems (Qiu *et al.*, 2004). One of the primary salt-detoxifying mechanisms in cells is calcium activated SOS3-SOS2 protein complex, which activates SOS1 (Na^{+}/H^{+}-antiporter) on the plasma membrane and causes Na^{+} to be expelled from the cells (Qiu *et al.*, 2002). Inhibiting HKT1 (low-affinity potassium transporter), which transports Na^{+}ion under high-salt conditions, is another function of the SOS3-SOS2complex (Laurie *et al.*, 2002).

Additionally, according to Quan *et al*. (2007), this complex is thought to control both the extrusion of Na^+ ions (by controlling SOS1) and the compartmentalization of Na^+ ions in the vacuole (by activating NHX-type transporters that pump Na^+ ions into the vacuole). Under salt stress, SOS2 also functions to activate H^+-ATPases and aids in restoring ionic homeostasis (Vitart *et al*., 2001). Based on the increasing calcium ion content found in this study, *Solanum okadae* could be considered a salt-tolerant line. Future research should focus on finding the elusive salt-stress sensors and other signalling elements that help salt stress control the expression and activity of ion transporters in *Solanum okadae*.

Magnesium (Mg), the amplest free divalent cation in a cell, is a key macronutrient in the growth and development of all organisms, including humans. According to Gerendás and Führs (2013), Mg is an essential part of the chlorophyll (Chl) pigments that make up the light-capturing complex of chloroplasts and is consequently involved in the assimilation of CO_2 during photosynthetic processes. Much of the magnesium absorbed by plants (between 65 and 85 percent) is used for protein synthesis and other related biological processes, with only about 15 to 35 percent of it being fixed in chlorophyll pigments (Marschner, 2011). Additionally, more than 300 enzymes (including those involved in Chl biosynthesis and photosynthetic CO_2 fixation) use Mg as a cofactor (Chen *et al*., 2018). Several other enzymes, including protein kinases, RNA polymerase, glutathione synthase, adenosine triphosphatases (ATPases), phosphatases, and carboxylases, require magnesium to be activated (Marschner, 2011). Additionally, Mg takes part in numerous biological processes, including the transport of sucrose, energy metabolism, nitrogen utilisation, pollen development and male fertility, stress tolerance, interactions between plants and microbes, and many others (Ishfaq *et al*., 2021).

In this investigation, the Mg concentration increased as the salt concentration increased, with the greatest value (measured at a NaCl concentration of 150mM) being observed. Similar results were reported by Assaha *et al*., (2013) in *Solanum scabrum* Mill. (huckleberry) and *S. melongena* L. (eggplant), under salinity stress. Usually, the impact of salinity on Mg concentration is determined by Ca-Mg interactions at the root level, where high substrate Ca causes an increase in leaf Ca synchronously with a reduction in Mg concentration (Grattan and Grieve 1999). It is therefore possible that in the current study, this competition was diminished or eliminated, leading to an increase in leaf Mg concentration in *Solanum okadae* plantlets, which was particularly enhanced in the plantlets under the 150mM salt treatment (Table 3). As Mg is a structural component of chlorophyll, this increase was certainly advantageous for

plantlets under the 150mM treatment, as evidenced by the corresponding increase in chlorophyll concentration (Table 2).

Melatonin treatment significantly increased the K^+, Ca^{2+}, and Mg2$^+$ ion concentrations while decreased the Na^+ ion concentration in 200mM NaCl-stressed plants when compared to plantlets in 200mM NaCl without exogenous melatonin application. These results are similar to those reported by Jiang *et al*., (2021) and Zhang *et al*., (2022) in salt stressed cotton and wheat respectively, where melatonin application significantly lowered the Na^+ ion and increased the K^+ ion concentration. Plants use an effective strategy to resist salt stress by maintaining ion homeostasis and reducing ionic toxicity. Maintaining a high $[K^+]/[Na^+]$ ratio in cells is an important strategy for increasing salt tolerance (Flowers and Läuchli, 1983). For plants to tolerate salt, ions must be taken up and compartmentalized because too many salt ions in the cytoplasm disrupt ion homeostasis and prevent plant growth and therefore, under conditions of high salinity, plants move excessive salt ions from the cytoplasm into the vacuole or compartmentalize them into different tissues (Yuan *et al*., 2016). The salt-induced Na^+/H^+ antiporter in the tonoplast is responsible for compartmentalizing cytoplasmic ions into vacuoles in order to reduce cytoplasmic ion concentrations (Yang *et al*., 2010). In this study, melatonin application decreased Na^+ concentrations, increased the $[K^+]/[Na^+]$ ratio induced by NaCl stress alone, and increased Ca^+ and Mg^{2+} concentrations. This indicated that melatonin may have a beneficial effect on the salt stressed plantlets. In a signalling network involving H_2O_2 and Ca2+, the ion transport system including the ionic equilibrium of $[K^+]$ and $[Na^+]$ and $[K^+/[Na^+]$ is frequently considered. Kaya *et al*. (2019) reported that melatonin treatments increase plant growth in Cd-stressed wheat plants due to increased Ca^{2+} and K^+ in the leaves and decreased MDA, H_2O_2.

Ion homeostasis is crucially maintained by melatonin. Melatonin enhanced MdNHX1 (the malus vacuolar Na^+/H^+ antiporter gene), which improved the salt tolerance of M.26, a significant apple (*Malus domestica*) dwarf rootstock (Li *et al*., 2010). The relative rates of Na^+ and K^+ uptake under high salinity are mediated by the inward-rectifying channel AKT1 (Arabidopsis K^+ transporter 1) (Shao *et al*., 2014). In M.26, MdAKT1 (Malus inward-rectifying channel AKT1) was observed to have the same effect and was highly expressed in leaves. Melatonin protected apple cells from the harm caused by high salt levels by preserving ion homeostasis by regulating the expression of MdNHX1 and MdAKT1. Melatonin is thought to affect ion regulation and partitioning via the ABA biosynthesis and signalling pathway. Melatonin application under salt stress, however, can affect the breakdown and synthesis of

ABA. The precise role of melatonin under salt stress is that it maintains ion homeostasis by up-regulating the transporter genes NHX1 and AKT1(Li *et al.*, 2011). Melatonin application also increased K^+ accumulation, decreased Na+ accumulation, and maintained the higher K^+/Na^+ ratio to induce salinity tolerance in maize seedlings (Jiang *et al.*, 2016). Melatonin treatment similarly increased the expression of NHX1 and SOS2 in rapeseed seedlings, which was correlated with a lower Na^+/K^+ ratio (Zhao *et al.*, 2018). Moreover, it is believed that the interaction between Ca^{2+}/CaM ($Ca^{2+/}$Calmodulin) and melatonin plays a role in salt stress resistance. Interaction between Ca and melatonin induces long-distance signalling, resulting in salt stress tolerance in *Dracocephalum kotschyi* (Vafadar *et al.*, 2020). However, further research is necessary to elucidate the mechanism by which melatonin under salt stress induces salt tolerance.

4.4 Proline content and Total protein

Proline is the most prevalent endogenous osmolyte accumulated in response to salinity and other abiotic stresses in plants (Slama *et al.*, 2015). Numerous studies demonstrate that salt stress causes genes involved in proline biosynthesis to be switched on, which results in proline accumulation (Kim and Nam, 2013). Also, ion accumulation necessitates the accumulation of solutes in the cytosol, which plays a role in osmoprotection and osmotic adjustment under abiotic stress (Munns and Tester, 2008). This accumulation of osmolytes, particularly proline, is a common occurrence in plants. Proline, in addition to its role as an osmolyte, helps to scavenge ROS, stabilise subcellular structures, modulate cell redox homeostasis, supply energy, and function as a signal (Sharma *et al.* 2011).

According to biochemical studies, plants under salt stress accumulate a variety of metabolites that are referred to as compatible solvents because they do not interfere with plant metabolism (Sivakumar *et al.*, 2000). Proline is widely distributed in plants and accumulates in salt-stressed plants in greater amounts than other amino acids (Ashraf and Foolad, 2007). One of the most common modifications caused by salt and water stress in plants is proline accumulation, which is frequently thought to be involved in stress tolerance mechanisms (Ashraf & Foolad, 2007). In present study, salt treatment raised proline concentrations at 150mM salt stress relative to the control, but these concentrations reduced dramatically in severely salt stressed plantlets (200mM NaCl). The results of this study are in contrast with those reported by Pravin *et al.*, (2019), in *Solanum lycopersicum L.*, where the proline content declined significantly by 68% in the plants treated with 150mM NaCl. Under salt stress, proline content increased in all potato

cultivars' shoots and calluses, however, the basic proline content of the control seedlings from salt-tolerant cultivars was higher than that of the sensitive ones, but this correlation was not seen in the callus tissue (Rahnama & Ebrahimzadeh, 2004). On the other hand, according to some studies (de Lacerda *et al*. 2001), sensitive cultivars always had higher salt-induced increases in proline concentrations than tolerant ones. As a result, in potato plants, proline accumulation and salt stress tolerance are not always related.

The high proline accumulation observed in salt stressed *Solanum okadae* was most likely due to (a) increased glutamine synthetase activity (responsible for the synthesis of glutamine which can be converted to proline via glutamate); (b) decreased proline degradation; (c) increased proline biosynthesis; (d) decreased protein synthesis or proline utilisation; and (e) increased protein hydrolysis (Chen *et al*., 2001). Similar to the findings of current study, tomato showed increased proline accumulation and tolerance to salinity (Kahlaoui *et al*., 2015). According to Gharsallah *et al*., (2016), the degree of proline accumulation varies between tolerant and sensitive genotypes in tomato, even though it is a common response to salt stress. When compared to the most sensitive genotype, they also reported that proline accumulation increased significantly in the tolerant genotype, primarily in the leaves. In order to preserve the level of chlorophyll and cell turgor and safeguard photosynthetic activity under salt stress, proline is preferentially accumulated in leaves (Silva-Ortega *et al*. 2008). Additionally, proline may be used to scavenge ROS-related products (Soshinkova *et al*. 2013). Proline builds up in stressed plants either because proline biosynthesis genes (P5CS, P5CR) are expressed more frequently or because its degradation pathway genes are suppressed (PDH silencing) (Marco *et al*., 2015).

Plants will enhance the production and storage of osmotic adjustment substances (proline, soluble protein, etc.) under adversity stress and the response to adversity stress (Khan *et al*., 2019). In tomato seedlings under temperature stress, treatment with melatonin significantly increases the content of proline, polyamines, and sucrose, up-regulates the expression of proline biosynthesis genes (P5CS) and reduces the negative effects of temperature stress on tomato plants (Ding *et al*., 2018). Melatonin significantly decreased the proline content of the plantlets in the NaCl+metatonin treatment compared to the NaCl treatment alone. This contrasted with the findings of Jiang *et al*., (2021) in salt stressed cotton where melatonin application significantly increased the proline content. Also, Meng *et al*., (2014) showed that melatonin reduced the osmotic stress of grapes under water stress, suggesting that proline improves plant's ability to withstand salt by raising the content of proline. Proline

decomposition brought on by melatonin's stress-relieving properties may be the cause of the observed decrease in proline content (Park *et al.*, 2021). Similarly, exogenous melatonin treatment decreased proline content in fenugreek (*Trigonella foenum-gracum L.*) under drought stress, according to Zamani *et al.* (2019). Proline degradation is caused by the enzymes proline dehydrogenase (PDH) and '-pyrroline-5-carboxylate dehydrogenase (P5CDH), which are catalysed by darkness and stress relief. Given that melatonin reduces the oxidative damage brought on by salt stress, proline degradation may occur when stress is reduced (Zamani *et al.*, 2019). Additionally, growth and development under salt stress depend on proline degradation (Park *et al.*, 2021).

Plants can respond to and adapt to salt stress through the synthesis of specific proteins that can modify cell metabolism, and the synthesis of stress-induced proteins is part of that stress tolerance mechanism. In addition to the accumulation of proline, which is one of the most common metabolic responses of higher plants to salinity (Heuer, 2003), plants are also able to respond to and adapt through the metabolism of specific proteins (Veeranagamallaiah *et al.*, 2007). Numerous salt-induced proteins have been found in various plant species and are divided into two groups: (1) salt stress proteins, which accumulate only as a result of salt stress, and (2) stress-associated proteins, which also accumulate in response to heat, cold, drought, water logging, and exposure to high and low amounts of mineral nutrients, and may be involved in osmotic adjustment (Ashraf and Harris, 2004; Veeranagamallaiah*et al.*, 2007).

In salinity-damaged *Solanum okadae* plantlets, the protein content declined significantly (Table 4). Similarly, the soluble protein content of shoots and calli decreased as NaCl levels increased in salt stressed potato seedlings as reported by Rahnama & Ebrahimzadeh, (2004) and Faried *et al.*, (2016). The lower protein content could be attributed to a decrease in K^+ content, which plays an important role in protein synthesis (Heuer and Nadler, 1998). As a result, the decreased protein content was primarily due to suppressed protein synthesis, and protein degradation may have played a role in the NaCl-induced proline accumulation in *Solanum okadae* plantlets.

Comparing melatonin treated (T_{SM}) and untreated salinity damaged (T_4) plants, the application of 100 μM melatonin increased protein content by 10%. This research showed that protein accumulation under salinity stress conditions was positively impacted by exogenous melatonin in *in vitro* raised *Solanum okadae*. Similar results were reported by Park *et al.*, (2021) in *Brassica juncea.* Plants produce proteins and other metabolites that maintain cell balance to

change the osmotic pressure (Kavi *et al.*, 1995). Salinity stress in the current study reduced the amount of soluble protein in the plantlets. However, plantlets damaged by 150 mM NaCl showed a slight rise in protein content. This increase can be attributed to the plants' coping mechanisms and salt stress adaptation (Chi *et al.*, 2019). Melatonin has been found to stimulate protein biosynthesis and inhibit degradation, maintaining cellular balance and physiological functions (Chen *et al.*, 2021).

4.5 CAT activity and H_2O_2 content

Reactive oxygen species (ROS) are effectively scavenged by antioxidant systems under normal conditions. However, when plants are subjected to environmental stresses involving salinity, ROS formation exceeds the capacity of the antioxidant system (Yazici *et al.*, 2007). The oxidative damage to lipids, proteins, and nucleic acids caused by these cytotoxic ROS (Parida & Das, 2005) can severely disrupt normal metabolism. In response to oxidative stress, plants have developed defence mechanisms to scavenge reactive oxygen species (ROS), including low-molecular-mass antioxidants (glutathione, ascorbate, and carotenoids) and ROS-scavenging enzymes such as SOD, CAT, POX, APX, and GR (Parida & Das, 2005). ROS-scavenging enzymes can prevent oxidative damage to cells. Superoxide dismutase (SOD), which dismutates superoxide anion to hydrogen peroxide (H_2O_2) and oxygen (O_2), is primarily used to neutralise ROS injury. Catalase (CAT) and peroxidase (POD) then break down H_2O_2 into H_2O and O_2 (Wang *et al.*, 2018).

The most effective enzyme for avoiding oxidative damage is catalase (CAT). Increasing salt stress concentrations resulted in a considerable increase in total CAT activity in the leaves of *Solanum okadae* plantlets. Similar to the findings in this study, increased CAT activity was observed in salt-tolerant *Cassia angustifolia L.* (Agarwal &Pandey, 2004), maize (de Azevedo Neto *et al.*, 2006), *Jatropha curcas* (Gao *et al.*, 2008), and *Sesamum indicum (*Koca *et al.*, 2008*)*. Also, under salinity stress, the activities of antioxidant enzymes catalase (CAT), along with ascorbate peroxidase (APX), superoxide dismutase (SOD), and peroxidase (POX) were significantly increased in all potato genotypes with more increase in salt-tolerant genotypes than in salt-sensitive genotypes (Sanwal *et al.*, 2022), which is consistent with the findings in this study. All these findings indicate that *Solanum okadae* could be a potential salt-tolerant potato species.

Melatonin inhibits oxidative stress in several ways to protect plants from salt stress (Szafrańska *et al.*, 2016). By increasing the TaSNAT transcript, which encodes essential enzymes in the

melatonin biosynthesis pathway, exogenous melatonin application causes the accumulation of endogenous melatonin under salinity stress in wheat (Ke *et al*., 2018). Melatonin increases the expression of genes associated with antioxidants under salinity stress. For instance, melatonin was found to upregulate the transcripts for the enzymes ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD) in salt-stressed *Arabidopsis* (Chen *et al*., 2018).

H_2O_2 is indicated under histochemical (DAB) staining by dark brown spots (Figure 4). Melatonin application reduced the content of H_2O_2 in the leaf tissues of *Solanum okadae*. The findings of this study are similar to those reported by Ali *et al*., (2021), in tomato cultivars. According to Naveed *et al*., (2020), salt-induced oxidative damage may be responsible for plasma membrane disruption, electrolyte leakage, lipid peroxidation, and nutrient uptake inhibition, as evidenced by elevated oxidative stress indicators (H_2O_2) during salt stress (Figure 4). These findings are consistent with the findings of Manai *et al*., (2014), who concluded that oxidative injury is increased in tomato tissues in a saline environment. However, a significant decrease in H_2O_2 after melatonin application could be due to decreased Na^+ uptake, improved plasma membrane stability, and less exposure of roots to a saline environment (Ali *et al*., 2021).

4.6 Salt Tolerance Trait Index (STTI)

Within certain parameters, plants can adapt to salt stress by triggering a number of physiological reactions. These include the hormonal regulation of numerous stress responses as well as the regulation of water and ion homeostasis, sodium and chloride uptake/accumulation via exclusion and compartmentalization mechanisms, the detoxification of stress-induced reactive oxygen species (ROS), and so on (Rana *et al*., 2019). The majority of research point to less effective mechanisms in domesticated plants when compared to their wild relatives and in commercial cultivars when compared to landraces, which weren't bred for commercial traits (Morton *et al*., 2019). Two tomato landraces demonstrated higher salinity tolerance in terms of yield when compared to a commercial tomato variety, which was associated with a higher K^+/Na^+ ratio in landrace leaves (Massaretto *et al*., 2018). The consensus is that high-yielding traits have replaced adaptive/resilience traits in commercial crops during the breeding process. To save these adaptive traits, it has become fairly common practice to look for salt-tolerant traits in wild species and/or small traditional/landraces (Cuartero *et al*., 2006).

The majority of physiological processes and cellular metabolism are hampered by a high salt concentration in the root zone, which eventually results in decreased growth and crop

productivity (Isayenkov & Maathuis, 2019). To enable crop cultivation in salinized areas, it would be essential to improve plant tolerance to salt stress (Wu *et al*., 2018). The stress tolerance trait index was utilised to identify the maximum amount of salt *Solanum okadae* could tolerate and ultimately deduce its salt tolerance level based on the ratio of non-stress to stress indices. The improved performance of salt-tolerant potato genotypes under stress circumstances was linked to increased photosynthetic activity, osmotic regulator accumulation, and higher activities of antioxidant enzymes that reduce ROS formation (Sanwal *et al*., 2022) which is similar to the findings of this study as per the STTI values for the respective traits in *Solanum okadae*, which could probably deem it to be a salt tolerant species. There are comparatively few studies that describe the traits that remained in current potato cultivars that might be a starting point to improve potato tolerance to salt, despite the abundance of studies on salt stress related to model plants and crops. Although wild potatoes in the Andes are relatively tolerant to various stresses, extensive breeding, and selection for traits other than abiotic stress tolerance has resulted in cultivars that are considered moderately salt tolerant (Jaarsma *et al*., 2013).

5. Conclusion

A plant's ability to withstand moderate or high concentrations of salt in the water in its leaves or in the soil within reach of its roots is frequently referred to as its salt tolerance (Mawa *et al*., 2021). Tissue culture system can be utilised to identify stress-tolerant genotypes among existing cultivars, and it is the simplest and quickest method for determining the salinity tolerance of various potato cultivars. The morphological and biochemical characteristics of the wild potato species examined in the present study varied significantly. The superior performance of *Solanum okadae* under salt stress was attributable to a more robust antioxidant defence system, as indicated by higher catalase (CAT) activity, and better osmotic adjustment which was also observed in salt tolerant potato cultivars by Sudershan *et al*., (2012). Therefore, we can conclude that *Solanum okadae's* ability to withstand higher NaCl (150mM) concentrations by maintaining K^+ homeostasis along with deploying osmoprotectants like proline and antioxidant enzymes could make it a better candidate for salt tolerant species. This research established the baseline for *Solanum okadae's* salt tolerance but further screening with additional markers and field study are needed.

The results of the current study also, successfully demonstrated that melatonin stimulated salt tolerance. The findings of this study showed that exogenous melatonin application significantly

boosted the recovery of nearly 4-week-old salinity-damaged wild potato, *Solanum okadae* plantlets *in vitro*. Melatonin accelerated the leaf, shoot and root formation in salt stressed *Solanum okadae* plantlets along with increasing the K^+ and protein concentration and decreasing the toxic Na^+ and H_2O_2 content. Exogenous melatonin can effectively increase *Solanum okadae* plantlets performance and yield in either unstressed or stressed (salinity) conditions in an environmentally friendly way for more sustainable agriculture, according to the results of this study. Future research is required to shed light on the genetic mechanisms and metabolic pathways involved in the salinity-damaged *Solanum okadae* plants' ability to recover while being treated with different doses of melatonin.

CHAPTER 4: *In vitro* Micropropagation, Callus induction and Regeneration of *Solanum okadae*

1. Introduction

The potato (*Solanum tuberosum L.*) is an annual Andean solanaceous plant. Its commercial product, the tuber, is the underground stem designed to serve as a reserve source and a means of reproduction (Prado de Morias *et al.*, 2007). It is a crop of exceptional global significance due to its socio-economic effects and status as one of humanity's primary food sources. Potato is the most important non-cereal food crop in the world (Basera *et al.*, 2018), and is farmed in about 150 nations (Nikitin *et al.*, 2018). It is the most important vegetable crop in the world and the fourth most important crop overall. This crop is carefully managed and requires numerous inputs to provide profitable yields. In addition, rigorous quality requirements must be maintained throughout harvest and storage. Potato improvement is contingent upon the availability of genes that, when packed into a superior cultivar, will provide a crop of dependably high quality, preferably with less inputs than are currently necessary. In Europe (Ross, 1986) and North America (Douches *et al.*, 1996), the gene pool for potato cultivars is very limited.

On a commercial scale, potato is propagated vegetatively; nevertheless, this kind of multiplication can add several systemic fungal, bacterial, and viral infections that result in yield and vigour reductions in the plants (Nikitin *et al.*, 2018). Thus, the utilisation of virus-free propagative material with excellent phytosanitary, physiological, and genetic quality is crucial for maximising the production potential of a plant. In this context, plant tissue culture can be used to improve potato production by maintaining germplasm banks, facilitating genetic exchange, studying the particular species' interactions with biotic and abiotic factors, producing genetically modified plants, and producing seed potatoes that are free of pathogens.

1.1 Biotechnological Development to Improve Potato Cultivation

Potato is an excellent crop for introducing traits through biotechnology. It was one of the first crops to be genetically engineered, following virus resistant tobacco (China in 1992) and the FlavrSavr tomato (US in 1994); it was commercially cultivated as NewLeaf TM by Monsanto in 1995 (Halterman *et al*, 2016). Aside from the abundance of available traits for potato development, potatoes can be easily produced via tissue culture, making it simple to incorporate specific genes and recover plants from transformed tissue (Chakravarty *et al.*

2007). Some cultivars are more receptive to tissue culture than others, but with suitable protocol changes, most are capable of *Agrobacterium tumefaciens* mediated transformation and plant tissue regeneration. The most common way of stable transformation in potato is the use of *Agrobacterium* to transfer genes of interest, while alternative approaches including as particle bombardment, protoplast transformation, and microinjection have been successful. Regardless of the process, regulatory approval of the new potato variety is required before wide-scale release and production. In the United Kingdom, regulatory clearance can involve up to two federal agencies: Department for Environment, Food & Rural Affairs (DEFRA) and Animal and Plant Health Agency (APHA). New technologies for selectively editing portions of plant genomes are also being developed, which may give a mechanism for genetic improvement that is independent of the usual regulatory process (Waltz, 2012).

1.2 Potato Improvement using Cell and Tissue culture

It is anticipated that the adoption of high-quality, disease-free seed potatoes will boost the availability of potatoes for human use (Mohapatra and Batra, 2017). Tissue culture is the science of cultivating isolated plant cells, tissues, or organs on artificial media (Muthoni and Kabira, 2014). In recent years, tissue culture techniques have become an alternative and highly appreciated method for the vegetative multiplication of plants (Nuwagira *et al*., 2016). It has played a significant role in the production of disease-free planting materials for vegetatively propagated crops within the horticulture industries of numerous nations (Tegen and Mohammed, 2016). The procedures are utilised globally to produce microtubers, which are pre-basic, virus-free seed potatoes (Dessoky *et al*., 2016). Tissue culture, a biological technology with promising potential for crop productivity and development under aseptic circumstances, is characterised by highly adaptable and rapid multiplication, resulting in a high rate of multiplication in a relatively brief time frame (Mohapatra and Batra, 2017).

Due to potato's strong amenability to *in vitro* modifications, numerous procedures have been refined over the years. This range of technologies consists of approaches of varying degrees of complexity. While some of these technologies, such as micropropagation and pathogen removal, have been deployed to boost potato production, others are currently being refined and improved. Potato's most prominent application is the employment of *in vitro* techniques for virus elimination (meristem culture) and clonal mass propagation (micropropagation). Potato clones made *in vitro* and propagated using standard techniques have become a vital part of seed production in several nations (Naik and Sarkar, 2000).

Plant tissue culture offers extraordinary prospects for *in vitro* propagation, plant quality enhancement, and the creation of plants with desirable agronomic quality and quantity (Ragavendran and Natarajan, 2017). Micropropagation of potato is advantageous for increased yield and uniformity in germplasm (Singh, 2012). To optimise domestic production of disease-free, enhanced seed potato varieties, it is necessary to build capacity along the entire seed multiplication chain. The National Academy of Agricultural Sciences has deemed the application of *in vitro* culture of isolated tissue for the assembly of potato plantlets followed by multiple cycles of multiplication into mini-tubers and seed potatoes to be an appropriate large-scale intervention (FAO, 2021).

1.3 Techniques for Producing and Multiplying Seed Potatoes

The most effective scientific and technical knowledge transfer among potato-based agri-food systems around the world is predicted to accelerate the innovation process, as will the identification of the most significant constraints and the appropriate selection of target areas for potato research and innovation. Regarding seed potato production, it is projected that high-quality seed production and distribution will increase productivity both in industrial-based systems with greater local production and in rural-based systems where local agro-ecological circumstances permit excellent production. Devaux *et al.*, (2021) reported that by combining rapid multiplication techniques, potato seed production systems could provide access to high-quality seed tubers of improved varieties (e.g. aeroponics, sand hydroponics, or apical cuttings).

Some of the other important multiplication techniques include:

1.3.1 Early generation seed (EGS)

Early Generation Seed (EGS) is defined as "planting material produced in tissue culture laboratories by specialist entities (e.g., micro tubers, *in-vitro* plantlets) or under protective structures, such as greenhouses (e.g., cuttings, mini-tubers)". EGS is used by seed multipliers to deliver quality-assured or certified seeds for farm use (Fornkwa *et al.*, 2021).

The production of EGS began in the laboratory, where its multiplication will be completed by tissue culture or micropropagation. To create a constant supply of massive stocks, it was necessary to get rid of viruses by growing sprouts from contaminated tubers using meristem culture and to make a lot of virus-free plantlets quickly using nodal culture (Fornkwa *et al.*, 2021).

1.3.2 Methods for the multiplication of potato seeds

In traditional methods, seed potato tubers are used for production and multiplication (Struik, Wiersema, 1999), which may be ineffective, have a poor rate of multiplication, and have a significant risk of infection by numerous diseases caused by organisms including fungi, viruses, and bacteria (Otroshy, 2006).

To accumulate and multiply disease-free seed potatoes, numerous methods and technologies are being explored. For the assembly of disease-free micro-tubers, for instance, tissue culture was utilised with ovaries, anthers, apical stem cuttings, sprouts, shoots, axillary buds, stem, leaf, and tubers (Singh, 2012; Srivastava *et al*., 2012; Tsoka *et al*., 2012; Karp *et al*., 2013).

The three main steps of the apical rooted cutting (ARC) technique are as follows: Tissue culture (to create disease-free plantlets); Cutting production (involves two crucial stages: mother plant multiplication and ARC generation for planting); and Seed production (to provide seeds for field planting) (International Potato Centre, 2021). All of these operations, however, require well-trained personnel and close follow-up in order to produce the desired results. Tissue culture, which permits the production of disease-free agricultural planting material, is the *in vitro* regeneration of plants from disease-free plant components (tissues, cells) (Pradhan *et al*., 2021). It is necessary to develop relatively simple techniques, have a high multiplication rate with a high degree of reproducibility, and provides a high survival rate of micro shoots or plantlets upon transfer to *ex-vitro* conditions in order to advance tissue culture technology to large-scale propagation (Purohit *et al*., 2011).

1.3.3 Seed potato preservation and germplasm conservation

There is a desire to conserve primitive potato cultivars and associated wild species that cannot be stored as seeds in normal propagation, as there is always a risk of loss (Westcott *et al*., 1977). Micropropagation permits pathogen-free potato cultivars to be multiplied asexually on a vast scale. Every 21 days of sub-culturing, at least three nodal cuttings are produced from a single microplant. Hence, theoretically, a single virus-free explant can produce 3^{15} (43 million) microplants in a single year. Numerous strategies for generating a large number of microplants on nutritional media under aseptic conditions have been devised. Initial shoot multiplication is mostly accomplished by nodal segment culture, in which axillaries and terminal buds develop into new plants. Nodal explants of disease-free micro plants are cultured using a semisolid or liquid culture media. The impact of the culture media's physical, hormonal, and nutritional components on the growth of explants have been the subject of much investigation. For potato

micropropagation, Murasighe and Skoog (MS) media is most frequently employed. Although liquid medium promotes a greater development rate of potato micro shoots, semi-solid medium is employed for initial nodal segment propagation (Rosell *et al*., 1987).

Micropropagation is not only an excellent alternative for plant species that are resistant to conventional bulk propagation practices but also is the most economically viable tissue culture technique. It has a huge benefit over traditional methods in that a large number of plants may be grown from a single individual in a very short amount of time and space, regardless of the seasons (Hajare, 2021). On a large scale, potatoes may be micropropagated via shoot-tip cultures and meristem culture or via axillary shoots established from *in vitro*-cultured nodal cuttings, and adventitious shoot growth either directly from explants or indirectly via a callus phase.

In *Solanum* species, the combination of cytokinins [6-benzylaminopurine (BA) and kinetin (KIN)] and auxins [1-Naphthaleneacetic acid (NAA)] have been reported to produce better results in micropropagation studies and plant regeneration via callus compared to the use of auxins or cytokinins alone, (Kumlaya & Ercisli, 2015). In this study, we report a micropropagation method for a wild diploid potato species *Solanum okadae.* According to Subramanian (2017), some accessions of this species have an unusually high dry matter content. This species has some accessions that carry the Rpi-oka1 gene, which confers late blight resistance. Additionally, Watanabe (2011) noted *that Solanum okadae* is drought-resistant. This suggests that *Solanum okadae* should be a reliable source of drought and late blight resistance. Exploiting the wealth of micropropagation studies in *Solanum* specie*s,* this project has developed an effective procedure for micropropagation and callus induction from nodal segments, as well as rapid plant regeneration from callus, for *Solanum okadae* which is the first such report for *Solanum okadae*.

2. Materials And Methods

2.1 Plant material & Media preparation

Seeds of *Solanum okadae* (accession: OKA7129) were obtained from the Commonwealth Potato Collection (CPC) maintained by The James Hutton Institute (JHI, Invergowrie, Scotland). Initial germination was performed in small propagator trays by sowing the seeds in Levington M3 compost. After 2 weeks, the seedlings were transferred to 6” pots containing Levington CNSC (Container Nursery Stock Coarse) compost and kept in a controlled glasshouse environment with a 16 h photoperiod and 22 °C±2°C to be grown to maturity.

For tissue culture experiments, typically 1x MS (4.3g/L MS salts, 3% sucrose, pH-5.7; 0.8% (w/v agar) was used.

For shoot induction for sterile genetic stock, MS medium was supplemented with a combination of different concentrations of BA (2, 2.5 and 3 mg/L), NAA (2 and 3 mg/L) and KIN (1mg/L).

For callus induction, MS medium, containing different concentrations of 2, 4-dichlorophenoxy acetic acid (2, 4-D) (1, 2, and 3 mg/L) was used.

For plant regeneration via callus, MS media was supplemented with BA (2.5 and 3 mg/L) in combination with NAA (2 and 3 mg/L) and KIN (1 mg/L).

Media devoid of plant growth regulator (PGR) was used as a control (C). All PGRs utilised in this study were procured from (Sigma-Aldrich), and were supplemented in the media prior to the autoclaving (121 ºC for 15 minutes) as they all were heat tolerant

2.2 Explant Sterilization & Micropropagation

The first five internodes of the 4 week old glasshouse grown plants were excised and washed under running tap water to eliminate any dust/compost. The explants were then transferred to a laminar air flow cabinet and treated with 70% ethanol for 30 seconds, before being rinsed three times with sterile Millipore water (for 3 minutes each time). They were then treated with 5% (v/v) sodium hypochlorite solution (Sigma-Aldrich) for ten minutes before finally being rinsed three times with sterile Millipore water for three minutes.

Sterilized nodal segments (2 cm), were excised with a sterile scalpel and forceps and transferred into magenta boxes (Fisher Scientific) containing media for direct shoot induction for sterile genetic stocks. Cultures were incubated at 22 ± 2 ℃, 16-h photoperiod and 2500 lux light intensity and sub-cultured every week on fresh media. Following 4 weeks of culturing, each variation in growth was accurately examined and recorded.

2.3 Callus induction & Plant regeneration

Young and healthy plantlets from sterile genetic stock were aseptically cut into 1 cm segments and cultured on a callus inducing, full-strength MS medium (4.34 g/L). The culture plates were sealed and placed in dark at 23 ± 2 ℃. All the inoculated explants were sub-cultured on fresh media every 5 days. The development of callus was observed and recorded after 2 weeks.

The healthy calli were transferred to plant regeneration media. 30 ml of sterile culture medium was dispensed into magenta box and after transferring of the calli in the media, the boxes were sealed with Para film and incubated at 22 ± 2 ºC, and a 16-h photoperiod and 2500 lux light intensity in a controlled growth room. All the inoculated explants were sub-cultured on fresh media every 7 days. Following 8 weeks of culturing, any variation in growth was accurately examined and recorded.

2.4 Acclimatization

Plantlets with well-developed roots were thoroughly rinsed with sterile water to remove excess agar before being planted in autoclaved CNSC compost in 10 cm diameter plastic pots. Before being transferred to the glasshouse, potted plants were kept in the controlled growth chamber (22± 2 ºC; 70% relative humidity) for four weeks.

2.5 Statistical analysis

All trials were conducted in a completely randomised fashion. Each treatment consisted of at least 30 explants that were replicated three times. The analysis of variance (ANOVA) was used to establish significance, and the difference between the means was examined using Duncan's multiple range test (DMRT) using the SPSS (Version 21) programme.

3. Results

3.1 Micropropagation

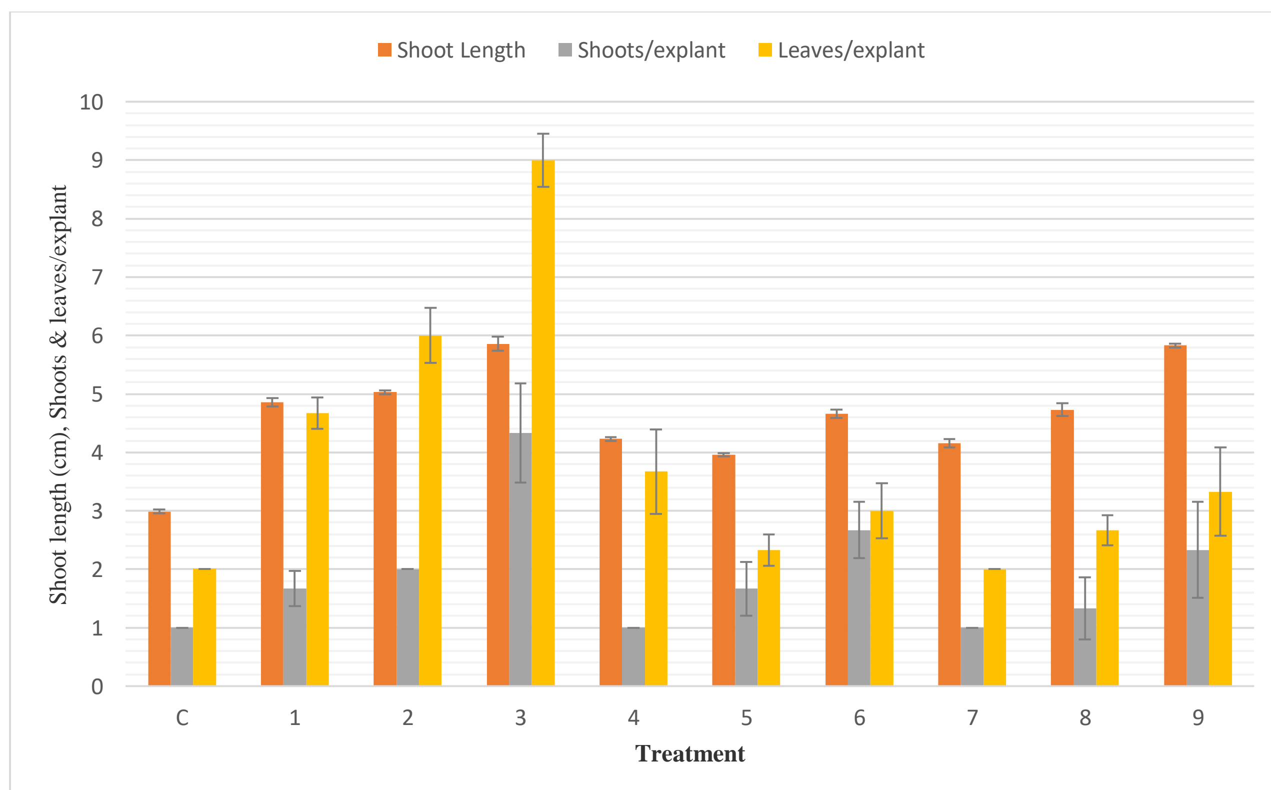
The effect of varying BA, NAA, and KIN concentrations on *in vitro* micropropagation of *Solanum okadae* shoot cultures was evaluated using nodal segments. Characteristics studied included shoot length, shoot number/explant, and leaf number. The response and development of nodal cuttings to *in vitro* micropropagation on MS medium with exogenous plant growth regulators (PGRs) are shown in Table 1 below. The maximum response was shown in nodal cuttings cultivated on MS media with a combination of 3 mg/L BA+2 mg/L NAA+1 mg/L KIN (T3), which had the longest shoot length (5.86 cm), the most shoots per explant (4.33), and the most leaves per explant (9.00) after 28 days (Figure 1).

Table 1: The effect of various concentrations of BAP, NAA and Kinetin on *in vitro* micropropagation of *Solanum okadae* from internode explants at 28 days.

Treatment	BA (mg/L)	NAA (mg/L)	Kin (mg/L)	Shoot length (cm)	Shoots/explant	Leaves/explant
C	0	0	0	2.97 ± 0.0^{e}	1.00 ± 00^{e}	2.01 ± 0.0^{d}
T1	2	2	1	4.86 ± 0.0^{bc}	1.67 ± 0.3^{ed}	4.67 ± 0.2^{bc}
T2	2.5	2	1	5.03 ± 0.0^{bc}	2.00 ± 0.0^{dc}	6.00 ± 0.4^{b}
T3	**3**	**2**	**1**	$\mathbf{5.86 \pm 0.1^{a}}$	$\mathbf{4.33 \pm 0.8^{a}}$	$\mathbf{9.00 \pm 0.4^{a}}$
T4	2	3	1	4.23 ± 0.0^{d}	1.00 ± 0.0^{e}	3.67 ± 0.7^{dc}
T5	2.5	3	1	3.96 ± 0.0^{d}	1.67 ± 0.4^{dc}	2.33 ± 0.2^{d}
T6	3	3	1	4.66 ± 0.0^{c}	2.67 ± 0.4^{b}	3.00 ± 0.4^{dc}
T7	2	2	0	4.16 ± 0.0^{d}	1.00 ± 0^{e}	2.00 ± 0.00^{d}
T8	2.5	2	0	4.73 ± 0.1^{c}	1.33 ± 0.5^{ed}	2.67 ± 0.2^{d}
T9	3	2	0	5.83 ± 0.0^{a}	2.33 ± 0.8^{bc}	3.33 ± 0.7^{dc}

All values represent the means ±S.E. Different letters within a column indicate significant differences at $P < 0.05$ by Duncan's multiple range test

Figure 1: Effect of different treatments of PGRs on shoot length, number of shoots and leaves per explant of *Solanum okadae*



3.2 Callus induction and Regeneration

The effects of three different concentrations of 2, 4-D on callogenesis in *Solanum okadae* via internodal segments are presented in Table 2. The degree of callus development, texture and colour of the callus, callus induction percentage (%), and callus weight (g) were observed characteristics in the callus induction experiment, 14 days following the explants' inoculation. It was found that the best callus development was attained on a MS medium containing 2 mg/L 2, 4-D (Figure 2a). For shoot and root regeneration, calli derived from internodal segments of potato were sub-cultured on MS media supplemented with varied amounts of BA, NAA, and 1 mg/L KIN. Shoot regeneration percentage (%), length (cm) of shoots, and number of shoots per callus and leaves were observed in the shoot regeneration experiment, while root induction percentage (%), number, and length (cm) of roots were observed in the root induction experiment 8 weeks after inoculation of calli on regeneration media (Table 3).

Table 2. The effect of various concentrations of 2, 4-D on *in vitro* callus formation in nodal segments of *Solanum okadae* at 14 days.

2,4-D	Weight (g)	% Callus induction	Colour	Texture
0	-	-	-	-
1	1.86 ± 0.11^{b}	37.66 ± 1.7^{c}	Light yellow	Watery & Soft
2	2.04 ± 0.83^{a}	77.66 ± 4.2^{a}	Light green	Friable
3	1.91 ± 0.33^{b}	51.66 ± 2.6^{b}	white	Loose & Friable

All values represent the means ±S.E. Different letters within a column indicate significant differences at $P< 0.05$ by Duncan's multiple range test

The number of leaves per plantlet, number of shoot/callus and shoot length increased when MS media used for shoot regeneration was supplemented with a combination of 3 mg/L BA+3 mg/L NAA+1 mg/L KIN. Due to the ability of light green and compact calli to regenerate into shoots, they were chosen for the shoot regeneration process. The regeneration of shoots began within 7 days of culturing in this study, and other shoot characteristics were recorded at regular intervals. The best shoot as well as root regeneration percentage was observed on MS medium containing 3 mg/L BA+3 mg/L NAA+ 1mg/L KIN (Figure 2b). The findings showed that a nutritional medium containing low levels of NAA combined with high levels of BAP and KIN encouraged multiple shoot growth and increased the number of nodes and leaves per plantlet.

Table 3. The effect of various concentrations of BAP, NAA and Kinetin (mg/L) on *in vitro* shoot regeneration in callus of *Solanum okadae* at 28 days.

BA	NAA	Kin	% shoot regeneration	Shoot/callus	Shoot length	Number of leaves
2.5	2	1	80.33±0.8^{b}	2.00±0.00^{c}	4.33±0.4^{b}	7.33±1.1^{b}
3	2	1	84.00±1.5^{ba}	2.33±0.4^{c}	5.33±0.5^{a}	8.33±0.5^{b}
2.5	3	1	75.00±1.5^{c}	3.33±0.5^{b}	4.67±0.5^{b}	5.33±0.4^{c}
3	3	1	87.67±0.3^{a}	5.67±0.5^{a}	5.33±0.4^{a}	10.67±0.3^{a}

All values represent the means ±S.E. Different letters within a column indicate significant differences at $P< 0.05$ by Duncan's multiple range test

Figure 2: Plant regeneration via callus (**a.**) Internode derived callus on MS + 2, 4-D (2 mg/L), after 2 weeks ; (**b.**) Plantlets regenerated via callus on MS 3 mg/L BA+3 mg/L NAA+ 1mg/L KIN, after 4 weeks; (**c.**) Callus derived *Solanum okadae* plant in glasshouse after being subjected to acclimatization.

(a.)

(b.)

(c.)

4. Discussion

Potatoes can be reproduced sexually through seeds or asexually through tubers. Seed potato tubers are primarily used for propagation and production. This approach has a lot of drawbacks, including a poor rate of multiplication and a vulnerability to numerous illnesses. In recent years, the plant tissue culture technique has gained popularity as an alternate approach for vegetative multiplication of plants. Plant tissue culture, as a modern-day technology, has enormous potential to fulfil the world's ever-increasing need. It has made important contributions to the advancement of agricultural sciences, and it is now a vital instrument in modern agriculture. With tissue culture, we can now micro-produce many plants with certain desirable features from a single seed or explant; minimise the amount of area necessary for field experiments; and grow disease-free plants through rigorous selection and sterile procedures.

The findings of this study regarding the effect of auxins and cytokinins on potato micropropagation are consistent with those of Koleva *et al*. (2012), who concluded that the composition of MS media with cytokinin and auxin (MS + 2 mg/L BA + 1 mg/L NAA) had the optimum impact on nodal segments of *Solanum tuberosum* for micropropagation. Several studies suggest that nodal cuttings are the best source of explants in either liquid or agar solidified media (Mohapatra & Batra, 2017). Ahmad M *et al*., (2012) also concluded that BA in combination with KIN gave the best results for shoot multiplication using nodal segments of *Solanum tuberosum*, with 2.0 mg/L BA + 0.1 mg/L KIN being the best combination. Sarker & Mustafa (2002) found that in the sprout buds of potato types "Lal Pari and Jam Alu", the BA exhibited greater response in terms of shoot per explants, shoot length, number of nodes, and leaves. On MS basal medium containing 2.0 mg/L BAP and 0.5 mg/L IAA, Hussain *et al*. (2005) acquired the highest regeneration percentage from potato nodal explants. Abeuova *et al*. (2020) discovered that stem internodal explants of *Solanum tuberosum* cultivated on MS medium supplemented with 1 mg/L zeatin, 0.1 mg/L IAA, and 7.0 mg/L GA3 were optimal for direct shoot regeneration and multiple shoot development. The results of this study are in contradiction with the findings of Hajare *et al*., (2021) who found the best shoot initiation on the MS medium supplemented with BAP and NAA at the concentration ranged between 1.5 mg/L BA and 3.0 mg/L NAA in *Solanum tuberosum* 'Gudiene' and 'Belete' varieties.

In the current study, the samples had the greatest averages for shoot length and leaf number when the culture media had low NAA and high BA, along with KIN. When the medium included low NAA and high BA without KIN, the average leaf number decreased (by 67%),

but the average shoot length remained high. This might be attributable to the mechanism of action of cytokinins such as KIN, which is involved in the control of plant cell cycle (i.e., cell division), shoot development, and plant senescence delay (Othman M *et al.*, 2016). To encourage the initial development of plant explants, auxins such as NAA are virtually always necessary. For example, George *et al.* (2008) stated that during the tissue culture cycle, a small concentration of auxin is often advantageous in combination with high levels of cytokinin, especially at the multiplication stage when shoot multiplication is required, though cytokinin alone is sufficient in some cases. As indicated by Badoni & Chauhan (2009), the combination of KIN and NAA produced best results in terms of increasing potato shoot length, where auxin (0.1 mg/L NAA) and cytokinin (0.01 mg/L KIN) at low concentrations resulted in better growth of whole plantlets from potato meristem tips.

The findings of this investigation regarding the callus induction are consistent with those of Khatun *et al*. (2003), who determined that MS+2.5 mg/L 2, 4-D was the optimal combination for callus production in nodal segments of *Solanum tuberosum* 'Diamant'. AL-Hussaini *et al.*, (2015) found that MS media with 2 mg/L 2, 4-D was good for callus proliferation in *Solanum tuberosum* cv. Burren and Riviera. Also, the highest degree of callus formation in internodal segments of *Solanum tuberosum cv. Almera* on MS medium within 7-12 days supplemented with 2.0-5.0 mg/L of 2, 4-D was observed by Khalafalla M *et al.*, (2010). Both of these studies are in agreement with the present investigation. Explants cultivated in MS media without growth regulators, on the other hand, did not create any callus. These findings corroborate those of Abdelaleem K., (2015), and Yasmin *et al.*, (2003). According to Castillo *et al.*, (1998) auxin 2, 4-D has been often used alone or in combination with cytokinins to enhance callus induction and maintenance. Additionally, multiple researchers have found 2, 4- D as the optimal auxin for monocot and dicot callus induction (Jaiswal & Naryan, 1985; Chee, 1990; Khan S *et al.*, 2011).

Internode explants for early callusing generated considerably greater callus diameters and produced elongated shoots, according to Huda *et al*. (2013), hence internodal segments were chosen as the callus induction explants in this study. They also noted that the size of leaf derived callus may be less than that of nodal explants because leaf explants of shorter thickness may dry rapidly depending on light and temperature. Abu Kawochar *et al.*, (2017), reported maximum callus initiation of internode of *Solanum tuberosum* in 3 mg L-1 NAA. In contrast, Haque *et al*. (2009) found that callus generated from leaf explants had the best diameter and weight when cultured on 1.0 mg/L 2, 4-D + 0.25 mg/L KIN. In experiments using potato leaf

explants, Yasmin *et al.*,(2003) reported the maximum percentage of callus (95%) was generated with 2.5 mg/L NAA + 2 mg/L BA. Additionally, Dhital *et al.* (2011) discovered that callus formation was more prevalent from leaf explants than from internode and petiole explants.

For *in vitro* plant tissue cultures, cytokinin is known to play a crucial role in encouraging shoot development, and a high cytokinin to auxin ratio stimulates shoot production from callus (Skoog and Miller, 1957). BA is good for potato callogenesis and organogenesis; when utilised in modest quantities, it significantly improves multiple shoot inductions Sarker & Mustafa (2002). It has been hypothesised that NAA is required for callus initiation and induction, and that MS medium with a mix of NAA and BA generated the best callus formation and shoot regeneration from internodal explants of potato in a short period of time (Nasrin *et al*, 2003). The combination of KIN and NAA proved to be more effective for potato shoot regeneration but, combination of 3 mg/L BA and 1 mg/L NAA proved to be best for regenerating shoots from internodal calli of potato (Shirin *et al.*, 2007), which is similar to the present study. According to Yasmin *et al.*, (2003), MS medium with 2 mg/L BA+2 mg/L NAA had the best results for plant regeneration using *Solanum tuberosum* leaf derived calli.

The results in this study also demonstrate that MS medium with higher concentrations of NAA in combination with lower or equal concentrations of BA and 1 mg/L KIN produced multiple roots per explant and increased the root length when compared to the media containing lower levels of BA and KIN. As a consequence, the results indicated that NAA concentrations in the lower range were necessary for the growth of shoots, whereas concentrations over the threshold resulted in the production of roots. This finding is consistent with Skoog and Miller's (1957) findings, which established how cytokinin and auxin interact to create distinct morphological responses depending on endogenous hormone concentrations and the ratio of exogenous auxin to cytokinin. Pawar *et al.*, (2019) observed maximum plant regeneration via *Solanum tuberosum* callus on MS media with 3 mg/L BA+2 mg/L NAA.

The relevance of addition of NAA (auxin) and BA or KIN (cytokinins) in MS media for *in vitro* potato proliferation has been well documented. According to Yousef *et al.* (1997), the number of nodes and shoot length increased in the medium treated with both NAA and BA. Badoni and Chauhan (2009 & 2012) found that the medium containing NAA along with low KIN concentration provided the best growth and development (shoot and root length as well as node number) of potato plantlets, whereas the medium containing NAA with higher KIN

concentrations yielded the lowest shoot length and number of nodes. The interaction between auxins and cytokinins affected the rate of endogenous auxin production by preventing excess IAA from being oxidised, allowing the optimal quantity for initiating shoot morphogenesis to be maintained (Mohapatra & Batra, 2017).

The leaves of *in vitro* grown plantlets are known for their low water retention capacity due to poorly operative stomata. Since these plantlets lose a lot of water through their leaves, it is critical that their new root systems are established as quickly as possible after being transferred to *ex vitro* settings. In this study, auxin (NAA) treatment boosted the rooting percentage, the quantity of roots, and the speed and synchronisation of rooting in plantlets, despite the fact that auxin is sometimes unnecessary (De Klerk G., 2002). In order for potato plantlets to adapt to *ex vitro* settings and take up water and nutrients, Sanavy & Moeini (2003) underlined the importance of root length and number growth. In this investigation, the rooted plantlets had morphologically consistent and typical shape, growth pattern and leaf form after acclimatization (Figure 2c). All of the plants that were transplanted to *ex vitro* environments had a high level of homogeneity, with no morphological evidence of somaclonal variation.

5. Conclusion

Techniques for growing plants via tissue culture are frequently used as an alternative to the scarcity of high-quality potato propagation material. Micropropagation stands out among these methods because its clonal multiplication protocols are well known and its method is regarded as the primary method of producing pathogen-free seed potatoes. Generally, *in vitro* potato multiplication has been shown to be a very effective method for producing good quality, robust plantlets with normal photosynthetic capability. Shoots, roots, and stem explants with nodes may regenerate readily under optimal circumstances, even without PGRs. Exogenous PGRs, on the other hand, can speed up *in vitro* potato micropropagation by increasing the number of plantlets produced in a shorter period. This study reports direct shoot regeneration and callus induction from internodal explants as well as plantlet regeneration via callus of a diploid potato species (*Solanum okadae*) for the first time.

As per the findings of current investigation, it may be concluded that among the various PGR treatments, MS medium containing 3 mg/L BA+2 mg/LNAA + 1 mg/L KIN improved the multiple shooting capacity of the diploid potato studied and resulted in the maximum improvement in the observed parameters (i.e. shoot length, number of leaves and number of shoots). Whereas MS media enriched with 3 mg/L BA+3 mg/LNAA + 1 mg/L KIN,

regenerated whole plantlets directly from callus tissue. In general, the data provided here also demonstrates an improvement over earlier callus induction results utilising various PGR combinations in *Solanum tuberosum* (Omidi & Shahpiri, 2003; Ashrafzadeh & Leung, 2017)

We have reached a critical juncture in the development of potato breeding. Hybrid breeding, genomic selection, transformation, and gene editing are all techniques that may be used to enhance inbred diploids in a targeted manner. Developing these strategies will depend on trait discovery and as *Solanum okadae* is certainly a source of late blight and drought resistance, current study has established a rapid callus induction and regeneration protocol for *Solanum okadae*, which would aid in subsequent *Agrobacterium*-mediated transformation experiments.

CHAPTER 5: Designing CRISPR-Cas9 construct for S-RNase gene knockout in *Solanum okadae*

1. Introduction

Genome editing is a cutting-edge approach that can be used to improve crops by knocking out genes and introducing insertion/deletion mutagenesis (Hameed *et al*., 2018). It generates double-stranded breaks (DSBs) at specific sites in the genome and repairs DNA repair mechanisms, specifically non-homologous end joining (NHEJ) or homologous recombination (HR). In the past, protein-guided nucleases such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases facilitated this system (TALENs). The focus is now on a new RNA-guided nuclease known as clustered regularly interspaced short palindromic repeats (CRISPR) associated (Cas) (Nadakuduti *et al*., 2018). The TALENs and ZFNs require specialised knowledge, longer timelines, and higher costs than the CRISPR/Cas system. Indeed, it has been reported that CRISPR/Cas has made tremendous strides in its application to agricultural crops. CRISPR/Cas has been demonstrated in potato for the improvement of tuber quality, disease resistance (late blight and potato virus Y), phenotype, and other traits (Hameed *et al*., 2020; Hofvander *et al*., 2021).

CRISPR/Cas is a versatile, simple, effective, and cost-effective system that does not require any protein engineering steps in its development (Lino *et al*., 2018). As a result of these advantages, CRISPR/Cas is a more useful genome-editing system than ZFNs and TALENs. However, CRISPR/Cas still has some drawbacks and limitations, such as PAM restriction and the need for more diversity in CRISPR tools to mediate multiple catalytic activities at the same time (Veillet *et al*., 2019). The decision to use the CRISPR/Cas tool and the general editing strategy is influenced by several factors, including cell type, cellular environment, agent type, and method of delivery, which all impose different constraints and tendencies for unwanted genome modification events (Anzalone *et al*., 2020).

The inbred line M6 was created from the wild tuber-bearing species *Solanum chacoense* to create diploid self-compatible (SC) potato lines (Jansky *et al*., 2014). In M6, a dominant allele of the S-locus inhibitor (*Sli*) renders the gametophytic SI system inactive, resulting in self-compatibility (Hosaka and Hanneman, 1998). *Sli* introgression into other germplasm takes time and runs the risk of linkage drag and fixing undesirable traits, like the donor *S. chacoense's* high tuber glycoalkaloid content. Genome editing can be used to speed up the production of SC diploid lines by focusing on genes involved in SI as a substitute for *Sli* introgression.

Gene knockouts (KOs) of potential agronomic trait-related candidate genes in significant crops have been produced by using the CRISPR-associated protein 9 (Cas9) system(Jaganathan *et al*., 2018). Cas9 causes double-strand breaks (DSBs) in the DNA at the target site, causing endogenous cell repair mechanisms to be activated. Non-homologous end joining (NHEJ), which can result in insertions and deletions in the coding region and a KO of gene function, is one of the cellular mechanisms to repair DNA damage (Bortesi and Fischer, 2015). A single guide RNA (sgRNA) carrying a 20bp target site complementary to the region next to a protospacer adjacent motif (PAM), 5′-NGG-3′, recognises the target DNA, leading to the generation of a DSB that can be used to create DSBs in target genes (Doudna and Charpentier, 2014).

1.1 Construct design for CRISPR/Cas-based genome editing

CRISPR systems, which have been used in a wide range of plant species, are currently the most popular genome editing technology. In many dicot and monocot species, effective genome editing has been accomplished using various CRISPR-Cas systems for fundamental research and crop improvement, and over the past few years, the use of CRISPR-Cas technology in plants has increased significantly (Huang and Puchta, 2021). CRISPR-Cas nucleases, base editors, and prime editors are the three classes of CRISPR technology currently available for genome editing in plants (Zhu *et al*., 2020).

The prospect of choosing a tool for the desired application can therefore be intimidating, especially for researchers who are new to the genome editing technology. This is especially true given the rapid discovery and development of diverse CRISPR toolboxes. In addition to choosing the best CRISPR tools, it can be difficult to deliver CRISPR reagents to plant cells. In some systems, such as mammalian cells, a zygotic cell can receive both the gRNA and a purified protein or mRNA of a Cas protein at the same time. In this way, by regulating the dosage of Cas proteins and gRNAs, targeting possibility can be enhanced. Although this strategy has also been demonstrated to be effective in plants, there are still some major obstacles to be overcome (Hassan *et al*., 2021). Therefore, the most common method of delivering CRISPR reagents to plants is through a construct that contains a Cas gene, at least one gRNA, and the elements needed for their expression (such as a promoter and terminator) through *Agrobacterium*-mediated transformation or particle bombardment. Therefore, a crucial step in carrying out the CRISPR experiment is construct design.

1.2 Design and selection of gRNAs

The choice of an appropriate target site and the design of a potent gRNA against that target are crucial for the success of a CRISPR experiment. Each gRNA is made up of two components: a spacer containing CRISPR RNA and a scaffold sequence known as a trans-activating CRISPR RNA (tracrRNA). For each new target, the spacer sequence is changed. Because this region is the first to bind to the target DNA after PAM recognition, a portion of the spacer sequence close to the PAM site is known as the seed sequence. This region is crucial for target recognition and binding (Shibata *et al*., 2017). Less tolerance is shown for gRNA mismatches in their seed region (i.e., a mismatch between the crRNA and the target site in the seed region might abolish the CRISPR activity). For Cas9 family proteins, the length of the seed sequence is approximately 8 to 12 nucleotides, whereas for Cas12a nucleases, it is 5 to 6 nucleotides. The Cas9 family of proteins' seed sequence has been further trimmed down to 5nt in some studies (Wong *et al*., 2015).

The purpose of the editing determines the site within a targeted region that is chosen for gRNA design. For instance, based on premature termination codon (PTC) induced by NHEJ-generated indels, targets located at earlier exons of a gene have a higher likelihood of producing knockouts. Exons that are very close to an ATG or an intron-exon junction should be avoided, though, as it is typical for PTC to not result in function loss in these areas. This is due to the fact that the PTC is more effectively destroyed by non-sense mediated decay when it is 50–55nt away from the intron–exon junction rather than close to the ATG or intron–exon junction (Popp and Maquat, 2016). The two main factors that affect gRNA efficacy are its associated secondary structure and its nucleotide characteristics. Effective gRNAs typically have a GC content of 30 to 80 percent, no mismatches to the intended target, and a seed region that targets the non-transcribed strand (Zhang *et al*., 2019).

A variety of web-based software can be used to create gRNAs. There are almost 30 websites with tools for creating gRNAs (Gerashchenkov *et al*., 2020). As a result, choosing a website for gRNA design can be challenging. Users must consider a number of factors when choosing a website for designing gRNAs. The type of input that the programme accepts is the first criterion. A user can avoid manually entering the exon sequences of protein-coding genes by using websites that allow them to provide the transcript identifier (from RefSeq or Ensemble), as is the case with CHOPCHOP, which only supports uploaded sequences on some occasions. Many tools create gRNAs that cover multiple transcripts, while others design gRNAs that cover multiple exons of the same gene. This is useful for creating gRNA libraries because it reduces

the possibility that all selected gRNAs will hit an exon that is only moderately expressed (Hanna and Doench, 2020). Another factor users should think about is the variety of genomes that the web tools support, as the vast majority of websites do not allow users to design gRNAs for plants. Fortunately, some websites, like CRISPyWeb, let users create gRNAs using genomes that they supply (Blin *et al.*, 2016). Another crucial element is the variety of Cas enzymes that are supported. Not all online resources enable the creation of gRNAs for Cas proteins that recognise different PAMs. Another crucial aspect to consider is the prediction of both on-target and off-target activity. Users can identify genome-wide off-target mutations using some tools, such as CRISPR, but not others.

When designing and/or choosing gRNAs, the planned downstream experiments are also crucial. For instance, if 'G' or 'A' is the first base of the gRNAs, transcription is significantly improved when a gRNA is expressed using a U6 or U3 promoter respectively. Some programmes (like CRISPR-P) automatically create gRNAs with the U6 promoter's starting codon of "G" or the U3 promoter's starting codon of "A." Additionally, some software enables the creation of gRNAs that either destroy or produce a restriction enzyme after editing, which is helpful for quick analysis of editing events. Researchers suggest using CRISPOR (Concordet and Haeussler, 2018), CRISPR-P (Liu *et al.*, (2017), RGEN Cas designer (Park *et al.*, 2015), or CHOPCHOP (Labun *et al.*, 2019) for CRISPR-Cas nucleases for different design programmes, depending on the editing goals. Although software is typically used to create gRNAs, it is not uncommon for an experienced user to create gRNAs manually that are tailored to meet particular requirements, such as making it simple to detect edits using a restriction fragment length polymorphism analysis.

1.3 Selecting GREs for Cas protein and gRNA expression

Cas protein and gRNA expression levels significantly affect the outcome of CRISPR/Cas-mediated genome editing. Expression at a high level improves editing efficiency, whereas expression at a lower-level decreases efficiency (Feng *et al.*, 2018). To express the Cas gene and gRNA(s) in plants, promoters with strong and constitutive expression patterns are typically used. Most commonly used promoters for *Cas* gene expression in plants are isolated *CaMV* and *NOS* or housekeeping genes (e.g., *UBIQUITIN*, *ACTIN*). Cas protein expressed from constitutive promoters isolated from housekeeping genes of plants, such as *UBIQUITIN* or *ACTIN*, results in higher mutation rates in both monocots and dicots compared to viral constitutive promoters, such as CaMV35S, according to some studies (Hu *et al.*, 2018). In

certain circumstances, tissue-specific, inducible, or developmentally regulated promoters are preferable. When CRISPR reagents are delivered to plant cells via *Agrobacterium*-mediated tissue culture, tissue-specific, inducible, or developmentally regulated promoters have also proven to be extremely effective.

Selecting a promoter to express gRNAs is simpler than selecting a Cas protein. To express gRNAs in plants, RNA polymerase III promoters, such as those found in small nuclear RNA (snRNA) genes U6/U3, are most commonly used. Endogenous U6/U3 promoters are chosen over exogenous U6/U3 promoters, as they have shown to produce better editing results in different plants (Sun *et al.*, 2015). It should be noted that U6/U3 promoters come in a variety of versions. Users must choose the appropriate variants for their experiments because their expression patterns might differ. Also, terminators, like promoters, can influence the stability of Cas and gRNA transcripts, reducing the editing competence of CRISPR/Cas systems (Wang *et al.*, 2015). Several studies have thoroughly assessed the effect of different terminators on the efficiency of the CRISPR/ Cas9 system in plants and concluded that the rbcS-E9 terminator from *Pisum sativum* is the best terminator for Cas9 expression cassette in plants (Ordon *et al.*, 2020).

Previous research in tomato wild relatives showed that missense mutations and gene loss inhibit S-RNase ribonuclease activity in *S. peruvianum* and *S. pennellii*, resulting in self-compatibility (Li and Chetelat, 2015). Given that S-RNase is the gametophytic SI component directly involved in RNA degradation in self-pollen tubes, inhibiting S-RNase function is a simple strategy for conferring self-compatibility in potato. Ye *et al.*, (2018) used CRISPR-Cas9 to generate SC diploid lines with stable self-compatibility in T_0 and T_1 generations in *Solanum phureja*. Enciso-Rodriguez *et al.*, (2019) added to our understanding of SI in diploid potatoes by describing three new S-RNase alleles and generating stable SC KO lines in two diploid lines (DRH-195 and DRH-310). Using a similar approach, this study tried to use CRISPR-Cas9 to knock out the S-RNase gene in a wild diploid potato species, *Solanum okadae*. This chapter, discusses in detail, the construction of two different CRISPR-Cas9 vector systems pRGEB31 (promoter: Rice snoRNA U3 and dual 35S) and pKSE401 (promoter: AtU6-26p), using single (So_1 allele specific) guide RNA with respect to HVb region of S-RNase in *Solanum okadae*.

2. Materials & Methods

2.1 Binary vector selection & Design of single guide RNA

Two binary vectors with different promoter systems were used in this study (Table 1). Both the vectors were procured from Addgene (Massachusetts, USA).

Table 1: Comparison of the two CRISPR-Cas9 plasmids used in this study.

Vector	Addgene catalog no.	Bacterial Resistance	Promoter	Selectable markers
pRGEB31	51295	Kanamycin	Rice snoRNA U3 and dual 35S promoter	Hygromycin
pKSE401	62202	Kanamycin	AtU6-26p	Neomycin/Geniticin

As hypervariable (HV) regions are accountable for determination of specificity of S-RNase and probably interact with the male determinant of the SI response, the 20-nt single guide RNA sequence for So_1-RNase was selected manually from the hypervariable region (HVb: 5'-TTGATCAAGCTTCTGCTCTTAGGA-3') of *Solanum okadae* and used for BLAST analysis against the reference genome for potato (*Solanum tuberosum*, taxid: 4113) in NCBI. This target sequence was retrieved from an amino acid sequence of So_1-RNases from *Solanum okadae* (Acc. KX641173) (Fig 1, Dzidzienyo *et al*, 2016). To avoid knocking out genes that shared the 20bp target site or extremely similar sites, presumed off-target sites were searched using the Cas-OFFinder platform (Bae *et al*., 2014), which is accessible at http://www.rgenome.net/cas-offinder/.

Figure 1: Alignment of amino acid sequence of the 5 S-RNases cloned from *S. okadae*, with hypervariable regions, HVa and HVb.

Suitable adaptor sequences were inserted for cloning at the *BsaI* site. The reverse primer contained an adaptor of 5'-AAAC-3' for both the vectors while the forward primer included an adaptor with 5'-GGCA-3' for pRGEB31 and 5'-ATTG-3' for pKSE401 (Xie *et al.,* 2014). The final guide RNA oligos designed for pRGEB31and pKSE401 binary vectors were purchased from SourceBioscience (https://www.sourcebioscience.com/) and are illustrated in Table 2 below.

Table 2: Oligos designed for respective vectors

Vector	Oligos	Sequence (5'→3')	T_m (°C)
pRGEB31	pR-Hvb_F1	GGCATTGATCAAGCTTCTGCCTAA	58.7
	pR-Hvb_R1	AAACTTAGGCAGAAGCTTGATCAA	55.5
pKSE401	pK- Hvb_F1	ATTGTTGATCAAGCTTCTGCCTAA	54.5
	pK- Hvb_R1	AAACTTAGAGCAGAAGCTTGATCA	55.5

2.2 Binary Vector Construction

All the enzymes and buffers used for binary vector construction were purchased from New England Biolabs (NEB). The vectors were constructed according to the protocol reported by Xie *et al*., (2014).

2.2.1 Vector digestion

2ng of vector (pRGEB3/pKSE401) was digested using 2 µl of 10x Buffer 4, 2 µl of 10x BSA and 1 µl of *Bsa I* at 37°C for 4 hours. De-phosphorylation of these vectors was done by adding 0.5 µl calf intestinal alkaline phosphatase (CIP) and incubating for further 30 min at 37°C. The processed vectors were purified using the PCR purification kit (QIAquick) and stored at -20°C.

2.2.2 DNA oligo-duplex preparation

1µl of 100µM forward and reverse oligos were combined with 1µl of 10x T4 DNA ligase buffer and 0.5µl of T4 PNK. After making up the volume to 10µl, this mix was first incubated at 37°C for 1 h followed by 95°C for 10 minutes in a thermal cycler. The mixture was cooled down to 25 °C at 0.1 °C /sec and diluted to 1:200 using nuclease free water.

2.2.3 Ligation of diluted oligo-duplex into vector

Approximately 50 ng of *BsaI* digested vectors (after thawing on ice) were mixed with 1µl of their respective diluted oligo-duplex, 1µl of 10x T4 DNA ligase buffer and 1µl of T4 DNA

ligase. After adding water to make the total volume of 5µl, the mixture was incubated at 37°C for 4h.

2.2.4 Transformation of *E.coli*

Competent cells of *E. coli* DH5α (Thermofisher, cat no:18265017) were thawed on ice and transformed with 4µl of ligation product using the heat shock method. Almost 100µl of transformed cell mixture was plated on pre-warmed LB agar plates having kanamycin (50µg/mL) for transformant selection. Two random colonies were picked by means of sterile micropipette tip and inoculated in LB medium with 50µg/ml kanamycin and grown overnight on a shaking incubator at 37°C. Plasmids from the transformed DH5α cells were extracted and purified using a Plasmid Mini kit (QIAGEN). Putative cloned plasmids with respective gRNAs were subjected to restriction digestion using *BsaI* and *SacI* to check the integration of respective gRNAs in the vectors which was eventually confirmed by Sanger sequencing using M13R (-48).

2.3 Transformation of *Agrobacterium tumefaciens* (EHA105 and LBA4404) using Electroporation

2.3.1 Competent cell preparation

Frozen glycerol stocks of the *Agrobacterium tumefaciens* strains LBA4404 and EHA105 (kindly provided by Dr. Rupert Fray's lab) were spread on LB agar plates (supplemented with 25 µg/ml of rifampicin and 50 µg/ml kanamycin) and incubated for 2-3 days at 28 °C to produce bacterial lawn that covered the surface of the plate completely. A loop full of this culture was suspended in 25ml of liquid LB media and incubated at 28 °C overnight on a shaker at 280rpm. After the OD_{600} of this culture reached 0.7-1.0, it was centrifuged at 4000rpm for 5 minutes at 4 °C. The supernatant was discarded and the bacterial pellet was washed with 12mL ice cold 10% (v/v) sterile glycerol by centrifugation at 4000rpm for 5 minutes at 4 °C. The washing of pellet was repeated with subsequent halving of ice cold 10% (v/v) sterile glycerol each time until the total volume of culture reached 1ml after which the culture was separated into 100µl aliquots and stored at -80ºC.

2.3.2 Electroporation

Agrobacterium strains: EHA105 (Hood *et al*. 1993) and LBA4404 (Hoekema *et al*. 1983) were transformed with confirmed constructs, pRGEB31 and pKSE401 via electroporation. Tubes containing 100µl competent cells of LBA4404 and EHA105 were thawed on ice and 4µl of

each respective construct was added to these tubes (and mixed by stirring gently with pipette tip). This mixture from vials was transferred to electroporation cuvettes. Electroporation was carried out by setting the program on the electroporator (MicroPulser Electroporator, BIO-RAD) to 'Agro' which applied an electric voltage of approximately 2000 volts and 180 Ω for 1 sec. After that, 1 ml from LB media was added to the electroporation cuvettes and mixed by inverting. This mixture was transferred to new 2 ml tubes and incubated for 4 hours in a 28 °C shaking incubator. 50µl from each transformation tubes were spread on LB agar plates containing 50µg/ml of kanamycin and 25µg/ml rifampicin. The LB agar plates were inverted and placed in a 28 °C incubator for 2-3 days. To check the success of electroporation, 2 random colonies were subjected to colony PCR (https://www.sigmaaldrich.com/GB/en/technical-documents/technical-article/genomics/pcr/colony-pcr) using the primers, reagents and thermocycler conditions mentioned below (Table 3, 4 and 5 respectively).

Table 3: Primers used for Colony PCR

Vector	Primer	Sequence (5'→3')	T_m (ºC)
pRGEB31	pR-Hvb_F1	GGCATTGATCAAGCTTCTGCCTAA	57.7
	hSpCas9-R1	CGCTCGTGCTTCTTATCCTC	55.7
pKSE401	pK- Hvb_F1	ATTGTGATCAAGCTTCTGCTCTAA	55.4
	hSpCas9-R1	CGCTCGTGCTTCTTATCCTC	55.7

Table 4: Colony PCR master mix

Reagents	Volume (µl)
Template	Small colony picked with sterile pipette tip
10x MyTaq™ Reaction Buffer	10
Forward Primer (10µM)	1
Reverse Primer (10µM)	1
MyTaq™ DNA Polymerase	0.1
Water	7.9
Total	20

Table 5: Thermocycling conditions:

Step	Temperature (^{0}C)	Time (min)	Cycles
Cell lysis	94	3	1
Denaturation	94	0.5	
Annealing	55-65	0.5	30
Extension	72	1	
Final Extension	72	10	1
Hold	4	Indefinitely	

3. Results

3.1 Vector construction

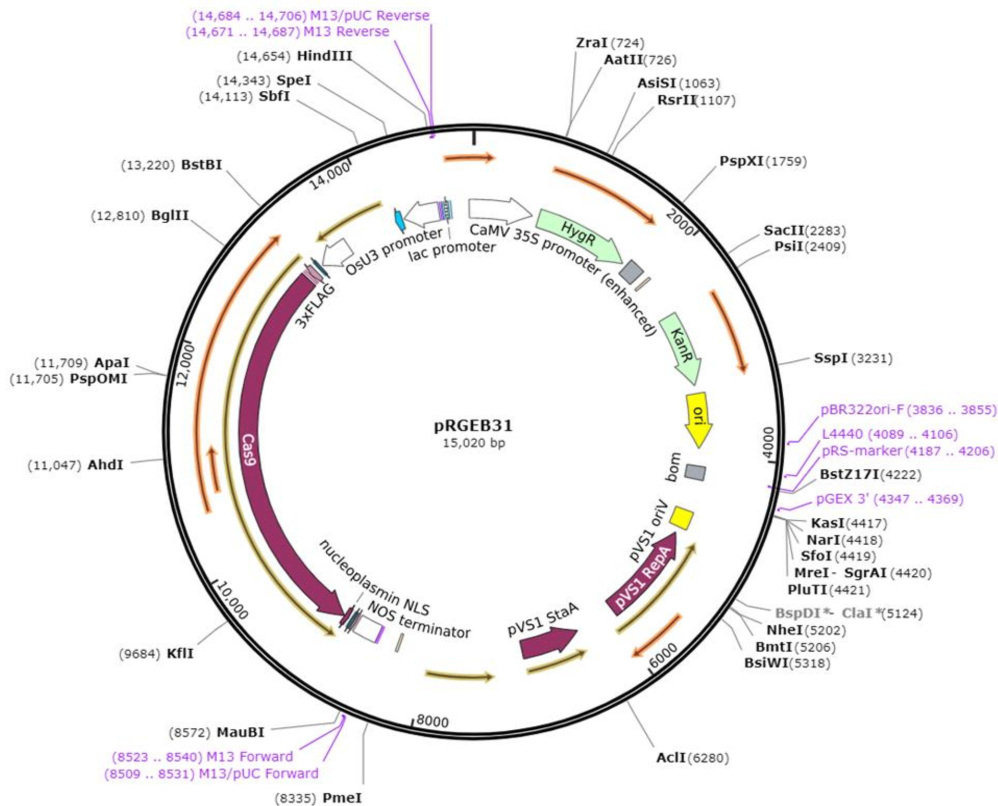
Two RNA-guided genome editing constructs (pRGEB31 and pKSE401) were developed for the expression of gRNA and Cas9 in *Solanum okadae* in order to adapt the CRISPR-Cas9 system for S-RNase knockout. In both the vectors, CaMV 35S promoter was used to control the expression of Cas9. In pRGEB31, the CaMV 35S promoter was fused with 3 FLAG tags. As shown in Figure 2A and 3A below, both the vectors had, (1.) a DNA-dependent RNA polymerase III (Pol III) promoter (rice snoRNA U3 and AtU6-26p promoter in pRGEB31 and pKSE401 respectively) that regulated the expression of designed gRNA in the plant cells, where transcription was stopped by a Pol III terminator (Pol III Term); (2.) a DNA-dependent RNA polymerase II (PolII) promoter (CaMV 35S promoter) that controlled the expression of Cas9 protein; (3.) a multiple cloning site (MCS) located between the PolIII promoter and the gRNA scaffold for inserting a 15-30-bp DNA sequence as gRNA (Figure 2B and 3B). pRGEB31 had a pCAMBIA1300 (hygromycin-resistance gene as a selectable marker) backbone whereas, pKSE401 had pCambia (neomycin-resistance gene as a selectable marker) backbone.

To introduce mutations in So$_1$-RNase, a single guide RNA was designed to target the hypervariable region 'B' (HVb) of the So1-RNase protein with the target sequence 5'-TTGATCAAGCTTCTGCTCTTAGG-3' because it contained 5'-AGG-3'. As this study aimed on using *S. pyogenes* Cas9, whose prospective target sites are both [5'-20nt-NGG] and [5'-CCN-20nt] which is the protospacer adjacent motif (PAM) succeeding the target DNA sequence that is vital for cleavage by Cas nuclease, targeting HVb of So$_1$-RNase was a valid choice.

The presence of gRNAs in constructs was confirmed by restriction analysis using putative transformed 3 colonies (Figure 4). Guide RNA insertion in pRGEB31 resulted in disruption of one of its two *BsaI* site, whereas in pKSE401, gRNA insertion resulted in disruption of its only *BsaI* site. Due to this disruption, positive clones that were subjected to restriction with the *BsaI* and *SacI* enzymes did not release 20bp and 1225bp fragments in pRGEB31 and pKSE401 respectively. Finally, each construct was validated by Sanger sequencing (Figure 5).

Figure 2: Sequence Map for pRGEB31 (Addgene plasmid # 51295) (**A.**) Schematic representation of the pRGEB31 vector that contains a U3 promoter-controlled gRNA production unit and CaMV 35S promoter controlled Cas9 expression cassette. (**B.**) The cloning site of plasmid pRGEB31 with designed gRNA (in red) along with gRNA scaffold.

A.



B.

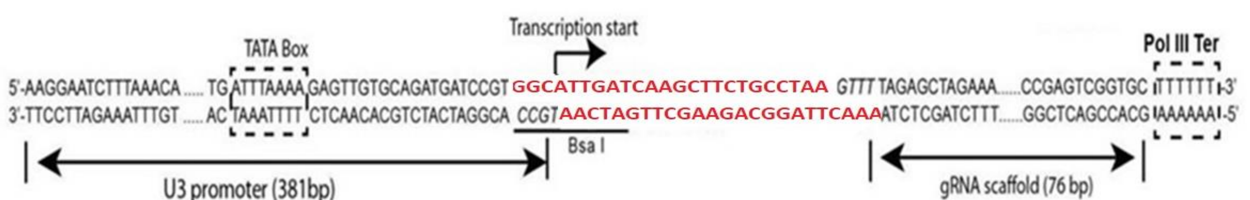
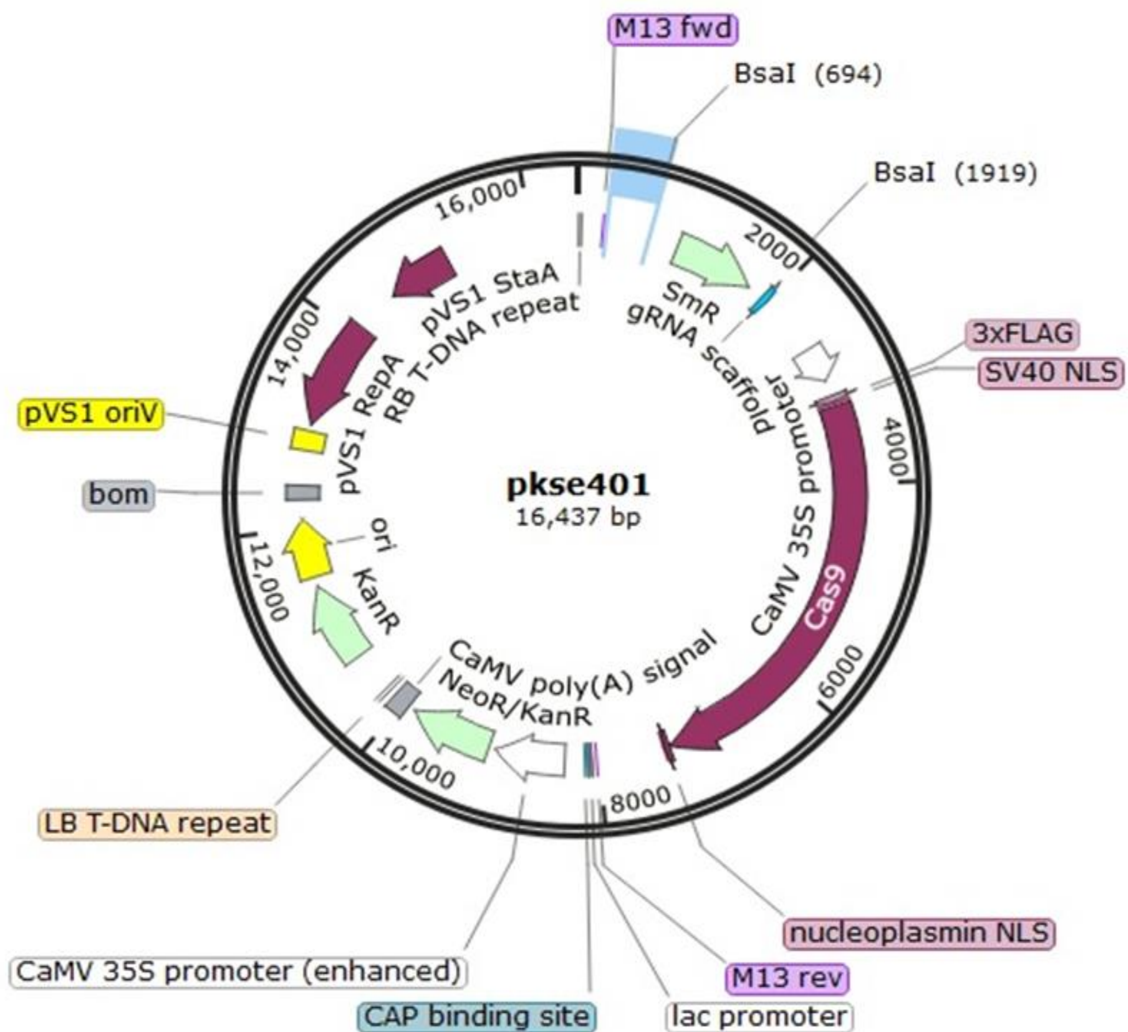


Figure 3: Sequence Map for pKSE401 (Addgene plasmid # 62202). (**A.**) Schematic illustration of pKSE401 vector showing restriction *BsaI* restriction sites and AtU6-26 promoter region highlighted in blue. (**B.**) The cloning site of plasmid pKSE401 with designed gRNA along with gRNA scaffold.

A.



B.

Figure 4 : Restriction analysis using *BsaI* and *SacI* double digest. **(1.)** pRGEB31 (1st band: 9564bp, 2nd band: 3423bp, 3rd: 2013bp, 4th band not visible due to very short (20bp) length; **(2.)** pKSE401 (1st band: 9654bp, 2nd band: 4458bp, 3rd band: 2009bp); **(3.)** pRGEB31 with gRNA (1st band: 9580bp, 2nd band: 3423bp, 3rd band: 2013bp); **(4.)** pKSE401 with gRNA (1st band: 9563bp, 2nd band: 6682bp); **(L)** 1kb DNA ladder

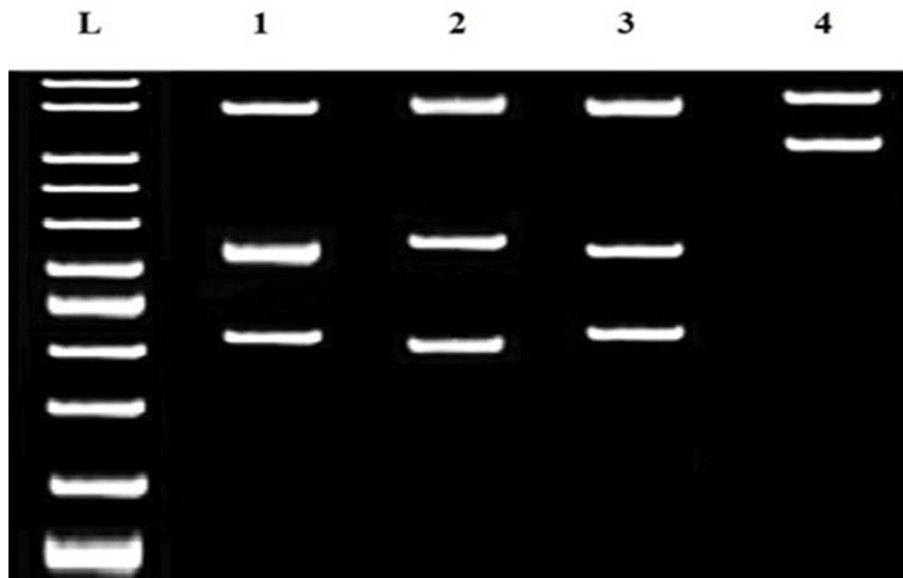


Figure 5 : Sanger sequencing results confirming integration of gRNA into the **(A.)** pRGEB31 and **(B.)** pKSE401, CRISPR-Cas9 constructs.

A.

```
5'  NNNNNNNNTCTTNNTTCTTAGATATGAAGATAATCTTCAAAAGGCCCCTGGGAATCTGAAAGAAGAGAAGCAGGCCCATTTATATGGGAA
3'  NNNNNNNNAGAANNAAGAATCTATACTTCTATTAGAAGTTTTCCGGGGACCCTTAGACTTTCTTCTCTTCGTCCGGGTAAATATACCCTT

    AGAACAATAGTATTTCTTATATAGGCCCATTTAAGTTGAAAACAATCTTCAAAAGTCCCACATCGCTTAGATAAGAAAACGAAGCTGAGT
    TCTTGTTATCATAAAGAATATATCCGGGTAAATTCAACTTTTGTTAGAAGTTTTCAGGGTGTAGCGAATCTATTCTTTTGCTTCGACTCA

    TTATATACAGCTAGAGTCGAAGTAGTGATTGTGATCAAGCTTCTGCTCTAAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGT
    AATATATGTCGATCTCAGCTTCATCACTAACACTAGTTCGAAGACGAGATTCAAAATCTCGATCTTTATCGTTCAATTTTATTCCGATCA

    CCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTGCAAAATTTTCCAGATCGATTTCTTCTTCCTCTGTTCTTCGGCGT
    GGCAATAGTTGAACTTTTTCACCGTGGCTCAGCCACGAAAAAAAACGTTTTAAAAGGTCTAGCTAAAGAAGAAGGAGACAAGAAGCCGCA
```

B.

```
5'  AGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACCGAGACCTCGGGGCATGATCAAGC
3'  TCGGAATAAAATTGAACGATAAAGATCGAGATTTTGGCTCTGGAGCCCCGTACTAGTTCG

    TTCTGCTCTAATCCTGCCACGGATCATCTGCACAACTCTTTTAAATCAGCTTTGATCTAT
    AAGACGAGATTAGGACGGTGCCTAGTAGACGTGTTGAGAAAATTTAGTCGAAACTAGATA

    GTGGATAGCCGAGGTGGTACTAATACTAGTCTTTGTTGTCGTCCAATTGCGTAATGGGCC
    CACCTATCGGCTCCACCATGATTATGATCAGAAACAACAGCAGGTTAACGCATTACCCGG

    GGCCCATACTGCAATACATGTCCTGAAAGGCTTCATGGCCCACTACGAAATGCTTTTCTC
    CCGGGTATGACGTTATGTACAGGACTTTCCGAAGTACCGGGTGATGCTTTACGAAAAGAG
```

3.2 Transformation of *Agrobacterium* strains

A Liquid culture-based preparation method was used to make electro-competent cells as it required less equipment, number of inoculations, and centrifugation steps dropping the possibility of contamination. Colony PCR was carried out on 3 colonies of the *Agrobacterium* strains carrying the desired plasmid in order to confirm the presence of the respective construct. To be extremely sure about *Agrobacterium* strains being transformed by correct constructs (containing gRNA) before commencing the plant transformation, colony PCR was done using the forward primers (pR-Hvb_F1 and pK-Hvb_F1: used in gRNA design) and reverse primer (hSpCas9-R1: part of vector backbone). Colony PCR produced a band of approximately 500bp which indicated the positive clones prepared for transformation (Figure 6).

Figure 6: Colony PCR for confirming gRNA integration **.** Lane 1:1 kb DNA ladder. Lane 2 and 3: Positive transformants of LBA4404 strain harbouring cloned pRGEB31 and pKSE401. Lane 4 and 5: Positive transformants of EHA105 strain harbouring cloned pRGEB31 and pKSE401.

4. Discussion

The vectors pCAMBIA1300/ 2300/3300 and their variants are among the most extensively used binary vectors for a wide range of plant species (Xing *et al.*, 2014), and several plant transformation techniques have been especially tailored using these vectors. As a result, creating pCAMBIA-based CRISPR/Cas9 binary vectors improves their compatibility with some optimal plant transformation techniques. The CaMV35S promoter is a constitutive promoter, and due to its capacity to drive expression in wide range of species, it has maintained its prominence, continuing to be utilised for modern approaches such as CRISPR-Cas9 (Bruce *et al.*, 2019).

In this study, to introduce target sites into the single gRNA vector pRGEB31(Rice snoRNA U3 and dual 35S promoter), a cloning strategy was used that ligated two annealed oligos into the backbone previously digested with a *Bsa1* restriction enzyme. It was quick, cheap and very efficient. A similar strategy was used by Xie *et al.*, (2014) for targeted gene mutation in rice. A second vector, pKSE401 was deployed separately as well just for its different promoter (AtU6-26p, 35s), which has shown its effectiveness in transforming diploid self-incompatible *Solanum tuberosum* group *Phureja* S15-65 (Ye *et al.*,2018). Also, previous research indicate that the RNA polymerase III promoter AtU6-26 has the strong ability to transcribe sgRNAs in dicot plant species (Li *et al.*, 2018). Other studies have also reported a significant improvement in editing results when a specific promoter rather than a constitutive one is employed (Nandy *et al.*, 2019).

In the CRISPR-Cas9 system, targeting of the nuclease to a specific locus depend on the guide RNA (gRNA), along with the company of a target DNA region of a protospacer adjacent motif (PAM) sequence. In this study, to introduce mutations in So_1-RNase gene, a single guide RNA was designed to target the hypervariable region ‘B’ (HVb) of the So_1-RNase protein as it contained 5’-AGG-3’ which is a PAM sequence. The gRNA designed in this study met all the defined conditions mentioned in the studies carried out by Mohr *et al.*, (2016). As we plan to generate a knockout in the genetic system, the importance of limiting off-targets on the same chromosome might compensate the significance of limiting off-targets on other chromosomes that can be ‘cleaned up' in succeeding back-crosses or outcrosses.

Numerous online tools make it promising to view the designs of gRNA in the perspective of a genome browser, as in several cases, picking a suitable gRNA design is vastly reliant on the location of the gRNA relative to exact features of the gene, such as within 500–50 bp of the

transcription start site (e.g. for CRISPRa) (Gilbert *et al.*, 2014), neighbouring the transcription start site (e.g. for CRISPRi), in a mutual coding exon (e.g. for NHEJ-mediated knockout) (Wang *et al.*, 2014), or within a precise intron, exon, protein domain-encoding sequence, or other. Also, in recent times, for NHEJ-facilitated knockout alleles, targeting of protein domains was revealed to result in a greater percentage of loss-of-function mutations, probably due to poorer tolerance of amino acid deletions or substitutions within protein domains (Shi *et al.*, 2014).

While the CRISPR/Cas9 system has shown great promise for site-specific gene editing and other uses, there are various aspects that influence its efficacy that must be addressed, particularly if it is to be employed for *in vivo* gene editing, irrespective of the species. Finest practices in a genetic system comprise of matching the phenotypes of autonomous CRISPR-generated knockouts either individually or in grouping to confirm a relationship between a phenotype and genotype. Researchers investigating gRNA design utilising an online tool developed by others should be aware of any guidelines for managing off targets and maximising efficiency used by a given gRNA design tool, as well as the precise species, delivery method, study, and application from which the rules were generated.

There is extensive use of molecular cloning protocols to create recombinant DNA molecules in molecular biology. Usually, cloning involves suitable restriction enzyme sites in the plasmid and DNA insert for ligation. But, when scientists desire to edit a small part of DNA, few hundreds of base pairs long, in a plasmid, suitable restriction sites adjacent to the area of interest are often inadequate; therefore, scientists frequently edit a bigger DNA fragment as an alternative (Carrigan *et al.*, 2011). In this study, we used a specific DNA cloning process that involved digesting a part of the backbone plasmid using *BsaI* restriction enzyme and ligating new DNA fragments in its gRNA scaffold. So far, limited restriction enzyme-independent protocols have been established by using the CRISPR-Cas system (Barrangou *et al.* 2007). Conversely, scientists can cut DNA in a region missing a suitable enzyme site via CRISPR endonucleases. Besides, it is easy to alter DNA target sites by just altering the guide RNA sequence and it is also inexpensive.

The most extensively utilized method for delivering CRISPR/Cas9 components into dicotyledonous plant cells is stable genomic integration of expression units via *Agrobacterium*-mediated transformation. When working with sexually propagated plants, such as tomatoes, the transfer DNA (T-DNA) can be eradicated in subsequent generations due to Mendelian

segregation, resulting in altered but transgene-free plants. This method, however, cannot be applied to vegetatively propagated and/or highly heterozygous plants, such as potatoes, because sexual reproduction would result in the loss of favourable features (Veillet *et al*., 2020). To overcome these constraints, Chen *et al*. (2018) established a technique based on *Agrobacterium*-mediated transformation of CRISPR/Cas9 reagents followed by a high-throughput screening protocol in tetraploid tobacco, yielding transgene-free mutants without selective pressure.

Due to its numerous advantages, the *Agrobacterium tumefaciens*-mediated transformation is frequently utilized to make transgenic plants. This method has advantages over direct gene transfer techniques like silicon carbide fibres, electroporation, and particle bombardment. The benefits include (1) steady gene expression due to the insertion of the foreign gene into the chromosome of the host plant, (2) low transgene copy number, and (3) the ability to transfer long DNA segments. This technique has been used to genetically modify a variety of crops, flowers, and trees that are significant from an agronomic and horticultural point of view (Ko and Korban, 2004; Lopez *et al*. 2004). Various strains of *Agrobacterium* are utilized to perform the transformation experiments. *Agrobacterium tumefaciens* strains LBA4404 and EHA105 carrying pRGEB31 and pKSE401 to alter diploid potatoes was the focus of the current investigation. To make things easier, the technique of the preparation of competent cells, this experiment used the liquid culture-based preparation method (Kámán-Tóth & Evelin *et al*. 2018) as it requires less equipment and media or a reduced number of inoculation and centrifugation steps (dropping the possibility of contamination). As the results presented by Kámán-Tóth & Evelin *et al*. (2018) showed that their method was efficient enough for routine plasmid transfer into *Agrobacterium*, this study adjusted and simplified it a little to prepare electro-competent cell preparation and perform their transformation.

The type of *Agrobacterium* strain utilized can influence the frequency of transformation. Bakhsh *et al*., (2014) reported that LBA4404 strain produced the most positive transformants in tobacco, followed by GV2260, EHA105, C58C1, and AGL1. Thus, it was determined that *Agrobacterium tumefaciens* strains vary in their capacity to transform tobacco plants and that LBA4404 continues to be the optimum strain for a system that works well for tobacco transformation. A wild potato species (*Solanum brevidens*) was transformed by Liu *et al*. (1995) and the transformation frequencies of the *Agrobacterium* strains LBA4404 and GV2260 were compared. In comparison to LBA4404, the GV2260 strain had a transformation efficiency of 11% as opposed to LBA4404's 1%. A virulent tumor-inducing plasmid makes AGL1 a more

virulent strain (Lazo *et al.*, 1991). Although LBA4404 and AGL1 both had similar transformation efficiencies in *Solanum tuberosum*, Davidson *et al.* (2004) argued that LBA4404 was better suited for potato transformation than AGL1 since a greater proportion of plants transformed with AGL1 had abnormal looks which was also later confirmed by Nadolska-Orczyk *et al.* (2007) in two diploid lines of *Solanum tuberosum*, DG 88-596 (3C) and DG 82-330 (10J). Therefore, the genotype of the potato, the *Agrobacterium* strain, the characteristics of the vector, the transformation method, and phenotypic evaluation of greenhouse plants must all be taken into account when determining if an *Agrobacterium* strain is suitable for the transformation of potatoes.

5. Conclusion

Self-incompatibility has been a limiting aspect in the creation of inbred/F1 hybrid potato cultivars since efforts including crossing with wild SC relatives result in the segregation of numerous unfavourable characteristics in the progenies. To reorient potato breeding toward an effective inbred/F1 hybrid production technique, we tried to utilise the S-RNase-based SI system in diploid potato species *Solanum okadae* and developed self-compatible KO lines by knocking out the S-RNase gene with CRISPR.

In this chapter, we demonstrated, (a.) the successful construction of two different CRISPR-Cas9 vector systems (pRGEB31b and pKSE401) using single guide RNA and, (b.) the transformation of two different strains of *Agrobacterium tumefaciens* (LBA4404 and EHA105) with the respective constructs. The arrangement of components within the plasmid was also taken into account. *A. tumefaciens* transports T-DNA from the right border to the left. To aid in the identification of events that contain the whole cassette, it is preferable for expression to have selectable/screenable markers near the left border and in some cases, a second selectable marker might simplify the process (Bruce *et al.*, 2019). In our case, the pRGEB31 vector had a single selectable marker (hygromycin), whereas kanamycin and neomycin were dual-selectable markers in pKSE401. The selectable markers were situated at the left border of the vector. We also confirmed the vector at each important step (i.e. *E. coli* transformation, *Agrobacterium* transformation) via restriction digestion and Sanger sequencing.

The main problems with tetraploid potatoes are the lack of reliable CRISPR/Cas arrays, target gene selection, effective plant transformation techniques, and few off-target mutants. It is a fact that polyploidy and clonal propagation make multigenic trait improvement more challenging than monogenic trait improvement, especially in potatoes. Despite this, significant progress has

been made in potato, largely by using the gene knockout or insertion/deletion techniques. Studies have indicated that the best strategy for potato is to combine *Agrobacterium*-mediated transformation with multiplexing SpCas9, which can handle one or more sgRNAs by targeting conserved regions. In addition, for genome editing research to be successful, public and governmental knowledge is required. Together, CRISPR-Cas offers quick potato breeding an efficient next-generation toolkit to achieve sustainable crop output.

CHAPTER 6: *Agrobacterium*-mediated transformation of *Solanum okadae*

1. Introduction

The cultivated potato is a highly heterozygous auto-tetraploid. Its genome is most likely the result of hybridizations between diploid wild potatoes that have undergone chromosomal duplication. To minimise inbreeding depression-related problems, such as lower fertility and yield, maintaining heterozygosity is essential. Potatoes are grown as an annual crop via vegetative reproduction utilising tubers as planting material, while reproduction by seed is restricted to breeding programmes only (del Mar Martínez-Prada *et al*., 2021).

Potatoes are vulnerable to a wide range of abiotic and biotic stressors, with defoliating insects being one of the most significant threats, lowering the tubers' yield and quality. The Colorado potato beetle (*Leptinotarsa decemlineata*) stands out among insect infections due to its destructiveness and pesticide tolerance. Other insects feed on tubers, causing plant deterioration, productivity losses, and in rare instances, plant death (Balaško *et al*., 2020). In addition, certain insects can be problematic as *vir*al pathogen vectors, particularly the aphid-borne potato leaf roll *vir*us (PLRV) and potato *vir*us Y (PVY), which can cause up to 80% yield losses (Kreuze *et al*., 2020). Nematodes and several bacterial species, including *Ralstonia solanacearum*, also harm potatoes (Charkowski *et al*., 2020). However, the oomycete pathogen *Phytophthora infestans*, which causes late blight disease and early plant death, is the pathogen that causes the most remarkable losses globally. *Alternaria solani*, several species of *Fusarium* and *Rhizoctonia*, as well as *Passalora concors*, which is responsible for the disease Cercospora leaf blotch, are other fungal pathogens of concern (Kirk and Wharton, 2014). Throughout the growth season, the plant needs multiple insecticidal and antifungal treatments to protect it against these pests and infections.

The potato's increasing sensitivity to pests and diseases is likely attributable to the loss of genetic variety caused by conventional breeding procedures. Selection of tubers with reduced bitterness, for instance, has resulted in lower quantities of insect-repelling glycoalkaloid compounds in the leaves, hence potentially aggravating insect damage (Nadakuduti *et al*., 2018). Due to the abundance of potato species and landraces, there is an abundance of germplasm available for enhancing varietal characteristics, particularly disease resistance

(Dangol *et al*., 2019). Related wild species offer a sizable gene pool that is ready to be utilised in breeding initiatives. Even though 40% of non-domesticated species possess desirable characteristics, their introgression into commercial cultivars via conventional breeding is challenging. Numerous wild varieties have different ploidy levels than cultivated potatoes, resulting in sexual incompatibility, phenotypic diversity, and undesirable impacts (Halterman *et al*., 2015). Therefore, the introduction of genetic engineering technology to potato could have a significant impact on its development (Halterman *et al*., 2015).

There are several copies of each gene in polyploid crops such as potato, necessitating the editing of all haploalleles. This raises the difficulty of gene editing since sgRNA must be designed based on a sequence that is conserved among the four copies. If a conserved sequence cannot be identified, each haploallele will require a distinct sgRNA (Zaman *et al*., 2019). Despite this, CRISPR/Cas9 is widely utilised due to its simplicity and adaptability relative to alternative approaches (Nadakuduti *et al*., 2018). While the improvement of cultivated potatoes depends on the identification and introgression of genes from wild species for features such as disease resistance, the polyploid nature of cultivated tetraploid potatoes impedes the fixation of favourable alleles in new cultivars. In a triplex or quadruplex allelic arrangement, for instance, the introgression of important dominant alleles such as the potato *vir*us Y (PVY) disease-resistance gene can take up to 15 years (Mendoza *et al*., 1996). Re-inventing the potato as a diploid inbred/F1 hybrid variety (2n = 2x = 24) would enable the adoption of effective breeding techniques, since inbred potatoes would accelerate the development of new varieties with advantageous allelic combinations targeting yield, tuber quality, and resistance qualities (Jansky *et al*., 2016). The presence of gametophytic self-incompatibility (SI) in most of the diploid potato germplasm is a substantial impediment to this method, restricting the capacity to develop diploid homozygous lines.

Genome-editing paired with the establishment of inbred diploid lines would represent a tremendous shift in the potential for genetic improvement and create opportunities for a more efficient potato breeding pipeline. Changing to diploid potatoes allows us to create hybrids based on selected inbred lines, allowing us to increase agronomic qualities such as disease resistance and eliminate compatibility hurdles. By knocking out the stylar ribonuclease gene (S-RNase), genome editing was effectively utilised to overcome gametophytic SI in diploid potato (Ye *et al*., 2018, Enciso-Rodriguez *et al*., 2019). The most effective application of genome editing to potato development will include F1 hybrids. In plant transformation, there is a requirement for a set of diploids, inbred, and self-compatible germplasm that forms tubers

with commercial form and appearance and a high regenerative capacity. Although some current diploid lines possess some of the requisite features, additional effort is required to develop germplasm that meets these standards.

An appropriate DNA delivery technology, a productive plant regeneration procedure, and an improved selection technique to identify transgenic cells are necessary for successful plant transformation (Bakhsh, 2020). There are several different DNA delivery techniques available for genetically modifying potatoes and although each system has benefits and drawbacks, a thorough analysis of the information reveals that *Agrobacterium tumefaciens*-mediated transformation is the most popular. Potato was one of the first crops successfully transformed using this approach, and many genetic transformation procedures based on *A. tumefaciens* have been created since then (Ooms *et al*., 1986). However, efficient direct DNA delivery techniques have also been described (Halterman *et al*., 2015). The *A. tumefaciens* approach is more effective than others and yields a higher proportion of plants with a single transgene copy insertion, reducing the likelihood of any negative effects (Gelvin, 2017). Stacking transgenes with *A. tumefaciens* was done successfully in many ways, including crossing individual transgenic plants, re-transformation with independent genetic constructs, use of gene combining constructs, co-transformation with double constructs, and use of polyprotein systems, among others (Romano *et al*., 2001; Rivero *et al*., 2012; Fernandez Bidondo *et al*., 2019).

Direct DNA delivery techniques, such as particle bombardment, protoplast transfection with polyethylene glycol (PEG), protoplasts electroporation and microinjection can be utilised to facilitate DNA delivery (Fehér *et al*., 1991; Romano *et al*., 2001; Valkov *et al*., 2011). These techniques would permit the coordinated integration and expression of several genes as well as the modulation of potato metabolic pathways (Craig *et al*., 2005). Even though these techniques are efficient (Weiland, 2003), problems such as intricate integration patterns, high copy numbers, transgene rearrangements, and gene silencing have been described (Sawahel, 2002). As an appealing alternative to stable transformation, *vir*us-induced gene silencing produces transitory loss-of-function to quickly evaluate the activity of genes (Dobnik *et al*., 2016).

The most well-known and recommended method for potato transformation is *A. tumefaciens*-mediated transformation. The best DNA delivery approach will depend on the experimental goal, such as transient or stable integration and/or nuclear or plastid expression. To contribute to the development of diploid inbred potato lines, this study attempted to establish self-

compatible (SC) diploid lines in wild potato species, *Solanum okadae*, by knocking out S-RNase gene with CRISPR-Cas9. This chapter discusses the protocol for *Agrobacterium*-mediated transformation of *Solanum okadae* in detail.

2. Materials & Methods

2.1 Plant material

To maintain sterile conditions, 4-week-old *Solanum okadae* plantlets (S_1S_5) that had been grown *in vitro* were employed for transformation experiments (Chapter 4). Establishing an effective regeneration system from leaf and stem explants for each new genotype utilised in a transformation experiment is a must before starting transformation. In addition to being genotype-specific, regeneration from explants is also influenced by the different combinations of plant growth regulators present in the culture media (Nadakuduti *et al.*, 2019), and therefore, a suitable regeneration protocol was optimised for *Solanum okadae* using nodal segments which is described in detail in Chapter 4.

2.2 Stock solutions

All solutions, containers, and equipment were sterilized by autoclaving at 121 °C and 1.05 kg/cm^2 (15-20 psi). All plant growth regulators (Sigma-Aldrich) were co-autoclavable and therefore added to the media directly prior to autoclaving and the antibiotics were filter sterilized.

a.) **1 mg/ml Kinetin:** 10 mg of kinetin was dissolved in 200 µl of 1 N NaOH. MilliQ water was added to make the total volume of 10 ml. This stock was stored at -20°C.

b.) **1 mg/ml Naphthaleneacetic acid (NAA):** 10 mg of NAA was dissolved in 200 µl of 1 N NaOH by shaking vigorously. MilliQ water was added to make the total volume of 10 ml. This stock was stored at 4°C.

c.) **1 mg/ml 6-Benzylaminopurine (BAP):** 10 mg of BAP was dissolved in 1 ml of 1 N NaOH by shaking vigorously. MilliQ water was added to make the total volume of 10 ml. This stock was stored at 4°C.

d.) **2 mg/mL 2,4-Dichlorophenoxyacetic acid (2,4-D):** 0.05 g of 2,4-D was dissolved in a few drops of 1 N NaOH and the total volume was made up to 25 ml with MilliQ water. The stock solution was filter sterilised through a 0.22 µm filter and stored at 20°C.

e.) **50 mg/ml Kanamycin (Kan):** 0.5 mg of Kan was dissolved in 10 ml of MilliQ water. This stock was filter sterilised through a 0.22 µm filter and stored at -20°C.

f.) **10 mg/ml Rifampicin (Rif):** 0.1 mg of Rif was dissolved in 10 ml of 100% methanol. This stock stored at -20°C.

g.) **50 mg/ml Geniticin (Geni):** 0.5 mg of Gen was dissolved in 10 ml of MilliQ water. This stock was filter sterilised through a 0.22 µm filter and stored at -20°C.

h.) **50 mg/ml Hygromycin (Hyg):** 0.5 mg of Hyg was dissolved in 10 ml of MilliQ water. This stock was filter sterilised through a 0.22 µm filter and stored at -20°C.

i.) **250 mg/ml Carbenicillin:** 2.5 g of carbenicillin was dissolved in 10 ml of MilliQ water. This stock was filter sterilised through a 0.22 µm filter and stored at -20°C.

j.) **74 mM Acetosyringone (3' ,5' -Dimethoxy-40 -hydroxyacetophenone) :**145.18 mg of acetosyringone was dissolved in 10 mL of dimethyl sulfoxide (DMSO). The stock was aliquoted in 1ml tubes and stored at -20°C.

2.3 Media preparation

All media were prepared in a 1 L volume and divided into two 1 L glass bottles, each carrying 500 mL, before being autoclaved for 20 minutes at 121°C.

a.) **Luria-Bertani broth (LB):** Tryptone (10 g), yeast extract (5 g) and NaCl (5 g) were added to 800ml distilled water and mixed well to ensure all the components dissolved properly. The total volume was made to 1 L and the media was then dispensed in small glass bottles (100 ml) and autoclaved. 1 g of agar was added to each 100 ml LB media bottle for preparing solid LB media. After autoclaving, the media was transferred to laminar flow hood and cooled down to 50°C before adding any antibiotic (if needed).

b.) **Stock plant media (SPM):** 500 ml of distilled water was added to a 1 L glass bottle and placed on a magnetic stirrer after adding a magnetic stir bar into the bottle. 30 g of sucrose was added to the bottle followed by 4.3 g of MS basal media and 1 ml of Gamborg's vitamin solution (Sigma-Aldrich, G1019). The total volume was made up to 1 L with distilled water. The pH was adjusted to 5.7±0.1 using 1 M HCl or 1 M NaOH. Prior to autoclaving, 0.8 g of agar was added per 100 ml liquid SPM media to prepare a solid SPM media. After autoclaving, the glass bottles containing media were transferred to laminar flow hood and cooled down to 50°C before pouring in individual magenta boxes GA-7 or petri plates.

c.) **Callus induction media (CIM):** SPM medium containing 1 ml of 2,4-D stock solution (2 mg/L) was used as CIM media. After autoclaving, the glass bottles containing CIM

media were transferred to laminar flow hood and cooled down to 50°C before pouring in individual petri plates.

d.) **Regeneration media (RM):** 4.3 g of MS basal salts, 1 ml of Gamborg's vitamin solution, 30 of sucrose, 3 ml of BAP stock solution (1 mg/L), 3 ml of NAA stock solution (1 mg/L) and 1 ml of kinetin stock solution (1 mg/L) were added to 500 ml of distilled water and mixed using a magnetic stirrer. The total volume was made up to 1 L after adjusting the pH to 5.7±0.1 using 1 M NaOH. 8 g of agar was added to this liquid media before autoclaving. Prior to pouring in individual petri plates, the glass bottles containing media were transferred to laminar flow hood and cooled down to 50°C before adding carbenicillin stock (250 mg/L) and appropriate selection agent (hygromycin for pRGEB31 and geniticin for pKSE401).

2.3 Transformation of *Solanum okadae*

Agrobacterium-mediated transformation of *Solanum okadae* was attempted according to the protocols described by Nadakuduti *et al*., (2019) in *Solanum tuberosum* and Ye *et al*., (2018) in *Solanum tuberosum group Phureja* S15-65, with some minor alterations. Four groups of experiments were set up (i.e. each of the two strains of *Agrobacterium* harbouring each of the two vectors, individually). All the procedures were performed in laminar flow hoods and biosafety cabinets to avoid contaminations.

2.3.1 Preparation of explants and pre-culture

Leaves and stem from *in vitro* growing healthy 4-week-old plantlets were excised. For internodal explants, thick stem internodal segments were cut and kept horizontally on SPM. Plates comprising of explants were wrapped with micropore tape and placed under lights for two days at 22±2ºC with a 16/8-hour light/dark photoperiod.

2.3.2 Inoculation, Co-cultivation and Regeneration

a.) A few days before the transformation experiment, a fresh plate of *Agrobacterium* stock from -80ºC was streaked onto a LB plate with appropriate antibiotic (Kan+Rif) and incubated at 28ºC for 2 days.

b.) The day before transformation experiment, 10 ml of LB broth was inoculated with a loopful of *Agrobacterium* colony from the plate above and incubated at 28ºC on a shaker incubator (200 rpm) until the OD_{600} reached 0.6-0.7.

c.) On the inoculation day, *Agrobacterium* culture was centrifuged for 15 min at 9000xg at room temperature. The pellet was re-suspended in 10 ml liquid SPM and this suspension was transferred to a sterile flask. 50µL of 74 mM acetosyringone was added to this suspension.

d.) The pre-cultured explants were carefully transferred to the flask containing *Agrobacterium* suspension. The flask was sealed with sterile cotton buds and incubated on shaker incubator at 90 rpm for 30-45 min at 22-26ºC. It was ensured that the *Agrobacterium* solution washed over all the explants.

e.) The *Agrobacterium* solution was then decanted into a disposal vessel and treated with chemgene.

f.) The explants were carefully transferred onto a clean petri plate with sterile filter pater using sterile forceps and gently blotted o remove excess of *Agrobacterium* solution. Approximately, 15 blotted explants were placed on to a fresh CIM plate, which was then wrapped with a sealing film and placed in dark at 22/26ºC for 2-3 days.

g.) After 3 days of co-cultivation, all the explants were collected into a sterile flask and rinsed with MilliQ water containing 50µL stocks of carbenicillin. Rinsing was continued till the water ran out clear.

h.) After rinsing, all the explants were blotted dry and transferred to the RM plates containing the appropriate antibiotics for each respective plasmid and carbenicillin to remove *Agrobacterium* from the culture.

i.) The plates were kept in growth rooms at 22±2ºC with a 16/8-hour light/dark photoperiod. The explants were sub-cultured on fresh RM every 7 days.

j.) Emerging shoots were excised and transferred into the magenta boxes having RM with appropriate selective agents (geniticin/hygromycin).

3. Results

To contribute to the development of diploid inbred potato lines, this study attempted to establish self-compatible (SC) diploid lines in wild diploid potato species, *Solanum okadae*, by knocking out S-RNase gene with CRISPR-Cas9 via *Agrobacterium*-mediated transformation. In total, 36 batches of *Agrobacterium*-mediated transformation experiments of *Solanum okadae* using callus cultures (20/experiment) and nodal segments (100/experiment) as explant material were performed. The combination of parameters applied are mentioned in table 1 below. The efficacy of all the antibiotics used as a selection agent was checked (Fig 1A and 1B) and the blackening of leaves in selection media showed that the selection antibiotics were potent. Unfortunately, the transformation of *Solanum okadae* proved to be ineffective since the shoots developing in the selection media were unable to survive and perished within a week of being excised and transplanted in fresh selection media. In some batches, The explants showed positive response at first but turned black just like control indicating unsuccessful transformation.

Table 1: Different parameters (constant and variable) used for transforming *S. okadae*.

<table>
<tr><th>Vector</th><th>Constant parameter</th><th>Variable parameter</th></tr>
<tr><td>pRGEB31</td><td>Hygromycin (Selection)
Carbenicillin</td><td rowspan="2">Agrobacterium strain: LBA4404, EHA105
Infection time: 30 min, 45 min
Co-cultivation time: 2 days, 3 days</td></tr>
<tr><td>pKSE401</td><td>Geniticin (Selection)
Carbenicillin</td></tr>
</table>

Figure 1: Transformation of *Solanum okadae*. (A.) Non-GM control to check efficacy of hygromycin as selection agent, (B.) Non-GM control to check efficacy of geniticin as selection agent, (C.) & (D.) Nodal segments subjected to transformation using *Agrobacterium tumefaciens* EHA105 harbouring pRGEB31 and pKSE401 constructs respectively.

4. Discussion

Potato is predominantly a polyploid crop, with autotetraploid genotypes dominating agricultural settings, breeding efforts, and genetic research. The majority of autotetraploid potato cultivars are of the subspecies '*tuberosum*' (*Solanum tuberosum ssp. tuberosum*) and are easily transformable using *Agrobacterium tumefaciens*-based plant transformation techniques (Halterman *et al*., 2015). However, autotetraploid potato types are genetically extremely heterozygous and are frequently quite sterile, making them unsuitable for genetic analysis and functional genomics research. It is extremely difficult to alter all four alleles using SSNs, and

chimerism observed in SSN-based main events in typical *Agrobacterium tumefaciens*-based protocols has further hindered genetic and genome editing efforts, respectively (Dangol *et al.*, 2019).

The vast majority of wild, diploid potato species are self-incompatible and incapable of inbreeding. However, self-compatible accessions of *Solanum chacoense* that are capable of setting selfed seed without artificial intervention have been discovered (Jansky *et al.*, 2014). A particular inbred line of *S. chacoense*, designated 'M6', was crossed with the homozygous, doubled-monoploid ('DM') potato (Xu *et al*, 2011) used to produce the potato reference genome. The resultant F1 hybrid (DMF1) is self-compatible and has been utilised to create the first F2 population in potato produced from an inbred line (Endelman and Jansky, 2016). The genome of M6 was also sequenced, offering additional genetic resources for M6-derived lines and highlighting the significance of inbred lines for functional genomics (Leisner *et al.*, 2018).

The most important factors affecting the transformation efficiency of potato are discussed in detail below. These factor could have either been individually or together been responsible for unsuccessful transformation of *Solanum okadae* in the current study.

4.1 Potato genotype

The capacity of specific *Agrobacterium* strains to transform plant cells is determined by their chromosomal and plasmid genomes, which must encode all the machinery required for attachment and DNA-transfer. However, plants produce a variety of inducer molecules with varying inducing ability and cellular concentration. This variation influences the level of *vir* gene expression in various hosts, hence influencing their susceptibility to *Agrobacterium* infection. A low level of *vir* gene expression can render a plant resistant to infection due to the bacterium's inability to generate and transmit sufficient T-strand DNA required for a successful infection (Karami, 2008).

The vulnerability of various plant species to *Agrobacterium* infection varies significantly (Cheng *et al.* 2004). Even within the same species, different cultivars or ecotypes may exhibit significantly varying degrees of susceptibility to tumerogenesis induced by specific *Agrobacterium* strains (Karami, 2008). These differences have been observed in maize and aspen (Ritchie *et al.* 1993), several legumes (Owens and Cress 1984), tomato (van Roekel *et al.* 1993), grape (Lowe and Krul 1991) and *Arabidopsis* (Nam *et al.* 1997).

There are approximately 4,000 landraces of native potatoes, most of which are located in the Andean Region, as well as over 180 wild potato species. Despite the large varieties of potato available, only a few cultivars are commercialised, chosen for their marketability and storage ability (Yang *et al.*, 2016). Early transformation assay favourite included the cultivars "Desiree" and "Bintje," but today most potato cultivars are tissue culture-friendly, and almost all commercially significant varieties can be successfully transformed with changes to the standard protocols (Kaur *et al.*, 2020). In 1988, the use of leaf discs to transform the four potato cultivars "Bintje," "Berolina," "Desiree," and "Russet Burbank" was reported as a genotype-independent technique (De Block, 1988). However, later studies revealed that transformation efficiency is genotype dependent (Conner *et al.*, 1992; Kumar *et al.*, 1995; Heeres *et al.*, 2002). Also, different plant cultivars belonging to the same genus and species might demonstrate drastically variable genetic transformation efficiencies. Nevertheless, the genetic reasons behind these disparities in transformation rate remain largely unidentified (Hisano and Sato, 2016). Several studies have been conducted to compare the regeneration and transformation efficiency of different genotypes using the same methodology, and the results show that the success in generating transgenic plants varies (Heeres *et al.*, 2002; Han *et al.*, 2015; Bakhsh, 2020). Furthermore, Heeres *et al.* (2002) demonstrated that regeneration and transformation efficiency are genetically distinct variables. Therefore, the success of transformation for a given genotype is dependent on a number of critical factors, including *Agrobacterium* strain, vector, mode of injury, infection time, explant type, pre-culture period, co-cultivation time, composition of the subculture media, and selection markers, among others (Kaur and Devi, 2019; Bakhsh, 2020).

The presence of inhibitors of the *Agrobacterium* sensory apparatus like 2-hydroxy-4,7-dimethoxybenzoxazin-3-one (MDIBOA), may explain the difference in sensitivity of genotypes to *Agrobacterium*. The presence of such inhibitors may explain the differential in resistance of agronomically important plant species to *Agrobacterium*-mediated genetic transformation, rather than insufficient activation of the *Agrobacterium* pathogenicity machinery by the exudates of the host cell (Karami, 2008). Natural inhibitors of signal reception by the *Vir*A/*Vir*G two-component regulatory system appear to play a key role in host defence (Zhang *et al.* 2000). Plants, on the other hand, may see *Agrobacterium* and the transferred transgenes as foreign particles and activate their defence mechanisms to combat infection and the expression of foreign genes.

4.2 Strains of *Agrobacterium tumefaciens*

Once the usage of a bacterial delivery system has been specified, the next criterion to be determined is the strain to employ, as it affects the efficacy of genetic transformation. In this regard, the LBA4404 strain is the most frequently utilised in potato transformation techniques (Heeres *et al*., 2002; Bakhsh, 2020; Rahman-Mollika *et al*., 2020). However, other strains, such as EHA105, GV2260, GV3101, and C58C1, have also been utilised with success (Kumar *et al*., 1995; Banerjee *et al*., 2006; Han *et al*., 2015; Ahmed *et al*., 2018; Craze *et al*., 2018; Bruce and Shoup Rupp, 2019; Décima Oneto *et al*., 2020). Despite multiple studies, there is no apparent link between the strain and the genotype to be transformed; therefore, the best appropriate strain must be experimentally determined for each genotype.

The inability of *Agrobacterium* to infect different genotypes or species has also been a key hindrance in the genetic transformation of elite agricultural cultivars. McCormac *et al*. (1998) discovered that only EHA101 permitted T-DNA transport into wheat when they evaluated the T-DNA transfer efficiency of two *A. rhizogenes* strains (LBA9402 and Ar2626) and two *A. tumefaciens strains* (LBA4404 and EHA101). The majority of crops have been genetically transformed using *Agrobacterium* strains LBA4404, EHA101, and their derivatives (EHA105 from EHA101, AGL0 and AGL1 from EHA101) (Cheng *et al*. 2004). Huang and Wei (2005) demonstrated that EHA105 outperforms LBA4404 in maize transformation. The insertion of the constitutively active *vir*G mutant gene in a binary vector has also been demonstrated to improve T-DNA delivery in both monocot and dicot species (van der Fits *et al*. 2000; Ke *et al*. 2001). These findings suggest that, using different strains and combining them with superbinary vectors or binary vectors containing a constitutively active *vir*G may increase transformation efficiency in several recalcitrant species.

Once the strain has been chosen, there are three primary elements that influence transformation efficiency: the optical density of the bacterial suspension, the addition of a centrifugation step prior to inoculation, and the length of the co-cultivation time. According to the reviewed research, the ideal optical density values range between 0.5 to 0.8, depending on the growth medium and the construct. Some experiments show that centrifuging the bacterial culture prior to inoculation may have an adverse effect on the viability of the bacteria, resulting in a significant decrease in transformation efficiency (Beaujean *et al*., 1998; Banerjee *et al*., 2006; Décima Oneto *et al*., 2020). The co-cultivation duration should be long enough to allow effective T-DNA transfer, but not so long that tissue damage and somaclonal variation occur.

As a result, the ideal time is between 1-4 days, with 2 days being the most commonly observed co-cultivation period (Millam, 2006). To acquire the best transformation efficiency, it is crucial to experimentally identify not only the ideal strain but also the co-cultivation conditions for each genotype. In our study, we selected the two most widely used *Agrobacterium* strains to transform potatoes and used the recommended optical density of 0.6-08 along with a 3 day co-cultivation period but, still couldn't achieve successful transformation. This could be attributed to the centrifugation of bacterial culture prior to inoculation, negatively affecting the bacterial viability.

4.3 Vectors

The vector to utilise based on the chosen transformation approach is another crucial decision. Among other considerations, the type, size, regulatory elements, selectable marker, cloning efficiency, cost, and availability must be considered.

The type and size of the vector are directly proportional to the transformation approach used, mostly whether a shuttle (Hellens *et al*., 2000) or non-binary vector is required. Regarding regulatory components, the ubiquitous and constitutive CaMV35S promoter is typically utilised to express transgenes in potato; nevertheless, there are numerous instances of other promoters successfully used for specialised purposes (Yevtushenko *et al*., 2004; Pino *et al*., 2007; Li *et al*., 2015; Nahirñak *et al*., 2019). In addition, well-characterized tissue-specific or inducible potato promoters have been identified for a cisgenic strategy (Naumkina *et al*., 2007; Almasia *et al*., 2010). Avoiding repetitive regulatory sequences to prevent possible silencing effects is an additional factor to consider in vector design.

Genes encoding antibiotic or herbicide resistance are commonly utilised as selectable markers for identifying altered plant cells (Bruce and Shoup Rupp, 2019). Most potato transformation procedures use the neomycin phosphotransferase II (nptII) gene, which provides resistance to the antibiotic kanamycin (Barrell *et al*., 2013). Barrell *et al*. (2002) assessed the efficacy of various selectable markers for potato transformation using identical vectors. The following rankings reflect the effectiveness of recovering transgenic lines: resistance to kanamycin is followed by resistance to hygromycin, phosphinothricin, phleomycin, and methotrexate.

Other indicators for potato transformation that use xylose or galactose as selecting agents rather than antibiotics or herbicides have been identified (Joersbo *et al*., 2003). Although their use needs further optimization, these systems provide alternatives to the traditional ones. Although to a lesser extent, visual marker genes like glucuronidase, luciferase, and green fluorescent

protein have also been used (Verhees *et al*., 2002; Rakosy-Tican *et al*., 2007). Potato transformation without the use of markers has also been documented. This method relies on the detection of transformed plants using PCR screening of plants that have been grown from seed again without the use of a selection system (De Vetten *et al*., 2003; Ahmad *et al*., 2008).

Regarding *Agrobacterium tumefaciens* binary vectors, consideration should also be given to the layout of expression cassettes in the plasmid. The selectable/screenable markers should be located at the left border to assist the selection of events comprising the whole cassette, as T-DNA is transferred from the right border to the left. Additionally, the cassettes' inwards opposite orientation should be avoided to minimise possible silencing effects and finally, before beginning the transformation experiment, it is critical to confirm the vector using DNA sequencing. (Nahirñak *et al*., 2022).

4.4 Explants

For a genetic transformation system to work, it is important to come up with a fast and effective way to regenerate plants. As explants, scientists have used leaves, stems, tubers, petioles, protoplasts, and microtubers to create transgenic potato lines.

Frequently, *in vitro* plants serve as the source of explant tissue for potato transformation (Barrell *et al*., 2013). Their availability of homogenous, pathogen-free material for transformation is their primary benefit (Newell *et al*., 1991) . Micropropagation of shoot cultures *in vitro* enables the year-round availability of healthy, vigorously growing plant material (Visser, 1991) . Since it is sterile and already acclimatised to grow under *in vitro* conditions, surface sterilisation of the plant tissue is unnecessary, minimising handling time, the risk of contamination, and plant stress caused by chemical treatments (Kumar, 1995).

The most commonly utilised explants are leaves and stem internodes from *in vitro* plants because they are easily available and simple to use. It has been claimed that stem internodes are rather strong and may be easily handled in greater numbers, in contrast to leaf explants, which are fragile and susceptible to damage during transformation, hence limiting the frequency of transformation and regeneration (Newell *et al*., 1991).

The somaclonal variance that might occur during the callus phase is the primary drawback of using stem and leaf explants in transformation tests (Visser, 1991). Utilizing tubers reduces the likelihood of somaclonal variation, which is favourable (Ishida *et al*., 1989). *In vitro*-grown microtubers have various advantages over soil-grown tubers since they are created from *vir*us-

free, aseptically-grown potato shoots, they can be produced quickly and in large quantities at any time, and they require less space for storage (Kumar, 1995).

The physiological status of the explant material is a crucial component regulating the frequency of callus and regeneration (Chakravarty and Wang-Pruski, 2010). To guarantee a successful transformation, young and healthy explants from the stock plant are desirable, as damaged tissue will reduce regeneration potential (Craze *et al*., 2018). Furthermore, the explant should be large enough to withstand *Agrobacterium* co-cultivation while retaining moisture during transformation procedures. The explant used for transformation is frequently dictated by the cultivar or by the expertise of each laboratory (Barrell *et al*., 2013).

To summarise, there are numerous alternatives for tissue susceptible to potato transformation; nevertheless, it must be a healthy material, sterile, transformable, and regenerative in order to achieve good and reproducible outcomes. In this study, even though the chosen explant material (internodal segments) from *Solanum okadae* were raised *in vitro* and showed excellent regenerative potential, it still couldn't be transformed which could be attributed to its susceptibility to *Agrobacterium* as several studies have revealed that different tissues, organs, and cell types within a plant may be more or less susceptible to *Agrobacterium* transformation (Karami, 2008). Therefore, in future, different parts of *Solanum okadae* could be used as potential explant materials to optimise its transformation. Also, deploying a reporter gene for testing *Agrobacterium* infection can be performed.

4.5 Antibiotics

For transformation, plant tissues are co-cultivated for two to three days in an antibiotic-free medium with a disarmed *A. tumefaciens* carrying the gene of interest. The bacterium must be suppressed (exterminated) after co-cultivation in order to prevent it from obstructing the development and growth of the transformed plant cells. The effectiveness of the plant regeneration systems is important for successful transformation using *Agrobacterium*, but it's also important that this bacterium is later eliminated from transformed cells. It is crucial to get rid of *Agrobacterium* because its continued presence can make it difficult to identify transformants, impede the growth and development of the transformed plant cells, or even lead to the death of the cultures. This removal of unwanted *Agrobacterium* is usually done by adding one or more antibiotics to the culture medium (Matzk *et al*. 1996). The two antibiotics that are most frequently used for this are carbenicillin and cefotaxime.

It is difficult to select the right concentration of selective agents in plant transformation to enable organogenesis on the one hand while avoiding escapes on the other (Holford and Newbury 1992). Antibiotics have the potential to inhibit cell wall synthesis whereas bacterial contamination slows regeneration and causes plant death. This effect may result in reduced transformation efficiency. As a result, having the proper antibiotic concentration is critical for achieving antibiotic protection/selection and adequate rates of organogenesis. There have been numerous reports on antibiotic toxicity to callus growth and shoot regeneration (da Silva and Fukai, 2003), but only a few on toxicity to somatic embryogenesis (Tang *et al.* 2000).

Antibiotics are limited in their ability to eliminate *Agrobacterium* because some antibiotics, such as carbenicillin and ticarcillin, can be inactivated by bacterial f3-lactamases, while others, such as cefotaxime, are highly resistant to f3-lactamases but inhibit plant regeneration (Ling *et al.* 1998). There have been several reports that another antibiotic, timentin (ticarcillin combined with clavulanic acid, a specific f3-lactamase inhibitor), can effectively eradicate *Agrobacterium* without hindering plant regeneration (Tang *et al.* 2000). However, Cheng *et al.*, (1998) had reported that, in Siberian elm (*Ulmus pumila L.*) and tobacco (*Nicotiana tabaccum*), carbenicillin and cefotaxime were as effective in subduing *A. tumefaciens* (LBA4404) as timentin at concentrations generally used in transformation experiments.

4.6 Plant growth regulators

Plant growth regulators (PGRs) are vital to the transformation of plants. The selection several PGRs is the most influential element of transformation efficiency, as evaluated by the frequencies of stable integration and transient expression. Competence for transformation may be missing or low in recalcitrant explants; however, PGR treatments can increase this competency. When pre-cultured on a medium containing PGRs, an explant becomes susceptible to *Agrobacterium* (Chateau *et al.* 2000).

In numerous plant tissues, PGR treatment stimulates cell division and de-differentiation. The acceleration of cell division by PGRs suggests that *Agrobacterium* transformation may be most effective at a particular point of the plant cell cycle. Villemont *et al.* (1997) studied the involvement of the plant cell cycle in *Agrobacterium*-mediated transformation of *Petunia* mesophyll cells synchronised with phase-specific cell cycle inhibitors. Non-cycling cells that had not been treated with PGRs were incapable of being transiently or permanently transformed to express a T-DNA-encoded gusA transgene. Similarly, cells treated with mimosine, which halts the cell cycle in late G1 phase, did not divide. In addition, the cells with the highest

transformation competence were those with a very high ratio of S and G2 phases:M phase. The authors came to the conclusion that *Agrobacterium*-mediated transient transformation requires S phase DNA synthesis since T-DNA could be taken up, transported to the nucleus, and expressed in cells performing DNA synthesis but not in the presence of cell division. T-DNA incorporation and transformation stabilisation required further cell division. Therefore, to distinguish between these two possibilities, a direct assay for T-strand absorption and nuclear translocation that does not rely on the expression of genes encoded by T-DNA is needed.

There are numerous forms of plant tissue culture media, the majority of which are based on Murashige and Skoog's MS Media (Murashige and Skoog, 1962). MS medium contains cytokinins, auxins, gibberellins, abscisic acid, and ethylene, as well as a range of minor salts, vitamins, PGRs and sucrose. Treatment of explants with zeatin or zeatin riboside with low levels of auxin often facilitates the callus induction stage, but the shoot induction stage often includes a reduction of zeatin and auxin, as well as the addition of gibberellin to encourage shoot outgrowth. Regeneration rates per explant are typically high, with the first shoots appearing around 4-6 weeks, depending on the genotype (Millam, 2006). It is required to modify and identify the ratio of growth regulators best suitable for de-differentiation and callus formation for each potato genotype, which also depends on the explant employed (Zhang *et al*., 2020).

4.7 Light

T-DNA transfer was inhibited in intact tobacco seedlings sprayed with *A. tumefaciens* and kept in the dark, according to Escudero and Hohn (1997). Clercq *et al.* (2002) discovered that continuous darkness for 24 hours decreased *Agrobacterium*-mediated transformation in bean compared to light for 16 hours each day. However, light is linked to a variety of physiological parameters, including plant hormone levels, cell proliferation, and cell cycle stage (Zambre *et al*. 2003). The effectiveness of T-DNA transfer is largely determined by how well *vir* genes are activated. Light has been demonstrated to increase the amount of the phenolic *vir* gene inducer in *Dendrobium* (Nan *et al*. 1997). Light had no effect on the transient expression of a CaMV p35SuidA gene supplied to *Picea* embryos or seedlings via particle bombardment (Gray-Mitsumune *et al*. 1996). As a result, light's stimulatory effect may be unique to *Agrobacterium*-mediated T-DNA delivery.

Numerous *Agrobacterium*-based plant transformation strategies for agricultural and model plant species employ dark co-culture conditions without quantifying the impact on

transformation rate. Incubation in the dark appears to enhance the morphogenic potential of callus or explants, primarily by retaining light-sensitive endogenous hormones or by avoiding the buildup of phenolic chemicals (Arezki *et al*. 2001). Compton (1999) discovered that a dark pre-treatment boosted the regeneration of shoots from cotyledonary node explants in diploid watermelon. It has been claimed that persistent darkness during co-culture increases transformation in carnation. In *Typha latifolia*, dark cultured callus exhibited greater transient activity than light cultured callus (Nandakumar *et al*. 2004). Dark preparation results in less vascular tissue and weaker cell walls, hence enhancing the penetration of *Agrobacterium* (Hartmann *et al*., 1997). Compton (1999) anticipated that incubating callus or explants in darkness would enhance their morphogenic ability.

According to some experiments, the influence of light on *Agrobacterium*-mediated transformation is primarily determined by photoperiod. According to Zambre *et al*. (2003), light greatly facilitated gene transfer from *A. tumefaciens* to plant cells in *Phaseolus acutifolius* (tepary bean), and the transgenic frequency was higher in co-cultivation under continuous light than in co-cultivation under a 16 h light/18 h dark regime. In *Arabidopsis*, *uidA* expression was strongly and positively associated with the light period employed during co-culture; it was significantly suppressed by darkness and increased more under continuous light than under a 16 h light/8 h dark photoperiod (Zambre *et al*. 2003). The difference in results between investigations of light impacts on transformation could be attributed to the interaction of light regime and other factors.

4.8 Temperature

Temperature has been identified as a factor influencing *Agrobacterium's* ability to transfer T-DNA to plant cells. Early research on *A. tumefaciens*-mediated tumorigenesis revealed that high temperatures inhibited tumour formation. Jin *et al*. (1993) revealed that the prevention of tumour growth was attributable to a conformational alteration in *vir*A generated at around 32ºC. Studies of bacterial conjugation in which an incompatibility group, Q plasmid, was mobilised by the T-DNA transfer machinery revealed that the best temperature for transfer was 19°C (Fullner and Nester 1996). Kudirka *et al*. (1986) showed that the temperature of co-cultivation of *Agrobacterium* and soybean explants was crucial where co-cultivation at 30°C for 48 hours inhibited transformation, but co-cultivation at 25°C promoted transformation. Transformation was only decreased if the heat treatment followed the 25°C co-cultivation, suggesting that

thermosensitivity was maximum during the T-DNA transferal from *Agrobacterium* to the host plant cell.

Dillen *et al*. (1997) studied the effects of temperature on gene transfer to plants using *Phaseolus acutifolius* callus and *Nicotiana tabacum* leaf discs. Soon after co-cultivation, the GUS reporter gene was employed to detect transient expression. The best temperature for gene transfer in both species was 22°C, and there was no significant difference between 19°C and 22°C for *Nicotiana tabacum*. As the temperature rose from 22°C to 25°C, the transient GUS expression decreased dramatically. In contrast to Dillen *et al*. (1997), Srivatanakul *et al*. (2001) found that shoot apex survival on selection medium was considerably larger at 25°C and 28°C than at 16°C and 19°C in dicotyledonous kenaf (*Hibiscus cannabinus*). Co-cultivation of cotton with *Agrobacterium* at a lower temperature substantially enhanced transformation efficiency (Jin *et al*. 2005). Salas *et al*. (2001) also observed the effect of co-cultivation period temperature on transient and stable T-DNA integration in *Nicotiana tabacum*. Co-cultivation of target tissues at four different temperatures (15, 19, 25 and 32°C) resulted in the maximum number of stably transformed plants at 25°C. Kondo *et al*. (2000) examined the effect of four temperatures, (i.e. 18, 20, 22, and 24°C), on the delivery of T-DNA using garlic calluses. At 22°C, 64% of all calluses exhibited transitory GUS activity, representing the greatest level of GUS expression recorded. At 20°C and 24°C, the proportion of GUS-stained calluses to total calluses dropped by 85 and 69 percent, respectively. When a conventional binary vector was used, a higher transformation frequency was reported in maize immature embryo transformation at 20°C than at 23°C (Frame *et al*. 2002). Transgenic maize plants were also produced from the elite inbred lines PHP38 and PHN46 by co-culture of immature embryos at 20°C followed by subculture at 28°C (Gordon-Kamm *et al*. 2002). All these findings suggest that the best temperature for T-DNA transfer may not be the best temperature for stable transformation for a specific species and explant. The ideal temperature for steady transformation should be determined for each individual explant and *Agrobacterium* strain.

5. Conclusion

Despite considerable success in genetically modifying plant crops, one of the primary technological problems in plant transformation is the improvement of methods for generating a high proportion of plants with stable and precise transgene expression. Many horticulturally and agronomically important species are now routinely transformed using *Agrobacterium*, and the list of species amenable to *Agrobacterium*-mediated transformation appears to be growing

all the time. Many economically significant plant species, or elite variations of specific species, are, however, resistant to *Agrobacterium*-mediated transformation. Necrosis and tissue browning caused by *Agrobacterium* infection remain significant barriers to genetic change.

In the current investigation, attempts to transform wild diploid potato species, *Solanum okadae* to produced inbred lines were unsuccessful even after a thorough review of literature and following appropriate protocols and precautions (i.e. vector, promoter system and *Agrobacterium* selection) which could be attributed to either one or more factors discussed above. A better understanding of the host (*Solanum okadae*) biological processes involved in transformation will reveal the principles that govern *Agrobacterium*-host cell interactions that result in the unique event of trans-kingdom gene transfer, provide novel insights into the cellular processes themselves, and aid in the development of new strategies for plant's efficient genetic transformation. The identification and molecular characterisation of *Solanum okadae's* genes involved in *Agrobacterium*-mediated transformation could open new possibilities for better understanding of the plant's response to *Agrobacterium* infection and such data could aid in the development of ways to increase its transformation efficiency. Although efficient transformation systems have been established in various potato genotypes, and several important factors influencing *Agrobacterium*-mediated potato transformation have been investigated and optimised, many genotypes like *Solanum okadae*, still require development and improvement of transformation parameters such as optimising inoculation, co-culture conditions and using readily available explants.

CHAPTER 7: Identification of putative SLF gene sequences in *Solanum okadae*

1. Introduction

Early economic interest in SI went beyond fruit trees and cabbages. Other agricultural plants included were the potato, sunflower, rye, cocoa and pomegranate. de Nettancourt (2001) published a thorough analysis of the early studies on SI in many different species. The fundamental molecular causes of SI were unknown until the middle of the 1980s. New techniques for modulating SI for the purpose of crop production and plant breeding have been developed as a result of later discoveries. Consistent evidence identifying the molecular causes of SI is now available for the Papaveraceae, Plantaginaceae, Solanaceae, Rosaceae, and Brassicaceae, however a plethora of investigations are ongoing in various other species.

1.1 Molecular Mechanisms of SI

Recent reviews of SI mechanisms offer extensive explanations of molecular and genetic mechanisms (Wang *et al*., 2019; Sehgal and Singh, 2018; Bedinger *et al*., 2017; Fujii *et al*., 2016). S-RNase-based gametophytic self-incompatibility (GSI) is found in families such as Solanaceae and Rosaceae. Surprisingly, the specific processes vary greatly despite the fact that various taxa use similar genes to control the specificity of pollen rejection. But in all families, the S-locus has at least two connected genes (although often many more). A single gene produces pistil-expressed glycoproteins with ribonuclease activity (S-RNases), which function as cytotoxin that result in pollen rejection when their single S-haplotype matches one of the two S-haplotypes of the diploid pistil (Xue *et al*., 1996). The other is an F-box protein gene, known as SLF or SFB depending on the family, that is only expressed in pollen. Importantly, the Rosaceae tribe Maleae (apple, pear) and the Solanaceae have a variety of 16–20 SLF genes that collectively contribute to pollen SI activities (Kubo *et al*., 2010; Williams *et al*., 2014).

F-box proteins are best known for their roles in the 26S ubiquitin/proteasome pathway (via the SCF complex formed with SKP1 and Cullin1 proteins), and the reported interaction between the AhSLF$_2$ F-box protein and self/cross S-RNases in *Antirrhinum* pollen suggested that cross S-RNases might be inactivated via this pathway (Qiao *et al*., 2004). These and other findings support a model in which non-self S-RNases are degraded in compatible pollinations, but self S-RNases evade degradation and degrade pollen RNA in self-pollinations. Ubiquitination and degradation of S-RNase are attributed to the collective action of an array of 16 to 20 SLF

proteins in *Solanaceae* (Kubo *et al*., 2010), but self-S-RNase is not degraded because it is not recognised (Kubo *et al*., 2015). This is known as the collaborative non-self-recognition model (the array of SLF proteins recognises non-self S-RNase), and it is the most widely accepted model at the moment. S-RNases, on the other hand, are sequestered in the pollen tube endomembrane system, which may also contribute to compatibility (Goldraij *et al*., 2006). In addition, the collaborative non-self-recognition model only addresses the roles of S-RNase and SLF proteins, and there is compelling evidence that pistil factors (modifiers) that do not contribute to S-specificity are nonetheless required for SI in *Solanaceae*.

An *in vitro* protein pull-down assay revealed that non-self-interactions between allelic variants of SLF and S-RNase were stronger than self-interactions, suggesting that the substrate of a SCF-SLF complex may be non-self S-RNase(s) for the particular allelic variant of SLF in the complex (McClure *et al*., 2004). This may help to explain why only self-S-RNase, which is not ubiquitinated or degraded in the pollen tube, can have a cytotoxic effect on the pollen tube. It is challenging to imagine how an allelic variant of SLF could interact with so many non-self S-RNases but not with a single self S-RNase given the large number of S-haplotypes found in *Petunia* and the high degree of sequence diversity displayed by allelic variants of S-RNase. When it was discovered that at least two SLF1 paralogous genes are also involved in pollen specificity, this puzzle was resolved (Kubo *et al*., 2010). According to the model called "collaborative non-self-recognition," pollen from an S-haplotype produces multiple SLF proteins that work together to recognise and detoxify all non-self S-RNases; however, none of these SLF proteins can interact with their own self-S-RNase (Kubo *et al*., 2010). Based on pollen transcriptome analysis, it has been determined that *Petunia inflata's* S2- and S3-haplotypes share the same 17 SLF genes as of yet (Williams *et al*., 2014). Additionally, eight additional *Petunia* S-haplotypes have been discovered to contain 16–20 SLF genes (Kubo *et al*., 2015). Eight of them have so far been proven to be involved in pollen specificity by an *in vivo* functional assay (SLF1, SLF2, SLF3, SLF4, SLF5, SLF6, SLF8, and SLF9) (Kubo *et al*., 2010, 2015; Williams *et al*., 2014).

1.2 Identification and Function of the *Sli* gene

Although the majority of diploid potato lines are self-incompatible (SI), there are self-compatible diploid potato lines that can be utilised to introduce SI into diploid potato breeding programmes. Hosaka and Hanneman (1998) isolated a dominant S-locus inhibitor (*Sli*) gene from a *Solanum chacoense* accession and used it to create potato inbred lines. Hosaka and

Hanneman proposed that *Sli* is a pollen-expressed gene with sporophytic activity and that homozygosity for *Sli* is lethal because homozygous *SliSli* genotypes were not present in the F8 population of *S. chacoense*.

Two independent groups have hypothesised that *Sli* is gametophytic which if true, could lead to segregation distortion among future generations. *PGSC0003DMG400016861* was ultimately identified as the candidate gene for *Sli* in various self-compatible diploid potato genotypes (Eggers *et al*., 2021; Ma *et al*., 2021). The gametophytic expression of *Sli* has been demonstrated by the observed segregation distortion for SC in the F_2 population of a cross between SI and SC. Eggers *et al*. (2021) fine-mapped the *Sli* interval from 628 kb to 12.6 kb using recombinant screening. This work resulted in the discovery of two genes, *PGSC0003DMG400016861* and *PGSC0003DMG400016860*, in *S. chacoense* (DS)-derived inbred lines. Ma *et al*. (2021) used a similar method to map the SC loci in RH, another self-compatible long-y adaptable line derived from *S. tuberosum* group *Tuberosum*. In various SC genotypes, these two investigations have discovered the two annotated genes *PGSC0003DMG400016861* and *PGSC0003DMG400016860* in the interval mapped for SC on chromosome 12. In addition, *PGSC0003DMG400016861* is exclusively expressed in pollen, as demonstrated by the transcriptome study of *in-vitro* germinated pollen from 10 SC and SI genotypes.

It took more accessible potato sequence information for the transgenic method to overcome SI to become a reality. However, using a transgenic method to overcome SI has become simpler thanks to the accessibility of *Sli* gene sequences. When an expression construct with the exons of the SC allele of *Sli* is introduced between the diploid SI potato's native promoter and terminator as used for transformation, it is able to overcome SI. Limited evidence for the loss of SI were provided by self-berry, seed set, and pollen tube penetration farther into the style after self-fertilization. If *Sli* is the main component of SC, then its functional shutdown could result in loss of SC. Consequently, losing SC and producing SI offspring after deleting the first exon of *Sli* in SC using CRISPR-Cas9 provides more proof that *Sli* alone is responsible for preventing SI in potatoes (Kardile *et al*., 2022).

The name given to *Sli* after its discovery reveals the namer's belief that *Sli* somehow prevented the S-locus from functioning (Hosaka *et al*., 1998). But it is now evident that SLF, a different active gene in the S-locus, shares similarities with its product. During pollen tube elongation, *Sli* may operate as SLF to interact with and detoxify S-RNases, giving self-incompatible lines

SC. It encodes the PP2-B10 F-box protein, which has an F-box domain connected to a lectin domain. Lectin domains have been shown to interact with carbohydrates and may do the same with proteins that have been glycosylated, such as S-RNase. The F-box domain's downstream motifs may give ubiquitination substrate selectivity. Both yeast two-hybrid experiments and firefly luciferase (LUC) complementation assays demonstrated that the majority of the StS-RNases interacted with the C-terminal region (Phloem Protein 2 domain) of *Sli*. *Sli* interacted with the majority of the 14 StS-RNases tested, with the exception of 3, 9, 10, and 13. However, StS-RNase 3, 9, 11, 13, and 14 did not interact with full-length *Sli* (F-box and PP2 domain). In contrast, the C-terminal portion of a male SLF gene from RHC01H2G1617 could only associate with StS-RNase9. This result shows that, contrary to what the collaborative non-self-recognition model predicts, one SLF typically interacts with a small number of non-self S-RNases. In order to out-cross and detoxify non-self S-RNases, solanaceous plants need a variety of SLFs, one for each S-haplotype (Kubo *et al*., 2010).

It was previously proposed, and in accordance with the two-step model (Fujii *et al*., 2016), that *Sli* developed as a novel SLF. *Sli* has been demonstrated to interact with self S-RNase as well as several types of S-RNases in order to outcross with other diploids. As a result, the presence of *Sli* may be an effective way to induce a fixed SC phenotype into S-RNase-based self-incompatible plants. *Sli*, on the other hand, does not interact with all types of S-RNases, implying that the *Sli* gene does not transfer SC to all self-incompatible lines. Furthermore, harmful mutations linked to *Sli* impede efforts to alleviate SI using this gene. As a result, using SC genes in breeding can successfully bypass the genetic bottlenecks induced by the detrimental mutations associated with these locations. Large potato germplasms can now be easily screened for new sources of SC thanks to the availability of SC genotyping utilising KASP^{TM} markers. The SC candidate region in potatoes has been identified using these markers. Rough purple chili has been identified as the source of *Sli* in all European and North American cultivars by pedigree research (Clot *et al*., 2020). However, distinguishing the SC haplotype across historical *S. tuberosum* cultivars and *S. chacoense* clones is difficult. *Sli* in S. *tuberosum* could be another example of gene flow between wild and cultivated potato germplasm, as has been observed for a number of other genes (Hardigan *et al*., 2017). However, commenting on the direction and timetable of a potential *Sli* introgression is problematic. Rough purple chili, the source of cytoplasm in present Chilean *S. tuberosum* cultivars, is thought to have crossed with the wild. *S. chacoense* or a more recent hybridization between self-compatible *S. tuberosum* dihaploids and the progenitor of M6 may have happened (Kardile *et al*., 2022).

The origin of the *Sli* locus in *S. chaocense* clone M6 is explained by K-mer analysis and distance estimate using Mash software. This investigation has demonstrated that *Sli* is a common occurrence, which may enable the use of several clones to breed SC diploid potatoes. By mating dihaploids from particular tetraploid kinds with M6 and other wild sources, it is suggested that SC can be introduced into *S. tuberosum* (Zhang *et al*., 2019). This data demonstrates that the SC haplotype is common among tetraploid varieties and that *Sli* must be present in the variety chosen for dihaploid induction. *Sli* has been discovered by KASP tests in the dihaploids of the potato cultivars "Atlantic," "Superior," and the breeding clone NY148, which is interesting (Kaiser *et al*., 2021). This demonstrates that *Sli* introgression is not necessary to extend the diploid SC gene pool.

1.3 The SCF-SLF Complex

Co-immunoprecipitation (Co-IP) followed by mass spectrometry (MS) revealed the presence of a typical Rbx1 (PiRBX1; a RING-finger protein), a pollen-specific Cullin1 (PiCUL1-P), and a pollen-specific Skp1-like protein in the SCF-SLF complex of *P. inflata* (Li *et al*., 2014). Similar elements have been found in the SCF complex of *Pyrus bretschneideri* and *Petunia hybrida* (Xu *et al*., 2013; Entani *et al*., 2014). According to phylogenetic analyses, the 17 SLF proteins of *P. inflata* form a monoclade, and the SSK1 and Cullin1 proteins implicated in SI form their own monoclades (Xu *et al*., 2013; Yuan *et al*., 2014). Additionally, the only F-box proteins that co-immunoprecipitated with PiSSK1 were SLF proteins (Li *et al*., 2014). Interestingly, tomato SpCUL1 is involved in unilateral incompatibility between tomato species and is also necessary for compatible pollination in *Solanum arcanum*, sharing 91% sequence identity with PiCUL1-P (Li and Chetelat, 2013). PiSSK1, PiCUL1-P, and SLF, three of the SCF-SLF complex's four parts, thus, seem to have developed specifically to work in SI.

The Co-IP followed by MS approach was successful in identifying three (SLF1, SLF4, and SLF13) of *P. inflata's* 17 SLF proteins as the F-box component of the SCF-SLF complexes (Li *et al*., 2014). An *in vivo* functional assay has confirmed the involvement of eight of these 17 SLF proteins in pollen specificity (including SLF1 and SLF4) (Sijacic *et al*., 2004; Williams *et al*., 2014a; Kubo *et al*., 2015). This assay involves raising transgenic plants to examine the effect of expressing a particular allelic variant of an SLF on the SI behaviour of the transgenic pollen. When SI breakdown is observed in transgenic plants, progeny from crosses with wild-type plants of appropriate S-genotypes must also be examined. The Co-IP-MS results suggest that this method may be a much quicker and less labour-intensive alternative to the *in vivo*

functional assay for assessing SLF protein SI function. If all SLF proteins are assembled into similar SCF complexes (Williams *et al.*, 2014), it begs the question of how all these sequence-divergent SLF proteins can be assembled into their respective SCFSLF complexes, and whether allelic variants of S-RNase taken into a pollen tube may favour the "selection" of specific SLF proteins that can interact with and detoxify the non-self S-RNases, especially when the complex's common components are limited.

1.4 S-Locus F-Box Proteins (SLFs)

Each of the *Petunia* SLF proteins studied thus far interacts with only one or a few of the S-RNases studied (Sijacic *et al.*, 2004; Kubo *et al.*, 2010). This pattern of interactions between SLF proteins and S-RNases is consistent with the collaborative non-self-recognition model's prediction (Kubo *et al.*, 2010). So, how does one SLF protein interact with a specific S-RNase but not others? How did all of these SLF proteins evolve so that pollen of a given S-haplotype has a complete arsenal to counteract the cytotoxic effect of all non-self S-RNases while avoiding interaction with their own S-RNase?

F-box proteins typically have two domains: the F-box domain at the N-terminus and a protein-protein interaction domain at the C-terminus (C-terminal domain or CTD; Wang *et al.*, 2004). Thus, it is reasonable to examine an SLF's CTD to identify the amino acids involved in its interaction with a specific S-RNase. Linking the amino acid sequences of SLF proteins that interact with the same S-RNase with the amino acid sequences of SLF proteins that do not would be one method for recognizing such amino acids. The amino acids in the CTD that are conserved among all SLF proteins that interact with the same S-RNase but divergent among those that do not are most likely important for that S-specific RNase's interaction. Knowing the interaction relationships between as many SLF proteins and S-RNases as possible will benefit this approach because the information can then be used to plan approaches to determine the biochemical basis for the different interactions.

Among the interaction relations recognised between SLF proteins and *P. inflata* S-RNases, S_2-SLF1 interacts with the most S-RNases, four, and all other SLF proteins interact with none or only one S-RNase (Sun and Kao, 2013). The SLF that interacts with more S-RNases than all other SLF proteins may have been the first to appear during the evolution of the SI system. If the first SLF could interact with a number of non-self S-RNases, pollen would be able to detoxify new non-self S-RNases as more S-haplotypes evolved without the need for a new SLF. However, there may be a practical limit to the number of non-self S-RNases with which

each SLF can interact, in which case a new SLF would be required to allow pollen to recognise and detoxify additional non-self S-RNases as more S-haplotypes evolved.

There would not be any selective pressure to produce another SLF with the same function if an SLF has evolved to interact with and detoxify a specific S-RNase. To protect pollen from the toxic effects of non-self S-RNases, it would be advantageous if multiple SLFs were able to detoxify a given non-self S-RNase. This will lessen the negative effects of mutations that prevent an SLF from interacting with and detoxifying a non-self S-RNase. Non-self-pollen must also be accepted by the pistil in order for a SI system to be sustained over an extended period of time. This is accomplished by all SLF proteins working together to detoxify all non-self S-RNases. It would be interesting to find out if pollen has actually developed a redundancy to deal with every non-self S-RNase during the evolution of the SLF genes. The findings from research on the impact of suppressing S_2-SLF1 expression in pollen of S_2S_3 transgenic plants are consistent with the existence of additional SLF proteins for detoxifying S_3-RNase, S_7-RNase, and S_{13}-RNases because transgenic pollen producing very low levels of, if any, S_2-SLF1 remained compatible with S_3-, S_7-, and S_{13}-carrying pistils (Sun and Kao, 2013). Additionally, Kubo *et al*. (2010) discovered that the same S-RNase, S_9-RNase, interacted with two SLF proteins produced by S_5 pollen of *P. hybrida*.

The SLF proteins found in pollen of a certain S-haplotype are hypothesised to detect all non-self S-RNases but not self-S-RNases and mediate their destruction in the pollen tube, allowing cross-compatible pollination. The utilisation of numerous SLF genes in SI is shared by other solanaceous species, implying that SLF gene diversification predates the division of Solanaceae genera. However, because the number of SLF genes is not preserved between various Solanaceae genera or between different S-haplotypes of the same species, the formation of novel types of SLF has happened following the separation of distinct Solanaceae genera (Williams *et al*., 2014; Sun *et al*., 2018). In light of this, we must ponder how the SI system was established, how many SLF genes were generated and spread, and why different S-haplotypes had varying numbers of SLF genes.

The *Petunia hybrida* S-locus has been physically localised to a sub-centromeric area of Chromosome 3, where recombination is thought to be heavily inhibited (Li and Chetelat, 2015), yet evidence of intragenic recombination based genetic exchanges at the *Petunia* S-locus has been reported. For example, various sections of the S-RNase gene have diverse evolutionary histories, implying that intragenic recombination contributed to allelic variation in the S-RNase

gene (Wang *et al*., 2001). *Petunia* has also been reported to have genetic exchanges between various alleles of the same SLF gene, as well as between distinct SLF genes of the same or different S-haplotypes (Kubo *et al*., 2015). To date, all assessments of SLF gene genetic exchanges have been conducted using their coding sequences, according to the scientific literature. The majority of the plant genome is made up of intergenic non-coding sequences, particularly transposable elements (TEs), which are crucial for genome evolution (Lisch, 2012). In order to address their potential roles in genetic exchanges that contribute to the spread of SLF genes and maintenance of the S-locus during its evolution, it may be helpful to examine the non-coding regions that border SLF genes. In the *Petunia* genome, SLF pseudogenes that resemble SLF genes in terms of sequence have been discovered. These genes either lack any open reading frames (ORFs) or have frame shift mutations that cause premature stop codons (Malla *et al*., 2016). The amount of SLF genes may fluctuate across various S-haplotypes as a result of pseudogenization, and studying the flanking areas of SLF pseudogenes may provide insight into the mechanism underlying this gene loss process. A self-incompatible solanaceous species' S-locus sequence should be useful for understanding the non-coding regions that surround SLF genes and SLF pseudogenes (Wu et el., 2020). It is still unknown whether there are any more protein-encoding genes present at the solanaceous S-locus, which already has an S-RNase gene and the full complement of SLF genes.

SLF genes in *Petunia* have been located using a variety of techniques. When looking for pollen-expressed genes of *P. inflata* that displayed S-haplotype-specific sequence polymorphism, RNA differential display was used to find the first two SLF genes, designated A113 and A134 (renamed SLF9 and SLF10) (Wang *et al*., 2003). By first screening a BAC library of the S_2S_2 genotype using the S_2-RNase gene as a probe, extending the region covered by the BAC clones to 328 kb, and then sequencing this contig, the first SLF gene (SLF1; formerly named PiSLF2) of *P. inflata* that was shown to be involved in pollen specificity was discovered (Wang *et al*., 2004). By employing S_2-SLF1 (without the coding sequence for the F-box domain) as a probe, pollen cDNA libraries of *P. inflata* were screened to find four more SLF genes (Hua *et al*., 2007). Since these four genes were thought to be SLF-like genes and unrelated to pollen specificity, they were first given the names SLFLa, SLFLb, SLFLc, and SLFLd. In order to find their orthologues and additional SLF genes in *Petunia hybrida* and *Petunia axillaris*, primers were created based on the sequences of all the aforementioned SLF/SLFL genes for 59 and 39 RACE (rapid amplification of cDNA ends) (Kubo *et al*., 2010).

As there are various SLF sequences available for *Petunia* and *Solanum tuberosum* on different databases, the SLF sequences for *Solanum okadae* could simply be amplified by PCR and sequenced using oligonucleotides designed on the data of the known SLF sequences as *Petunia* and *Solanum tuberosum* are related to *Solanum okadae* but as a single nucleotide change in the area where a particular oligonucleotide is meant to anneal could impede proper primer annealing and the generation of PCR products as synonymous mutations are frequent. Therefore, due to absence of genomic information on *Solanum okadae* and restricted time and budget, this study sequenced the putative partial SLF sequences from S_2 haplotype of wild diploid self-incompatible *Solanum okadae*.

2. Materials & Methods

2.1 Plant material

Leaves from approximately 6 weeks old *Solanum okadae* plant (S_2S_X) were used in this study. All the plants were grown in 6" pots containing Levington CNSC compost and kept in controlled glasshouse environment of 16 h photoperiod and 20 °C±2°C. Detailed protocol for micropropagation and plant maintenance is described in chapter 4.

2.2 Pollinations

Controlled cross- and self-pollinations were performed by selecting 5 flowers after visually examining the anther cones for the presence of pollen checking the flower's maturity. After sterilizing the gloved hands with 70% ethanol, the thumb of one hand was placed below the anther cone which was then tapped gently using the other hand until white pollen was separated from the cones. This pollen was then rubbed onto the stigma of the same flower, thus carrying out the self-pollination. Cross-pollinations were carried out in similar fashion, except that the pollen from one plant was used to pollinate flowers of other plants. A string tag with the type of pollination (self/cross) was placed around the pedicel of all pollinations. After 14 days of carrying out the manual pollinations, the flowers were scored for SI via fruit set.

2.3 Genomic DNA extraction

GenElute Plant Genomic DNA Mini-prep kit (Sigma-Aldrich) was used to extract DNA from leaves by following the manufacturer's protocol as follows: Approximately 150 mg of fresh leaves were collected into pre-labelled 2.0 ml centrifuge tubes. The collected leaves were ground to a fine powder using a pre-chilled mortar-pestle and liquid nitrogen (N_2). The tubes holding the powder were left in the liquid N_2. The tubes were then taken out of the liquid N_2

and 350µl of lysis A solution and 50µl of lysis B solution were added. A cloudy precipitate was formed by the addition of lysis B solution via shaking vigorously and inverting the tubes. The mixture was incubated at 65°C for 10 minutes with frequent inversion to disperse the resultant precipitation. 120µl of precipitation solution was added to this mix and mixed by inversion. These tubes were then placed on ice for 5 mins. The samples were centrifuged for 5 mins at 12,000 rpm to pellet polysaccharides, cellular fragments and protein. The supernatant was cautiously pipetted on to a GenElute filtration column (blue insert with a 2 ml collection tube). The samples were centrifuged at 12,000 rpm for 1 min. The blue filtration insert was discarded but the collection tube and the liquid was retained. 700µl of binding solution was added to the liquid and mixed by mild inversion. A GenElute mini-prep binding column was prepared. 500µl of the column preparation solution was added to each binding column and centrifuged at 12,000 rpm for 1 min. The flow through liquid was thrown away.700µl of the resulting mixture from the addition of binding solution step was then cautiously pipetted onto the prepared binding column and centrifuged at 12,000 rpm for 1 min. The flow through liquid was discarded and the collection tube was retained. This step was repeated for remaining of the lysate. The red insert was placed into a new 2 ml collection tube and 500µl of the diluted wash solution was applied to the column and centrifuged at 12,000 rpm for 1 min. The flow through liquid was discarded but the red insert was retained. Additional 500µl of wash solution was added to the column and centrifuged at 12,000 rpm for 2 min to dry the column (making certain that the binding column did not touch the flow through liquid). The binding column was shifted to a new 2 ml collection tube and 70µl (instead of 100µl recommended by the manufacturer) of pre-warmed (65°C) elution solution was added to it and centrifuged at 12,000 for 1 min. The elution step was repeated again and the eluate stored at -20 °C and DNA quantification and purity check was carried out using Nano-Drop Spectrophotometer (ND-1000 Spectrophotometer, NanoDrop Technologies, Inc., and Wilmington, USA).

2.4 Genotyping for allelic specificity

2.4.1 Primers and Polymerase chain reaction (PCR)

The primers for genotyping mentioned below in table 1 (Dzidzienyo *et al*, 2016), were procured from Sigma-Aldrich to allow S-RNase allele-specific amplification in *S. okadae*. The primers had an optimal melting temperature of 63 °C. PCR experiments were performed in a 20-µl reaction volume containing 1X PCR buffer comprising of 0.2mM dNTPs and 3mM $MgCl_2$ (Bioline), 0.5µM each of forward as well as reverse primers and 1 U of Taq DNA polymerase

(Mytaq, Bioline). PCR amplification was done using the following cycling conditions: an initial 2 min denaturation at 95 °C, followed by 35 cycles of 30 sec at 95 °C, 30 sec of annealing at 60°C, 1 min at 72 °C were implemented and then a final extension of 10 min at 72 °C in an automated thermal cycler (GeneAmp 9700, Applied Biosystems).

Table 1: Primers for S-RNase allele-specific amplification.

	Name	Primer Sequence (5'-3')	T_m (ºC)
S_{o1}-RNase	So1-F	GGATAAGGAGGGATCACAGC	65
	So1-R	TGTTGGCTTTGTATTTGTAGCA	63
S_{o2}-RNase	So2-F	TGCGAGTCCGAAGACAAGTA	66
	So2-R	AAGGGAAAGAAAACGGAAGC	63
S_{o4}-RNase	So4-F	TCGATTGGAGTTCTGCACTG	65
	So4-R	TTTCATCGCATGTGTTACCC	63
S_{o5}-RNase	So5-F	TGGTCGAAAGGAACAACCTT	64
	So5-R	TTCCAACCTGGTCATTCAAAG	63

2.4.2 Gel electrophoresis and Imaging

Agarose gel was prepared by mixing 1g agarose with 100 ml of 1xTBE buffer (UltraPure™ TBE Buffer, 10X, Invitrogen). The agarose was dissolved by heating in a microwave for 2-4 mins. The gel solution was cooled and 5µl of 1% ethidium bromide (EtBr; Fisher Bioreagents) was added. 10µl of the amplified product was mixed with 2µl (6X) loading buffer (Bioline, London, UK) and loaded into the wells of the agarose gel. In addition, 5µl of HyperLadder II (Bioline, London, UK) was added for size calibration and the gel was run at a constant voltage of 80 V for 1 hour. After the electrophoresis, the gel was visualized and photographed using a UV Trans-illuminator (Syngene, Cambridge, UK).

2.5 SLF sequencing

2.5.1 Primer design

The amino acid sequences for SLF1, SLF2 and SLF3 of S_2-haplotype of *Petunia integrifolia subsp. inflata* (Kubo *et al*., 2015) were retrieved from NCBI protein database. A protein-protein BLAST alignment was performed with the respective retrieved *Petunia* SLF sequence as a query against non-redundant protein sequence (nr) database and *Solanum* (taxid: 4107) as organism. On the result window, the results were filtered maximum percent (%) identity. This

was followed by viewing the first 10 alignments in the 'Multiple Alignment' mode, where an area of approximately 150-500 base pairs in length in more conserved region was targeted for positioning forward and reverse primers for optimal PCR amplification. Two sets of primers targeting the start and end of the target SLF sequence were designed to get a longer putative SLF sequence after sequencing. Each primer was 5 and 7 amino acids long equating to ~15-20 base pairs. The amino acid sequence chosen to design the concensus primers were converted into corresponding nucleotide sequence using EMBOSS Backtranseq tool (https://www.ebi.ac.uk/Tools/st/emboss_backtranseq/) with *Solanum tuberosum* as reference codon usage table. Figure 1 below provides a schematic representation of the local alignment search approach used in this investigation to design the primers.

Figure 1: The local alignment search method used in this work shown schematically

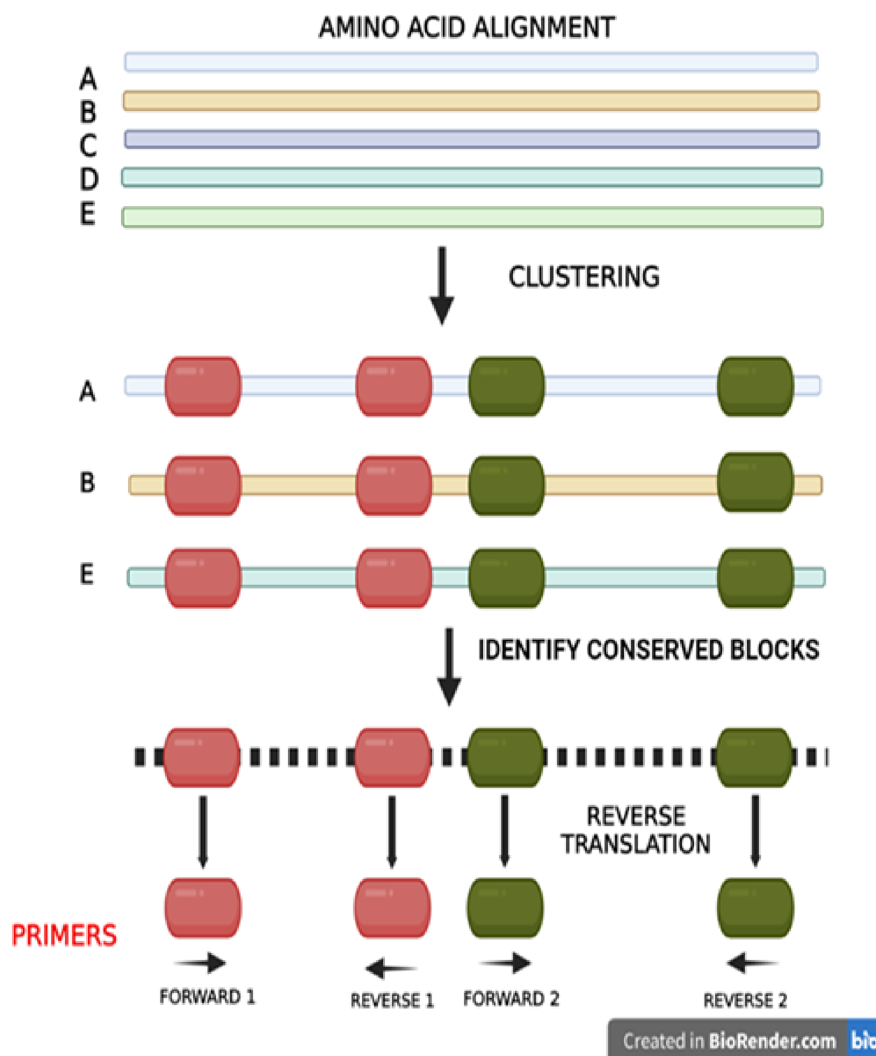


Table 2: Primers for sequencing putative SLFs from S_2-haplotype in *Solanum okadae* with expected product sizes.

Target	Primer	Sequence (5'-3')	T_m (°C)	Product size (bp)
S_2-SLF1	OF1_F1	ATTCAATCTACTACTTTTATT	55	291
	OF1_R1	TCTAGTAGCTGGATTAAA	56	
	OF2_F1	GCTACTAGAAATTTTAGA	56	507
	OF2_R1	TGGATCAATTGGAGACAT	62	
S_2-SLF2	O2SLF2_F1	ATGAAGAAGGTTCCTCAAGAT	61	342
	O2SLF2_R1	AATAAGTCCATGACAAGGTCC	64	
	O2SLF2_F2	ATTGGACCATGTCATGGACTT	59	399
	O2SLF2_R2	AGAAATATCAAAACAAAGAAT	54	
S_2-SLF3	O2SLF3_F1	AGATTTAAGTGTGTTACT	52	444
	O2SLF3_R1	AATTCTAACAACCTTATAAGT	55	
	O2SLF3_F2	ACTTATAAGGTTGTTAGAATT	55	480
	O2SLF3_R2	AATAGCAAGTGGAGATTCAAT	61	

2.5.2 Polymerase chain reaction for SLF gene amplification

DNA from a glasshouse grown *Solanum okadae* plant (S_2S_x) was extracted according to the protocol described in section 2.3 above, which was used as the template for the polymerase chain reaction. All the reactants were thawed on ice before use. 50µl of reaction was set up in PCR tubes according to the table below:

DNA template (10-50ng)	6µl
MyTaq Reaction Buffer (5X)	10µl
Forward primer (20 µM)	1µl
Reverse primer (20 µM)	1µl
MyTaq DNA polymerase	0.5µl
Water (dH_2O)	up to 50µl

The PCR tubes were transferred to an automated thermal cycler (GeneAmp 9700, Applied Biosystems) with the lid pre-heated to 105°C and the thermocycling procedure was started with following conditions:

Step	Temperature (⁰C)	Time	Cycles
Initial denaturation	94⁰C	2 min	1
Denaturation	94⁰C	30s	35
Annealing*	(50-65)⁰C	30s	
Extension	72⁰C	1 min	
Final extension	72⁰C	10 min	1
Hold	4⁰C	∞	

2.5.3 Gel purification & Sequencing

Gel electrophoresis was performed for the PCR products according to the protocol described in the section 2.4.2. The gel was visualised under UV gel trans-illuminator and by using a clean scalpel and forceps, the gel slice containing amplified DNA fragment was cut and collected in a clean pre-weighed 1.5 mL Eppendorf tube. The Eppendorf tube containing gel *Sli*ce was weighed to deduce the weight of gel slice. GeneJET gel extraction kit (Thermo Scientific™), was used to extract and purify the PCR product from the gel. The protocol for which was as follows:

1:1 volume of binding buffer was added to the gel slice (volume: weight). This gel mixture was incubated at 55-60°C until the gel was completely dissolved in the binding buffer. The gel mixture was vortexed in an analog vortex mixer (VWR international) and loaded on the GeneJET purification column followed by centrifugation for 1 min at 10,000 rpm. The column was reinserted into the same collection tube after the flow-through was discarded. Since the purified DNA would be utilised for sequencing, an extra binding step was performed by adding 100 µl of binding buffer to the column before centrifuging it at 10,000 rpm for 1 min. After discarding the flow-through, the column was put back onto the same collection tube. 700 µL of wash buffer was added to the GeneJET purification column and centrifuged for 1 min at 10,000 rpm. After discarding the flow-through, the empty purification tube was again centrifuged for 1 min at 10,000 rpm to fully remove leftover wash buffer. 40 µl of pre-warmed (65⁰C) elution buffer was added to the GeneJET purification column containing bound DNA

after transferring it to a fresh 1.5 mL Eppendorf tube. This was then centrifuged for 2 mins at 10, 000 rpm. The flow-through (purified DNA) was collected and stored at -20ºC.

The purified PCR products were directly sequenced via Sanger Sequencing SpeedREAD service provided by SourceBioscience (https://www.sourcebioscience.com/genomics/sanger-sequencing). Each putative SLF sequence was sequenced by using the same respective primers that were used for PCR.

2.5.4 Data analysis

The nucleotide sequences provided by Source Bioscience in .SEQ format were analysed using SnapGene® Viewer version 6.0.2 (GSL Biotech). The nucleotide sequences obtained post sequencing (forward & reverse) were aligned using ClustalΩ (Sievers F & Higgins DG, 2014). Once the sequences were aligned, they were downloaded and joined together to get one long sequence. This long nucleotide sequence was then translated into corresponding amino acid sequence using Expasy translation tool (https://web.expasy.org/translate/). A protein-protein BLAST alignment was performed with putative amino acid sequence as a query against non-redundant protein sequence (nr) database and *Solanum* (taxid: 4107) as organism. The putative amino acid sequence aligned with similar amino acid sequence in closely related *Solanum* species was viewed by clicking on 'Multiple Alignment' on the result window.

A phylogenetic tree was generated for the above multiple sequence alignment using Molecular Evolutionary Genetics Analysis (MEGA, version: 11, Tamura *et al*., 2021) software. The evolutionary history was inferred by using the Maximum Likelihood (ML) method and JTT matrix-based model (Jones *et al*., 1992). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 37 amino acid sequences. There were a total of 397 positions in the final dataset.

To test for purifying selection, the ratio of nonsynonymous to synonymous substitution rates (d_n/d_s) was calculated for putative SLFs without frameshifting indels or stop codons. d_n/d_s ratios close to one imply neutral sequence evolution, ratios less than one show negative or purifying selection, corresponding to significant selective restrictions, and ratios larger than one indicate positive selection, often indicating adaptive evolution. The d_n/d_s ratios were determined using

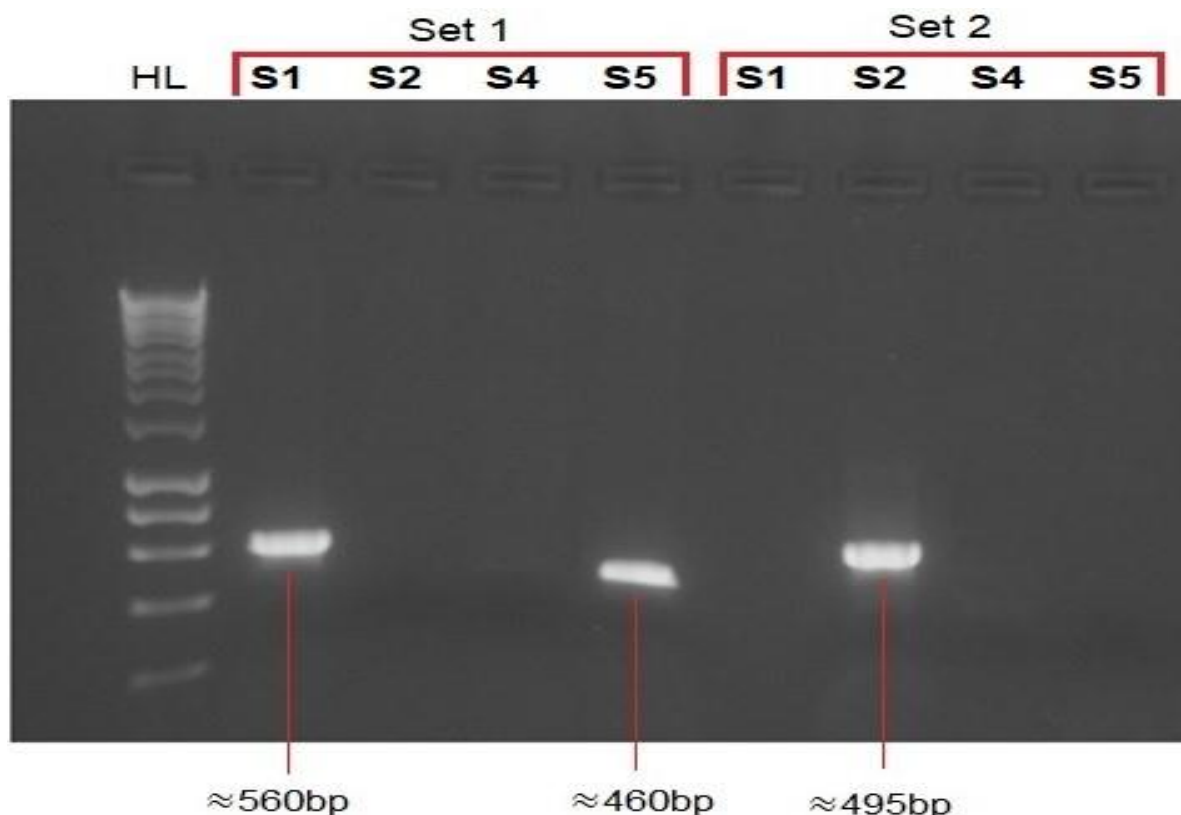
the modified Nei and Gojobori (1986) technique with the Jukes–Cantor adjustment, implemented in DnaSP (version 5, Librado and Rozas, 2009).

3. Results

3.1 Allele specific genotyping and SI status

In the current study, two packets (sets) of seeds of *Solanum okadae* (accession: OKA7129) were shipped from The James Hutton Institute out of which set 1 was positive for So_1 allele which was also confirmed by allele specific genotyping using PCR and S_{o1}-Rnase specific primers. The second S-RNase allele in set 1 and both the S-RNase alleles in set 2 were unknown, which were determined by allele specific genotyping using PCR and So_2/ So_4/ So_5-RNase specific primers.

Figure 2: DNA sample was amplified using allele-specific primers for the S_{o1}, S_{o2}, S_{o4}, and S_{o5} alleles, as shown. Set 1 was positive for S_{o1} and S_{o5}. Set 2 was positive for S_{o2} whilst the second allele in set 2 did not test positive for either of S_{o1}, S_{o4}, and S_{o5}.



As per gel image in Fig 2, the second unknown allele in set 1 was So_5 making the set 1 of genotype S_1S_5. One of the two alleles in set 2 was observed to be So_2, whereas the other allele in this genotype remained undetermined as neither of the four primer pairs produced an

amplified PCR product. However, when plants from set 1 were cross-pollinated with plants in set 2, there was a berry set (Fig 3b), indicating that the second unknown allele in plants from set 2 is a completely different allele causing a fully compatible reaction. Also, when the plants from set 1 and set 2 were self-pollinated, there was no berry formation in either direction, instead the pollinated flowers withered off (Fig 3a) indicating that both parent plants possess the identical pair of S-haplotypes, halting all pollen tube growth and causing an incompatible reaction demonstrating self-incompatibility in *Solanum okadae* under study. The seeds from the berries obtained from a cross between set 1 (S_1S_5) and set 2 (S_2S_x) were unable to germinate and therefore could not be genotyped but it can be inferred that the genotype could have been either one of the following combination of S_1S_2, S_1S_x, S_2S_5 or S_5S_x (Table 3).

Figure 3: Self and cross pollinated *Solamum okadae* plants. **(a.)** Shrunken flowers indicating parent plants possessing the matching pair of S-haplotypes, **(b.)** Berry set indicating semi-compatible or fully compatible reaction between parental genotypes.

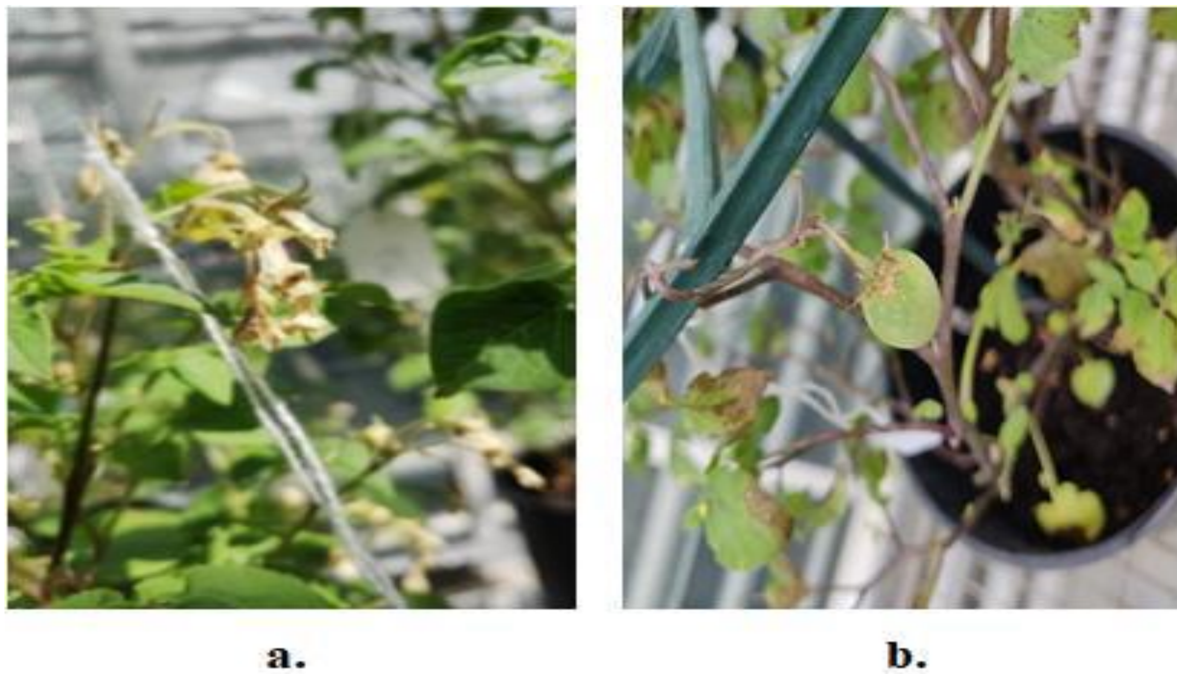


Table 3: Two inferred S-genotypes of diploid *Solanum okadae* using allele-specific primers used in the study and progeny analysis from resulting crosses

	Allele 1	**Allele 2**	**Inferred genotype**
Set 1	S_1	S_5	S_1S_5
Set 2	S_2	S_x	S_2S_x

Table 3 continued

	Parental genotypes	Berry set	Probable genotype
Set 1 x Set 1	(S_1S_5) x (S_1S_5)	x	-
Set 2 x Set 2	(S_2S_x) x (S_2S_x)	x	-
Set 1 x Set 2	(S_1S_5) x (S_2S_x)	✓	S_1S_2 S_1S_x S_2S_5 S_5S_x

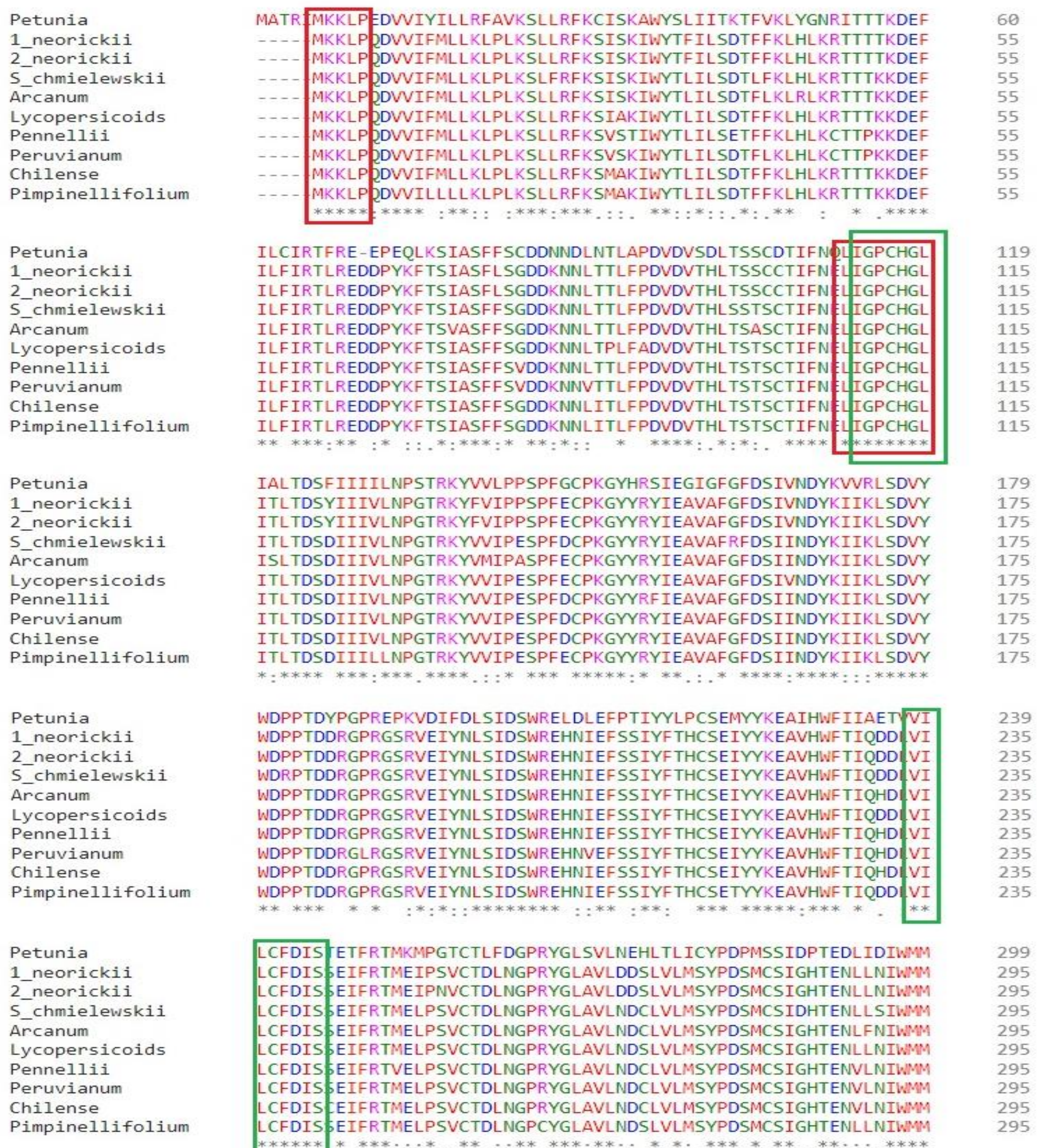
3.2 Putative SLF identification via PCR

The majority of polymerase chain reaction (PCR) applications are dependent on constructing primers that exactly match a specified target sequence. However, when isolating homologous sequences of a particular gene family member in organisms with unknown or insufficient genome sequence information, consensus primers designed from reverse-translated sequences of extremely conserved amino acid regions are frequently used. These regions are produced through several amino acid alignments or the synthesis of protein blocks based mostly on the sequences of distantly-related species (Al Mazrooei and Ghazala, 2018). In the current study, similar approach was used to identify the putative SLF sequences in *Solanum okadae*.

After reviewing the relevant literature, three amino acid sequences of S-locus F-box proteins (SLF) from *Petunia integrifolia subsp inflata* S_2-haplotype were chosen as the candidate gene references (SLF1: AAS79485.1, SLF2: AIK66497.1, SLF3: ABX82524.1). To design primers for identifying the corresponding SLF genes in *Solanum okadae*, the reference *Petunia* sequences were used as a query sequences for scanning the NCBI protein database for similar SLF sequences from other *Solanum* species (taxid: 4107) via the BLASTP tool. By comparing the *Petunia* reference sequences to the NCBI database, 10 sequences with the highest degree of identity to the reference were identified for each type of SLF. Interestingly, SLF1 sequence from *Petunia* had maximum % identity with SLF7 in other *Solanum* species (all tomatoes). Similarly, SLF2 and SLF3 shared maximum % identity with SLF23 and SLF12 in other *Solanum* species. After identifying these SLFs sequences in other *Solanums*, they were aligned along with one respective *Petunia* SLF reference using ClustalΩ (1.2.4). In the alignment section, at least three conserved places were located (Figure 4). The conserved regions were at

least five-six amino acids long. Furthermore, a number of factors like, a suitable annealing temperature, a suitable GC-content value, the location of non-degenerate bases at the end of primers, and a low degeneracy fold were taken into account to choose appropriate primers.

Figure 4: Multiple sequence alignment of S-locus F-box protein type-2 from *Petunia* with other *Solanum* SLFs. Red and green blocks highlight the highly conserved regions chosen to design forward and reverse primers for amplifying first and second part of the long amino acid sequence in *Solanum okadae*.



After performing the PCR using DNA from set 2 as template, interestingly, a single band was obtained (Figure 5) for each SLF per primer pair, which was sequenced directly. The nucleotide sequences obtained from both red and green block primers were joined together to get a longer putative SLF sequence which was then translated into its respective amino acid form. Comparisons between these putative SLF sequences from *Solanum okadae* with the protein database showed approximately 90-96% amino acid identity with SLF7, SLF12 and SLF23 sequence from *Solanum lycopersicoides, Solanum peruvianum, Solanum arcanum, Solanum chmielewskii, Solanum habrochaites and Solanum neorickii* (Fig 6b, 7b & 8b). On the contrary, the three putative SLFs from *Solanum okadae* showed 64.45-69.83% identity to SLF from *Petunia* (Fig 6a, 7a & 8a). Even though the primers were designed based on highly conserved blocks that included one *Petunia* SLF reference and 10 other *Solanum*s, the sequence similarity amongst the 10 chosen *Solanum* SLF sequences was highest when compared to their similarity with respective *Petunia* SLF and therefore, the concensus primers deduced SLF7, SLF12 and SLF23 (Appendix II) in *Solanum okadae* indicating an orthologous relationship between SLF7, SLF12 and SLF23 from *Solanum okadae* and SLF1, SLF3 and SLF2 from *Petunia integrifolia subsp inflata* respectively.

Figure 5: Gel image showing putative partial SLF sequences of *Solanum okadae* using primer pair mentioned in brackets with approximate product size. Lane 1 (O2SLF2_F2 & O2SLF2_R2; 399bp), lane 3 (O2SLF2_F1 & O2SLF2_R1; 342bp), lane 5 (OF1_F1 & OF1_R1; 291bp) and lane 7 (O2SLF3_F2 & O2SLF3_R2; 480bp). Lane L : 1kb DNA Hyper ladder.

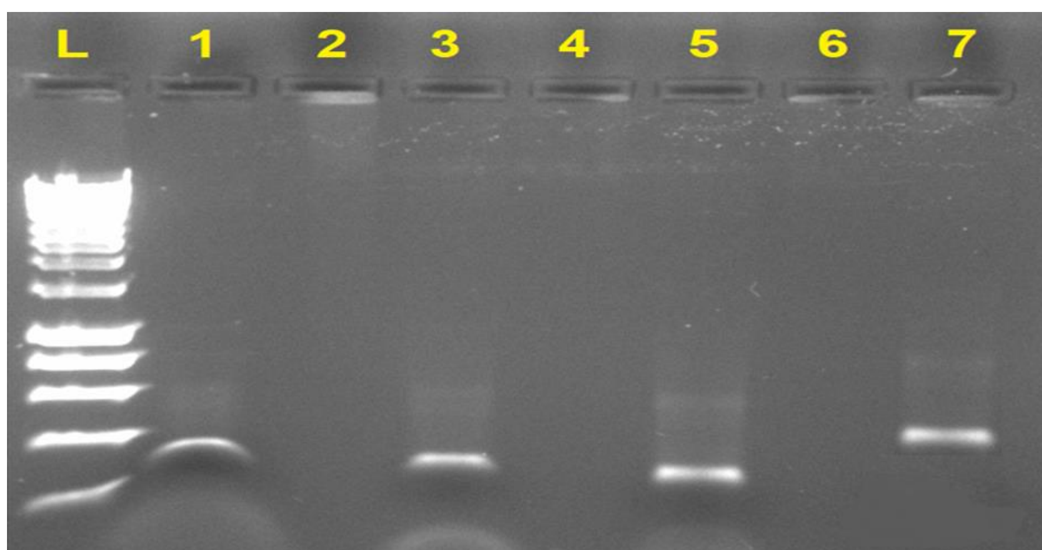


Figure 6: Putative SLF7 (Query) from *Solanum okadae* aligned with SLFs from *Petunia* (A) and SLF7 from *Solanum* (taxid 4017) (B). Nonsynonymous substitutions unique to *Solanum okadae* highlighted in yellow.

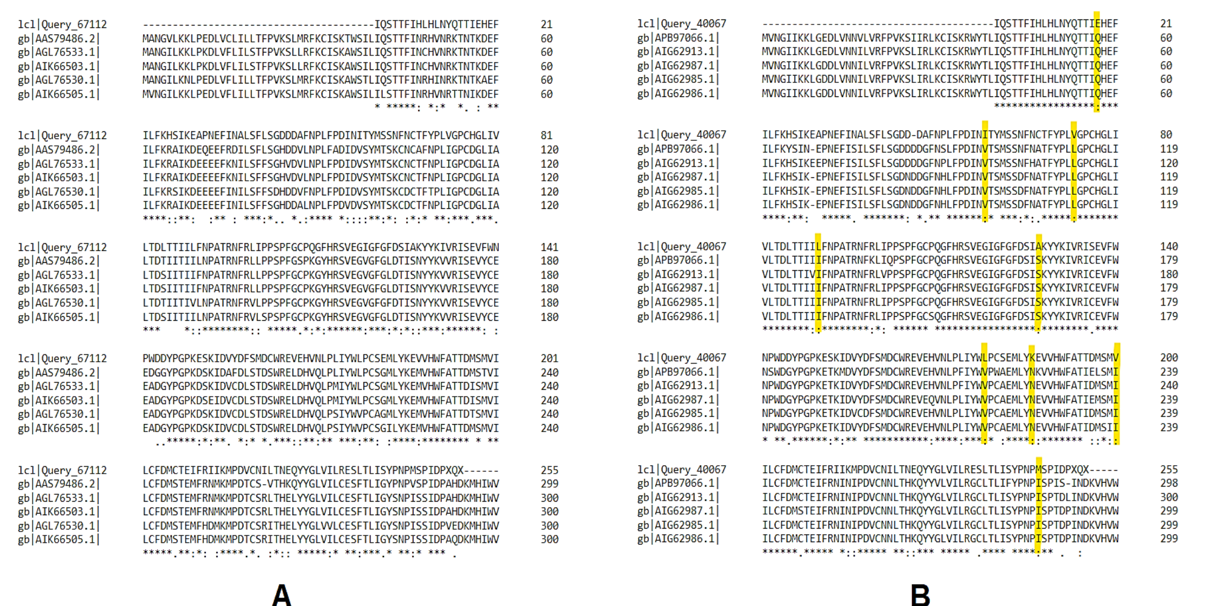
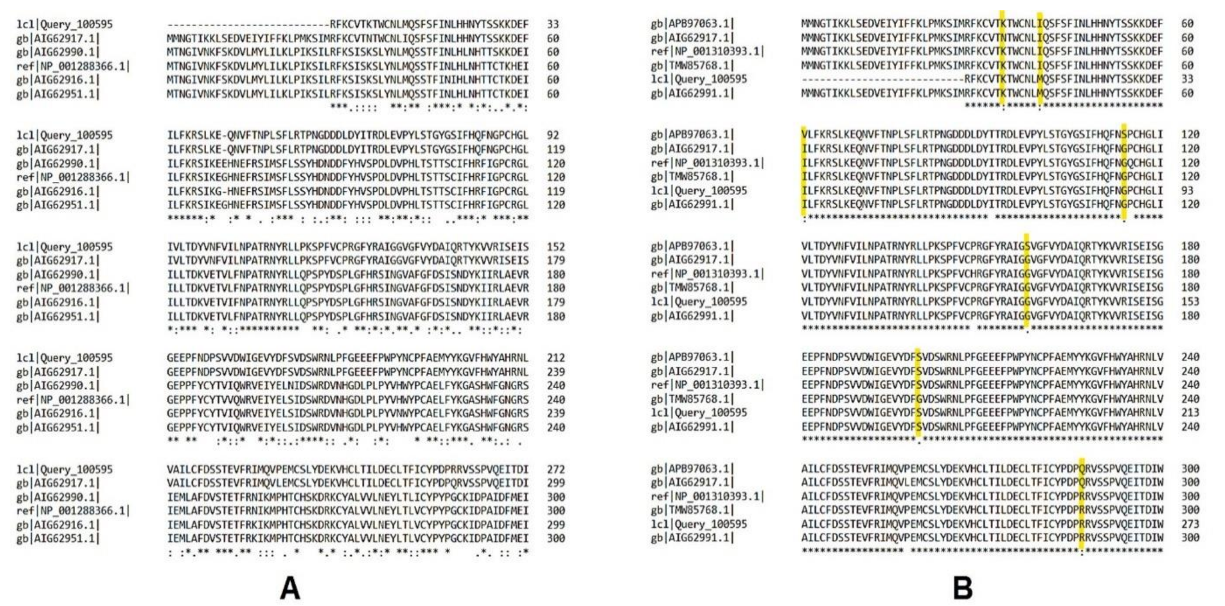


Figure 7: Putative SLF12 (Query) from *Solanum okadae* aligned with SLFs from *Petunia* (A) and SLF12 from *Solanum* (taxid 4017) (B). Nonsynonymous substitutions unique to *Solanum okadae* highlighted in yellow.



Single, fully conserved residue is denoted by (*), conservation among groups with highly similar properties is denoted by (:),conservation among groups with weakly similar properties is denoted by (.), no dot indicates no conservation.

Figure 8: Putative SLF23 (Query) from *Solanum okadae* aligned with SLFs from *Petunia* (A) and SLF23 from *Solanum* (taxid 4017) (B). Nonsynonymous substitutions unique to *Solanum okadae* highlighted in yellow.

```
gb|AIK66508.1|      -MANGILKKLPEDLVFLMLLTFPVKSLLRFKCISKAWSILIQSTSFINRHVNRKTNTKDE   59
gb|AIK66506.1|      -MANGILKKLPEDLVFLILLTFPVKSLMRFKCISKAWSILIQSTTFINRHVNRNTNTKDE   59
lcl|Query_355628    ------MKKLPQDVVIFMLLKLPLKSLLRFKSISKIWYTLILSDSFFELHLKRTTTTKDE   54
gb|ABX82528.1|      MTTRRMMNKLPEDVVIYILFRLPVKSLLRFKCTSKAWYTLILSDTFVKLHHNHATATKEE   60
gb|AIK66497.1|      -MATRIMKKLPEDVVIYILLRFAVKSLLRFKCISKAWYSLIITKTFVKLYGNRITTTKDE   59
gb|AIK66498.1|      ------MKKFHEDMMIYILLRFPVKSLLRFKCISKVYYTLILSNTFVKLHLNRIRTTKDE   54
                          ::*: :*::: :*: : :***:***. ** :  ** : :*.: : ::   **:*

gb|AIK66508.1|      FILFKRAIKD-EEEEFINILSFFSGHDD-VLNPLFPDIDVSYMTSKCDCTFNPLIGPCDG   117
gb|AIK66506.1|      FILFKRAIKD-EEEEFINILSFFSGHDD-VLNPLFPDIDVSYMTTKCDCAFNPLIGPCDG   117
lcl|Query_355628    FILFIRTLREDDPYKLTSIASFFSGDDKNNLTTLFPDVDVTHLTSSCCTIFNELIGPCHG   114
gb|ABX82528.1|      FILFIRTFRE-EPDLWKNVASFIYCDDNNDHNNLFPDLDLSHLTSSYCSIFGQLIGPCHG   119
gb|AIK66497.1|      FILCIRTFRE-EPEQLKSIASFFSCDDNNDLNTLAPDVDVSDLTSSCDTIFNQLIGPCHG   118
gb|AIK66498.1|      FVLFIRTFRE-EPDQLKSIASFFSCDDNNDLNTLSPDLDVSDLTSTCDTIFNQLIGPSHG   113
                    *:*  *:::: :     .: **:  .*.   . * **:*:: :*:.    *. ****..*

gb|AIK66508.1|      LIALTDSIITIILNPATRNFRVLPPSPFGCPKGYHRSVEGVGFGLDTISNYYKVVRVSEV   177
gb|AIK66506.1|      LIALTDSIITIILNPATRNFRVLPPSPFGCPKGYHRSVEGVGFGFDTVSNYYKVVRISEV   177
lcl|Query_355628    LIALTDSYIIIVLNPGTRKYFVIPPSPFECPKGYYRYIEAVAFGFDSIVNDYKIIKLSDV   174
gb|ABX82528.1|      LIALSDSIIIIILNPSTRKYVVLPPSPFGCPKGYHRSIEGIGFGFDSIVNDYKVVRLSDV   179
gb|AIK66497.1|      LIALTDSFIIIILNPSTRKYVVLPPSPFGCPKGYHRSIEGIGFGFDSIVNDYKVVRLSDV   178
gb|AIK66498.1|      LIALTDSFIIIVLNPATRKYIVLPPSPFGCPKGYHRSVEGIGFGFDSIVNDYKVVRLSDV   173
                    ****:** * *:***.**:: *:***** *****:* :*.:.**:*:: * **::::*:*

gb|AIK66508.1|      YCEEAGGYPGPKDSKIDVCDLSTDSWRELDHVQLPLIYWVPCSGMLYKEMVHWFA-TTEI   236
gb|AIK66506.1|      YCEEADGYPGPKDSKIDVCDLSTDSWRELDHAQLPSIYWVPCSGMLYKEMVHWFA-TTEI   236
lcl|Query_355628    YWDPPTDDRGPRGSRVEIYNLSIDSWREH-NIEFPSIYFSHCSEIYYKEAVHWFT-IKDD   232
gb|ABX82528.1|      YWDPPTDYPGPREPKVDIYDLSIDSWRELSEVEFPSIYYLPCSEMYYKEAVHWFSHIDMD   239
gb|AIK66497.1|      YWDPPTDYPGPREPKVDIFDLSIDSWREL-DLEFPTIYYLPCSEMYYKEAIHWFI-IAET   236
gb|AIK66498.1|      YWDPPTDYPGPREPKVDIYDLGIDSWREL-DVEFPPIYYLPCSEMYYKEAVHWFI-VTET   231
                    * :   .  **:  :::: :*. *****   . ::* **:  ** : *** :***

gb|AIK66508.1|      MVILCFDMSTEMFRNMKMPDTCSRLTHGLYYGLVILCESFTLIGYSNPVSPIDPLHDKMH   296
gb|AIK66506.1|      MVILCFDMSTEMFRNMKMPDTCSLITHELYYGLVILSESFTLIGYSNPISSIDPVEDKMH   296
lcl|Query_355628    LVILCFDISS--------------------------------------------------   242
gb|ABX82528.1|      VMILCFDIITEIFRTMKIPGDCTFL-EIPRYGLAILNECLTLISYPDPMCSDEPIEELIY   298
gb|AIK66497.1|      VVILCFDISTETFRTMKMPGTCTLF-DGPRYGLSVLNEHLTLICYPDPMSSIDPTEDLID   295
gb|AIK66498.1|      VVIFCFDISTETFRTMKMPGSCTFF-DGPRYGLAILNGHLTLICYPDPMSSIDPTEDLIE   290
                    ::*:***: :
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A

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gb|AUE24227.1|      MKKLPQDVVIFMLLKLPLKSLLRFKSISKIWYTLILSDTFFKLHLKRTTTKKDEFILFIR   60
lcl|Query_544385    MKKLPQDVVIFMLLKLPLKSLLRFKSISKIWYTLILSDSFFELHLKRTTTTKDEFILFIR   60
gb|AUE24214.1|      MKKLPQDVVIFMLLKLPLKSLLRFKSISKIWYTFILSDTFFKLHLKRTTTTKDEFILFIR   60
gb|AUE24217.1|      MKKLPQDVVIFMLLKLPLKSLLRFKSISKIWYTFILSDTFFKLHLKRTTTTKDEFILFIR   60
gb|AUE24223.1|      MKKLPQDVVIFMLLKLPLKSLLRFKSISKIWYTFILSDTFFKLHLKRTTTTKDEFILFIR   60
gb|AUE24218.1|      MKKLPQDVVIFMLLKLPLKSLLRFKSISKIWYTFILSDTFFKLHLKRTTTTKDEFILFIR   60
                    *********************************:****:**:********.*********

gb|AUE24227.1|      TLREDDPYKFTSIASFFSGDDKNNLTTLFPDVDVTHLSSTSCTIFNELIGPCHGLITLTD   120
lcl|Query_544385    TLREDDPYKLTSIASFFSGDDKNNLTTLFPDVDVTHLTSSCCTIFNELIGPCHGLIALTD   120
gb|AUE24214.1|      TLREDDPYKFTSIASFLSGDDKNNLTTLFPDVDVTHLTSSCCTIFNELIGPCHGLITLTD   120
gb|AUE24217.1|      TLREDDPYKFTSIASFLSGDDKNNLTTLFPDVDVTHLTSSCCTIFNELIGPCHGLITLTD   120
gb|AUE24223.1|      TLREDDPYKFTSIASFLSGDDKNNLTTLFPDVDVTHLTSSCCTIFNELIGPCHGLITLTD   120
gb|AUE24218.1|      TLREDDPYKFTSIASFLSGDDKNNLTTLFPDVDVTHLTSSCCTIFNELIGPCHGLITLTD   120
                    *********:******:*******************:*:.***************:***

gb|AUE24227.1|      SDIIIVLNPGTRKYVVIPESPFDCPKGYYRYIEAVAFGFDSIINDYKIIKLSDVYWDPPT   180
lcl|Query_544385    SYIIIVLNPGTRKYFVIPPSPFECPKGYYRYIEAVAFGFDSIVNDYKIIKLSDVYWDPPT   180
gb|AUE24214.1|      SYIIIVLNPGTRKYFVIPPSPFECPKGYYRYIEAVAFGFDSIVNDYKIIKLSDVYWDPPT   180
gb|AUE24217.1|      SYIIIVLNPGTRKYFVIPPSPFECPKGYYRYIEAVAFGFDSIVNDYKIIKLSDVYWDPPT   180
gb|AUE24223.1|      SYIIIVLNPGTRKYFVIPPSPFECPKGYYRYIEAVAFGFDSIVNDYKIIKLSDVYWDPPT   180
gb|AUE24218.1|      SYIIIVLNPGTRKYFVIPPSPFECPKGYYRYIEAVAFGFDSIVNDYKIIKLSDVYWDPPT   180
                    * ************.*** ***:******************:*****************

gb|AUE24227.1|      DDRGPRGSRVEIYNLSIDSWREHNIEFSSIYFTHCSEIYYKEAVHWFTIQDDLVILCFDI   240
lcl|Query_544385    DDRGPRGSRVEIYNLSIDSWREHNIEFPSIYFSHCSEIYYKEAVHWFTIKDDLVILCFDI   240
gb|AUE24214.1|      DDRGPRGSRVEIYNLSIDSWREHNIEFSSIYFTHCSEIYYKEAVHWFTIQDDLVILCFDI   240
gb|AUE24217.1|      DDRGPRGSRVEIYNLSIDSWREHNIEFSSIYFTHCSEIYYKEAVHWFTIQDDLVILCFDI   240
gb|AUE24223.1|      DDRGPRGSRVEIYNLSINSWREHNIEFSSIYFTHCSEIYYKEAVHWFTIQDDLVILCFDI   240
gb|AUE24218.1|      DDRGPRGSRVEIYNLSINSWREHNIEFSSIYFTHCSEIYYKEAVHWFTIQDDLVILCFDI   240
                    *****************:********* ****:****************:**********

gb|AUE24227.1|      SSEIFRTMELPSVCTDLNGPRYGLAVLNDCLVLMSYPDSMCSIDHTENLLSIWMMKEYGE   300
lcl|Query_544385    SS----------------------------------------------------------   242
gb|AUE24214.1|      SSEIFRTMEIPSVCTDLNGPRYGLAVLDDSLVLMSYPDSMCSIGHTENLLNIWMMKEYGE   300
gb|AUE24217.1|      SSEIFRTMEIPNVCTDLNGPRYGLAVLDDSLVLMSYPDSMCSIGHTENLLNIWMMKEYGE   300
gb|AUE24223.1|      SSEIFRTMEIPSVCTDLNGPRYGLAVLDDSLVLMSYPDSMCSIGHTENLLNIWMMKEYGE   300
gb|AUE24218.1|      SSEIFRTMEIPSVCTDLNGPRYGLAVLDDSLVLMSYPDSMCSIGHTENLLNIWMMKEYGE   300
                    **
```

B

Single, fully conserved residue is denoted by (*), conservation among groups with highly similar properties is denoted by (:),conservation among groups with weakly similar properties is denoted by (.), no dot indicates no conservation.

3.2.1 Using d_n/d_s ratios to detect Selection Signatures in SLFs

The deduced amino acid sequences of SLFs were examined according to the ML model of sequence evolution. Multiple sequence alignments were used to determine pairwise sequence distances using MEGA 11. In the current study, d_n/d_s comparisons of each type of SLF (SLF-7, SLF-12 and SLF-23) sequences from *Solanum* species along with putative SLFs from *Solanum okadae* with no frame-shifting and/or stop codons showed a strong indication of positive selection ($d_n/d_s>1$) with average d_n/d_s ratio of 3.91, 2.58 and 1.63 for SLF-7, SLF-12 and SLF-23 respectively (Table 4). When compared to SLF7 and SLF23 consensus alignments in the other 5 *Solanum* species, we found nine , seven and six nonsynonymous substitutions that are specific to SLF-7, SLF-12 and SLF-23 of *Solanum okadae*, respectively (Fig 6b, 7b and 8b).

Table 4: Ratio of Nonsynonymous to Synonymous substitutions in SLF-7, SLF-12 and SLF-23 between *Solanum okadae* (Oka) and other *Solanum* species.

Species 1	Species 2	d_s	d_n	d_n/d_s
Oka 7	Per 7	0.192	0.652	3.399
Oka 7	Hab 7	0.021	0.103	4.966
Oka 7	Pimp 7	0.201	0.649	3.233
Oka 7	Pen 7	0.021	0.100	4.799
Oka 7	Lyc 7	0.149	0.476	3.201
Oka 12	Per 11	0.111	0.351	3.164
Oka 12	Hab 11	0.294	0.701	2.384
Oka 12	Lyc 11	0.293	0.709	2.424
Oka 12	Chi 11	0.295	0.705	2.387
Oka 23	Neo 23	0.015	0.017	1.162
Oka 23	Arc 23	0.017	0.033	1.932
Oka 23	Lyc 23	0.027	0.033	1.230
Oka 23	Chm 23	0.020	0.038	1.933
Oka 23	Hab 23	0.020	0.040	2.026

(Per:Peruvianum, Hab:Habrochaites, Pimp: Pimpinellifolium, Pen:Pennellii, Lyc:Lycopersicum, Chi:Chilense, Neo:Neorickii, Arc:Arcanum).

3.2.2 Phylogenetic analysis

To investigate the evolutionary history of deduced SLF sequences in *Solanum okadae*, a phylogenetic analysis of known SLF sequences from *Petunia* and *Solanum* (taxid: 4107) sequences was performed. This study included 26 amino acid sequences (10 from *Petunia* and 16 from *Solanum*) with final dataset having a total of 406 sites. The ML tree, with the superior topology (−lnL=6932.59; Fig. 9) revealed three major clades. The first clade contained SLF-23 from *Solanum* species and SLF-2 from *Petunia*, the second clade contained SLF-7 from *Solanum* species and SLF-1 from *Petunia* and the third clade contained SLF-12 from *Solanum* species and SLF-3 from *Petunia.* To construct the ML tree, the deduced SLF sequences were subjected to BLAST alignment against the *Solanum* taxid and interestingly, all the hits (with maximum identity) belonged to wild/cultivated tomato SLFs. There was not a single hit belonging to the wild/cultivated potato SLFs even though the genome sequences for *Solanum*

tubersosum and *Solanum phureja* are available. This could due to lack of annotated potato SLFs. Also, it can inferred from the phylogenetic tree that *Solanum* SLF-7, SLF-12 and SLF-23 share an orthologous relation with *Petunia* SLF-1, SLF-3 and SLF-12 respectively due to a speciation event.

Figure 9: Maximum-likelihood phylogenetic tree with 1000 bootstrap runs and the highest log likelihood (-6932.59) for SLF-7, SLF-12 and SLF-23 from the *Solanums* and putative SLFs from *Solanum okadae* is shown along with respective *Petunia* orthologs. The percentage of trees in which the associated taxa clustered together is shown next to the branches.

In addition to the orthologous relationship of SLFs from *Solanum okadae, Petunia* and other *Solanum* species, the sequence divergence among the putative SLF paralogs *in Solanum okadae* was analysed. As previously indicated, the results demonstrated that each SLF ortholog is highly conserved between *Solanum okadae* and other *Solanum* species, with sequence identities ranging from 90% to 96% despite mutations at various sites. The results also showed that the deduced SLF paralogs in *Solanum okadae* had low similarity, with the percentage of sequence identity ranging from 73.5% to 68.8% at the nucleotide level and from 38.6% to 47.6% at the amino acid level.

4. Discussion

4.1 SI in *Solanum okadae*

In angiosperms, the SI mechanism allows the gynoecium of flower to identify and differentiate between self and non-self-pollen, allowing only non-self-pollen to fertilise the flower. Depending on the S-haplotypes expressed in the pistil and the pollen, different compatibility correlations might be seen in the GSI system. Therefore, when at least one of the expressed haplotypes in the pistil and pollen do not match, a compatible or partially compatible response will take place. If the two parents vary by a single haplotype, a semi-compatible reaction will take place, with all pollen tubes having the shared haplotype being rejected and those carrying the unique haplotype being allowed. In contrast, when both expressed S-haplotypes in the pistil and pollen match, an incompatible reaction occurs, halting the growth of all pollen tubes.

An effective genotyping technique is very essential for genome editing in plants. Plant biologists often need to learn about the genotype of more than one polymorphic loci. For multi-locus genotyping, many scientists use labour-intensive techniques, such as restriction fragment length polymorphisms (Harushima *et al*., 1988), cleaved amplified polymorphic sequences (Konieczny *et al*., 1993), and simple sequence repeats (McCouch *et al*., 2002). The crucial struggle in multiple genotyping with such genetic markers is that it needs a lot of labour and times since these studies require multi-step sample processing together with electrophoresis. Additionally, there are hardly any of the suitable polymorphisms in some regions of the genome. The allele-specific primer PCR (ASP-PCR) method was used in this study for allele analysis. The reliability of this method was demonstrated in wheat (Wei *et al*., 2009) and Arabidopsis thaliana (Drenkard *et al*., 2000).

Initially, the only parameter used to distinguish SC in potatoes was controlled self-pollination, followed by a berry and seed set (Hosaka and Hanneman, 1998). To study SC in diploid

potatoes, however, requires thorough phenotyping of many traits associated with reproduction because berries produced by selfing are produced by self-fertility rather than SC (Peterson *et al*., 2016). Infertility of either male or female gametes, a lack of fusion of gametes and embryo termination can all contribute to the absence of berry formation after selfing. In the diploid potato, spontaneous berry development is a rare occurrence (Johnson *et al*., 2019). Self-compatible diploids are uncommon, with SI being the most common cause.

Potatoes are typically propagated vegetatively, resulting in lower selection pressure for fertility traits. Ploidy reduction in diploids revealed harmful recessive mutations that worsen fertility issues in comparison to their tetraploid relatives. Therefore, the SC status of the potato could be determined by phenotyping other reproductive-related traits like pollen fertility and pollen tube growth after selfing. S-RNases are broken down, allowing pollen tubes to grow and perform self-fertilization. This mechanism, which can be examined 48 hours after self-pollination, has given scientists a new phenotyping tool that makes it easier to distinguish between SI and SC than with indirect or confusing markers like a berry set (Kardile *et al*., 2022). Based on research conducted by Clot *et al*., (2020), it is recommended that both pollen tube growth and berry set be recorded in order to explicitly identify the diploid SI and SC plants. Clones with 75% berry set and a significant number of pollen tubes reaching the ovaries, for example, may have been classified as SC. In order to prevent sterility from confusing the compatibility phenotype, a more strict SC phenotyping protocol may eventually incorporate berry and seed set from both self and cross-pollination as well as visualisation of pollen tube development in the style. Plants that produce multiple self-berries are categorised as SC, whereas plants that fail to produce self-berries after at least 10 self-pollinations, exhibit self-pollen tube growth arrest in the style, or produce berries after cross-pollination with substantial pollen are categorised as SI. An extensive collection of potato germplasm can be screened for self-compatibility thanks to improved extensive phenotyping. This method has discovered a new SC source, which has given us a way to thoroughly examine the underlying S-locus and S-locus inhibitor. Although SC phenotyping is robust, it requires a lot of work and exhibits quantitative variation. As a result, there is an urgent need to identify molecular markers linked to SC, which calls for a thorough examination of the genes that control SI.

The main genes controlling SI in potatoes are male determinant SLF and female determinant S-RNase. There have been reports of 49 S-RNase sequences in various potato species, mostly diploids (Eggers *et al*., 2021). Matching alleles are effectively rejected after pollination and prevented from developing into zygotes, according to allelic variants of the S-RNase gene. It

is anticipated that plants displaying the S-RNase-based GSI system will be heterozygotes carrying two distinct S-alleles, indicating the co-dominate nature of S-RNase (Ye *et al*., 2018). When compared to 70 S-RNases from *Solanum* species, the S-RNases of potato tuber-bearing species exhibit inter- rather than intraspecific similarities, indicating that these S-alleles have undergone trans-generic or trans-specific evolution (Ioerger *et al*., 1991). The fact that these similarities are interspecific suggests that S-RNase is primitive, descended from a single ancestor, and has been passed down to various genera. The Prunus genus of the Rosaceae family exhibits a comparable pattern (Sutherland *et al*., 2008). The observed S-locus polymorphism in angiosperms is a result of the age of S-alleles, diversifying selection, and the strongly linked S-locus genes that preserve allelic variation at the S-locus (Richman and Kohn, 2000). Extensive S-allele diversification can be seen in the *Solanum* genus as well as other genera like *Petunia* and *Nicotiana*. The ploidy of wild potatoes varies, ranging from diploid to hexaploid. Tetraploid potatoes suffer from inbreeding depression and are only partially self-compatible, in contrast to nearly all diploid potatoes, which are self-incompatible almost exclusively. Since there have been fewer S-RNase identified in potato genotypes (Ma *et al*., 2021) compared to other solanaceous plants, further research into this gene family is necessary.

4.2 Sequence and Phylogenetic analyses of putative SLFs

Unilateral incompatibility (UI) is a reproductive barrier linked to self-incompatibility (SI) in which pollen from one species or population is rejected on the pistils of a related species or population, although no pollen rejection occurs in reciprocal crosses. Pollen of SC species or populations is nearly always rejected by pistils of related SI species or populations, although pollen rejection is uncommon in reciprocal crosses (SC pollinated by SI). This unidirectional pollen rejection pattern is known as the "SI SC rule." (Lewis and Crowe, 1958). Two pollen factors from *S. pennellii*, *ui1.1* and *ui6.1*, have been previously reported by Li *et al*., (2010) to be necessary and sufficient to break through the UI barrier on allotriploid *S. lycopersicum x S. lycopersicoides* hybrids. A pollen specific Cullin1 (CUL1) protein is encoded at the *ui6.1* gene, and it is used by UI and SI to recognise pollen. On pistils expressing active S-RNases, pollen without *ui6.1* is incompatible; however, on pistils expressing a mutant S-RNase with no RNase activity, pollen lacking *ui6.1* is compatible. According to this discovery, *ui6.1*—and consequently, *ui1.1*—are necessary for pollen resistance to S-RNase-based rejection in the pistil.

According to Li *et al*., (2015), the *ui1.1* gene was located at the S-locus on chromosome 1 and mapped to a 0.43-cM, 43.2-Mbp interval, however positional cloning was impeded by a low recombination frequency. They anticipated that ui1.1 encoded an SLF protein or proteins that interacted with CUL1 and Skp1 to create a SCF-type (Skp1, Cullin1, F-box) ubiquitin E3 ligase complex. Following this, they discovered 23 SLF genes in the *S. pennellii* genome, 19 of which were also found in cultivated tomato. Expression analysis, recombination events, and sequence annotation revealed 11 *S. pennellii* genes as potential candidates. Genetic modifications revealed that just one of them, SpSLF-23, was required for *ui1.1* function. An evaluation of cultivated and wild tomato species found SLF-23 orthologs in all SI species but not in the SC species *S. galapagense, S. cheesmaniae* and *S. lycopersicum*, whose pollen lacked *ui1.1* function. These findings show that pollen compatibility in UI is regulated by protein degradation via the ubiquitin-proteasome pathway, which is similar to the mechanism that governs pollen recognition in SI. They also reported that, *Petunia* type-2 SLF protein, which has been demonstrated to function in SI by recognising S9-, S11-, and S19-RNases, shared a significant degree of amino acid sequence similarity with SpSLF-23, which is same as the findings in current study. SLF-23's sequence closeness to type-2 SLF in *Petunia* implies it may also operate in SI (i.e., in a SI ancestor of SC *S. pennellii* LA0716). However, this is not proof that SLF-23 is specialised for interspecific pollen rejection. In two separate allotriploid tester lines with various S-RNase genes, they demonstrated that SpSLF-23 provided pollen resistance to S-RNases.

Biologists utilise the d_n/d_s ratio to evaluate the evolution rate of gene sequences in molecular evolution (Yang and Bielawski, 2000). This assessment compares the sequences of two homologous protein-coding genes from two closely related species. The ratio of nonsynonymous (amino acid) substitutions per nonsynonymous site (d_n) to synonymous substitutions per synonymous site (d_s) reflects the rate of evolution between these two sequences. Only those observable genetic modifications that have been adopted into the gene sequences are considered substitutions in this context. Since d_s measures neutral evolution (without functional enhancements under selection pressure), the d_n/d_s ratio represents the pace of adaptive evolution relative to the rate of background evolution. This measurement has been widely used in the investigation of adaptive molecular evolution and is recognised as a standard method for determining the pace of sequence evolution in biology.

The ratio d_n/d_s estimates the rate of evolution by comparing efficient evolutionary changes to background evolutionary changes that are silent. This ratio indicates the pressure of natural

selection on the evolution of organisms. In cases where $d_n/d_s > 1$, nonsynonymous substitutions are fixed quicker than synonymous substitutions, indicating that positive selection fixes nonsynonymous amino acid changes faster than silent ones. While d_n/d_s is typically less than 1, there are instances where harmful substitutions are removed by purifying selection (negative selection) and the rate of amino acid change fixation is decreased. If $d_n=d_s$, the rate of fixation of these two types of changes is identical. Measuring a high d_n/d_s ratio shows that adaptive genetic variants were created and stabilised at a rapid rate (Hu and Banzhaf, 2008).

In phylogenetic reconstructions, shared ancestral polymorphism is frequently observed as a result of strong balancing selection, with alleles from various species and genera clumping together (Sonneveld *et al*., 2001). This suggests that S-alleles are frequently significantly older than the species from which they are taken. Coalescence times of S-locus polymorphisms are frequently predicted to be several tens of millions of years, which is significantly longer than the coalescence times of polymorphisms at loci not susceptible to balancing selection (Paape *et al*., 2008). Sequence divergence at S-loci is similarly substantial, with S-alleles frequently differing by 40% or more in amino acids. This reflects both their immense age and the rarity of recombination at recognised S-loci. In the current study, we found the d_n/d_s ratio for the three SLFs to be higher (Table 4) indicating a strong positive selection leading to diversification as diversifying alleles can leave unique signs of positive selection among amino acid positions in related species (Paape and Kohn, 2011). Higher estimated d_n/d_s ratios could be due to enhanced fixation of non-synonymous substitutions due to increased selection, or to fixation of more synonymous alterations in *Solanum* S-alleles due to their age. Numerous long-term balancing selection signatures can be seen in the long-term evolution of SI genes. The first characteristic is a high degree of sequence polymorphism, measured both in terms of the number of allelic lineages that are preserved and in terms of how different the lineages that segregate within species are normally (Castric & Vekemans, 2004). The second characteristic is a rapid evolution of the SI proteins. Rare mutants resulting from mutations should be promoted if they change the protein sequence and provide novel SI specificities, whereas synonymous mutations should follow a neutral pattern. This should result in an increase in the ratio of the SI genes' nonsynonymous (d_n) to synonymous (d_n) rates of evolution. As a result, multiple investigations have indicated higher d_n/d_s ratios for at least some SI gene codons in several species like *Arabidopsis* and *Brassica* (Castric & Vekemans, 2007), *Leavenworthia* (Herman, *et al*., 2012), *Prunus* (Vieira *et al*., 2007) and *Solanaceae*, (Paape & Kohn, 2011) which are in agreement with our findings. Additionally, the degree of d_n/d_s elevation for the

most extreme codons appears to differ between species, presumably indicating a variation in the rate of allelic diversification. In fact, the highest dn/ds values were found in *Leavenworthia* (Herman *et al.*, 2012, dn/ds = 3.49) and *Brassica* (Castric & Vekemans, 2007, dn/ds = 2.49 vs. dN/dS = 1.49 in *Arabidopsis*). Both are assumed to have experienced recent demographic bottlenecks that drastically condensed the number of allelic lines at the SI genes.

Markova *et al.*, (2017) discovered two nonsynonymous substitutions unique to all *S. chmielewskii* alleles at bp 118 and 532 of the consensus alignment, which change phenylalanine to leucine and proline to arginine, respectively, in their study to determine if the pattern of SI to SC transitions was common in other SC wild tomatoes. The second amino acid alteration occurred in the F-box protein interaction region of the SLF-23 sequence, which spanned bp 331 to 948. Similar to the findings of this study, their comparisons of five *S. chmielewskii* SLF-23 alleles revealed a tendency toward positive selection ($d_n/d_s = 2.55$). Whereas, on the contrary to our findings, comparing the d_n/d_s values of seven *S. neorickii* and five *S. arcanum* SLF-23 sequences showed a sign of purifying selection, with average d_n/d_s values of 0.67 and 0.349, respectively, within each species.

Positive selection has been estimated in different species using various methods, most notably the maximum likelihood phylogenetic approaches first described by Nielsen and Yang (1998). In our study, the similarities in amino acid sequences show that SLFs in each species are very different from each other. Sequence divergence within a species is on the same level as sequence divergence between species. In fact, it appears that some SLFs are more similar to SLFs from other species than to SLFs from the same species as depicted in Figure 9. For example, SLF 7 from *Solanum peruvianum* is more closely related to SLF 7 from *Solanum habrochaites* then to its own SLF 12 (Fig 9). Because population bottlenecks are predicted to reduce S-locus diversity, and because the S-locus is thought to be subject to negative frequency-dependent selection (i.e., new S-alleles that arise via mutation should be highly favoured when rare), it is anticipated that following a bottleneck, S-alleles will exhibit a strong signature of positive selection, particularly in genes encoding amino acids involved in recognition. Castric and Vekemans (2007) cited a bottleneck in their analysis of *Brassica* and *Arabidopsis* S-alleles to explain the higher positive selection operating on *Brassica* S-alleles. Such a result has similarly been observed in the S-RNase locus of the Solanaceous *Physalis* genus (the gene encoding the stylar specificity component of SI in this family), a taxon that has likewise experienced apparent recent diversification (Paape and Kohn, 2011).

In order to maintain gametophytic SI, SLFs cannot detoxify S-RNases from the same haplotype, and in order to allow for cross compatibility, SLFs must be able to detoxify S-RNases from other haplotypes, permitting cross-compatibility. The first limitation might be easily addressed by selection for an S-RNase that is outside the detoxifying range of the connected SLF if the ancestral S-locus consisted of a single SLF and S-RNase (Sakai, 2016). However, the chance of detoxification determines whether the second restriction may be satisfied. Only 25% of nonself styles would receive pollen if this chance was 0.5, as is the case with SLFs from *Petunia* (Kubo *et al*., 2015). It is challenging to imagine how the SI system might have developed given its high reproductive cost.

Expression of an SLF from a distinct S-haplotype in pollen will shield the pollen from its own encoded S-RNase, resulting in the elimination of incompatibility, a phenomenon known as competitive interaction (Sijacic *et al*., 2004; Zhao *et al*., 2021). The majority of these tests have been conducted with SLFs from the same species as the target S-RNase. According to recent findings by Zhao *et al*., (2022) , *Petunia* S-RNases can be detoxified by SLFs from various taxa in 22 out of 26 instances, or 0.85 times out of 26. A single SLF coupled to an S-RNase would be accepted by 72% of styles with such a probability (Kubo *et al*., 2015), resulting in a significantly reduced reproductive cost than with a probability of 0.5. As a result of their interspecific studies, they proposed that the ancestral system included SLFs with a high detoxifying probability. Their findings also point to a very dynamic future for SI's development. A single female and male component forming a close connection was likely necessary for the initial development of an ancestral GSI in angiosperms. To establish GSI with enough cross-compatibility, the original single SLF had a high detoxifying chance. While selection against self-S-RNase detoxification resulted in lower probabilities of detoxification within species (Li *et al*., 2019), selection for further increase in cross-compatibility led to duplication and divergence of the SLF inside the S-locus.

5. Conclusion

In this chapter, 3 putative partial SLF sequences (type 7, 12 and 23; Appendix II) from *Solanum okadae* were identified using concensus PCR and *Petunia* SLF sequences as references. It was observed that the *Solanum okadae* shared an orthologous relation with the *Petunia* SLF according to the phylogenetic analysis. These SLFs could be used in future SI breakdown experiments via competitive interaction route. This chapter also demonstrates that *S. okadae* SLFs are subject to substantially stronger positive selection, suggesting that this rapid rate of amino acid replacement is the product of an early bottleneck that drastically reduced the

quantity of S-alleles but did not result in the breakdown of SI. Strong bottlenecks have been shown to be associated with the transition to SC in mustard species (Foxe *et al*. 2009; Guo *et al*. 2009), and similar strong signals of positive selection were identified in the SLFs of SC wild tomatoes in this study. It would be fascinating to identify when such bottlenecks result in the loss of SI and when they allow SI and trans-specific and transgeneric polymorphisms to persist at the S-locus.

CHAPTER 8: General Discussion

Crop breeding is essential for producing high-quality food and enhancing global food security. In this context, increasing genetic variety is necessary to sustain the health of the agroecosystem and to offer prospects for further crop improvement. Monitoring changes in crop genetic diversity through time, brought on by biotic and abiotic restrictions as well as anthropogenic breeding techniques, is of utmost importance for achieving sustainable agricultural production in the face of upcoming difficulties like climate change. The cultivated potato (*Solanum tuberosum*) is an established crop for assisting with global food security because it is the most significant tuber crop in the world and the fourth most significant crop grown for human use (Devaux *et al*., 2021). This is because to its wide geographic distribution, extensive current cultivation to meet demand, and ease of use in cooking, particularly in developing nations with high levels of hunger, malnutrition, and poverty.

In modern potato breeding, the loss of natural genetic diversity is a complex challenge. Recent estimates indicate that during domestication, potato lost approximately 500 genes, including many R-genes important in disease resistance. There is a substantial allelic variety of R-genes and prospective alleles for enhanced abiotic stress tolerance, quality, and consumer-oriented features in wild potato species (Bashir *et al*., 2021). Enhancing potato germplasm involves transferring desirable traits from wild to cultivated potato (Maune *et al*., 2018). The linkage drag between desirable and unwanted traits complicates this transmission and severely limits the wild potato's donor potential. *De novo* domestication is a recently proposed concept for modern crop breeding that employs genome editing techniques to convert wild species into domesticated crops (Fernie *et al*., 2021). The idea is that 'wild' alleles of domestication genes can be artificially turned into 'domestic,' resulting in the production of a phenotype typical of the domestication syndrome. The concept was first presented and subsequently experimentally demonstrated for tomato (Zsögön *et al*., 2017). Two research groups demonstrated the successful introduction of domestication features into wild tomato using targeted knockouts of a few essential genes at the same time (Zsögön *et al*., 2018; Van Eck, 2018). Over the last five years, advances in genome sequencing and gene function research have enabled the identification of critical genes involved with major domestication-associated features in a variety of crops, including potatoes.

The application of the concept of *de novo* domestication necessitates suitable gene and genotype selection. There are over 200 wild potato species (Hawkes *et al*., 1990), with

numerous accessions in global gene banks. If *de novo* domestication is viewed as a method of developing novel domestic crops, selecting one or a few wild potato genotypes appears to be a difficult task. Another strategy is to use the concept of *de novo* domestication as a tool for improving trait donors. The selection of donor genotypes for beneficial features, as well as their modification to eliminate or rectify the most undesirable qualities, can help to speed up the process of elite potato germplasm augmentation.

The production of novel cultivars with viral, late blight, potato cyst nematode, and *Rhizoctonia* disease resistance is made possible via potato germplasm fortification. Other significant traits including high tuber solids, resistance to cold sweetening, and starch content in tubers can also be attributed to introgressions from wild relatives (Jansky *et al.*, 2021). Despite advancements in the creation of pathogen resistant potato cultivars, there aren't many wild genotypes used in potato breeding. It is estimated that, of the 228 wild potato species, only about 10% are engaged in breeding (Gavrilenko *et al.*, 2017). Many species are still understudied, and those with proven useful features cannot be easily crossed with cultivated potatoes (Yermishin *et al.*, 2021). Another issue is the high adaptive distinction and erratic phenotypic plasticity within species, as well as the large number of poorly characterised closely related accessions in germplasm collections. Wild potato germplasm must be phenotyped, genotyped, annotated, resistant genotype searched for, and conserved before being used in breeding.

This thesis examined one of the understudied wild Andean diploid potato species, *Solanum okadae*, to check for its potential to improve current cultivated potato. From the previous literature, it is know that *Solanum okadae* is drought resistant (Watanabe, 2011) and possess R2, R8, R9, and Rpi-blb2 genes that provide late blight resistance (Karki, 2020), but apart from this, there is no recorded data on its nutritional or any other beneficial trait. This thesis tried to fill in a major gap in information related to its nutritional value and also tested it for another abiotic stress tolerance (salt). For the first time, a protocol for in vitro micropropagation and regeneration of *Solanum okadae* was established in this theis which can be used for future transformation studies. This thesis also aimed at creating a S-RNase knockout for production of inbred lines of *Solanum okadae* and identified the putative S-Locus F-Box protein sequences.

1. Micropropagation and Regeneration

Tissue culture is a simple technique that has been widely used in potato plants for a variety of purposes, including the study of tuberisation and tuber dormancy physiology (Raspor *et al.*,

2020), tolerance to biotic and abiotic stressors (Queirós *et al*., 2009), and the production of transgenic plants (Conner, 2007). Due to the large number of explants produced from a single mother plant and the adaptation of micro-plants to conditions that favour slow development in order to preserve virus-free genetic material for extended periods of time, tissue culture has received special attention for potato breeding programmes (Novy, 2014). Furthermore, specialised methods, particularly somatic embryogenesis and protoplast culture, may be successfully used in potato breeding projects (Du *et al*., 2021). Callus cultures for shoot regeneration have been successfully adopted in potato plants; however, due to considerable somaclonal diversity, this approach is often utilised in breeding programmes targeting specific traits (Kumlay and Erci*Sli*, 2015).

Globally, the production of high-quality seed tubers (genetic purity, cleanliness, correct physiological age) is one of the most significant operations in potato farming (Halterman, 2016). The introduction of micro-propagation into the potato seed production chain sped up the development of healthy clonal types and enabled the production of seed tubers throughout the year and independent of climate. Despite discrepancies in tuber seed categorization and seed production methods utilised in different countries (Davidson and Xie, 2014), the micro-propagation technique is widely employed in the potato seed production area, particularly for pre-basic seeds. The major micropropagation technique used in commercial scale tuber seed production is *in vitro* cultivation of explants (apical meristems, shoot tips, or single-node cuts). The obtained explants (micro-plants) are employed in viral control experiments, *in vitro* regeneration of micro-plants, *in vitro* synthesis of micro-tubers, and in vivo manufacture of mini-tubers using this approach.

In potato breeding, wild potato species are a valuable source of genetic diversity. They can be incorporated into cultivated *S. tuberosum* via haploids that 'capture' genetic diversity and 2n gametes that transmit genetic diversity to cultivated forms (Peloquin *et al*., 1991). However, the existence of several barriers (self-incompatibility, genic-cytoplasmic male sterility, endosperm development failure) may occasionally interfere with the transfer of desirable new traits into cultivated forms.

Tissue culture competency disparities across potato cultivars and breeding lines have long been documented, as has the transmission of these competences in segregating populations. Shoot regeneration from leaf discs (Coleman *et al*. 1990), embryo development in anther culture (Sonnino *et al*. 1989), and p-calli creation following protoplast separation (Cheng & Veilleux

1991) have all been demonstrated to be genetically regulated. The first step in adopting these approaches for crop improvement is identifying plant lines that are tissue culture competent. Screening for competence in anther or protoplast culture is a time-consuming and costly operation, but screening for the capacity to regenerate shoots from calli, nodal segments and other explants is a rapid and simple method. Tissue culture, *in vitro* plant regeneration, and *Agrobacterium*-mediated transformation procedures are easily adaptable to tetraploid cultivars and certain diploid lines (Nadolska-Orczyk *et al*., 2006). These approaches, however, are genotype and procedure dependent.

A method for plant regeneration is necessary to create both genetically modified and genome-edited plants (Loyola-Vargas and Vázquez-Flota, 2006). Because the circumstances for regeneration vary widely depending on the cultivar, it is challenging to grow regenerated plants of potatoes. In this study, the ideal doses of plant growth regulators for micropropagation, callus induction and regeneration of *Solanum okadae* plantlets were identified by establishing the ideal amounts of auxins and cytokinins, two plant growth regulators crucial for the regeneration of potato plants. According to this experiment, the ideal doses of these plant growth regulators did not vary much depending on the type of technique. For micropropagation experiments via nodal segments, the best PGR concentration was found to be 3 mg/L BA+2 mg/L NAA+1 mg/L kinetin that produced longer shoots and higher number of leaves and shoots per explant whereas at the concentration of 3 mg/L BA+3 mg/L NAA+1 mg/L kinetin, the calli derived from nodal segments showed highest regeneration frequency with longer and greater number roots and shoots along with more leaves. On the other hand, the best calli was derived from nodal segments only with a single hormonal treatment (2mg/L 2,4-D).

The initiation and proliferation of shoots has been a persistent difficulty in potato tissue culture. Shoots were occasionally generated, but there was no easy-to-follow regeneration process. Evidently, unlike in other plant species, potato callus cannot be easily managed to regenerate shoots (Hajare *et al*., 2021). The findings of this investigation are consistent with those of Kikuta and Okazawa (1984), Jarret *et al*. (1980) and Hajare *et al*., (2021), who discovered that the MS medium supplemented with BAP and NAA at concentrations ranging from 1.5 to 3.0 mg/L promoted the best shoot initiation. Rout *et al*. (2001) discovered that BAP, kinetin, and ascorbic acid produced the greatest outcomes in potato for regeneration of multiple shoot development from apical shoots. Shah *et al*. (2001) discovered that an MS medium with 0.5 mg/l NAA produced the longest stem and the greatest single node in potato. A comparable study on the highest number of shoots induction with 2 mg/l BAP was previously published in

the PPR-1 mulberry variety (Rohela *et al*., 2018). As a result of the preceding experiments, it is obvious that protocol standardisation differs depending on the explants employed and the potato type.

In potato biotechnology, *in vitro* shoot multiplication techniques have been utilised effectively for about 60 years. Some of these procedures, including the cultivation of callus, roots, and shoots, are regarded to be established, well-developed, regular processes in which no significant advancements have been made for several years. In the present experiment, BAP, NAA and Kinetin were used to induce fast multiple shoot development from the nodal segments and 2,4-D was used to produce healthy calli. Lastly, our study demonstrates that plant tissue culture utilising nodal segments as an explant is efficient for *in vitro* potato micropropagation. An effective protocol for *Solanum okadae* micropropagation and regeneration was optimised. Under *ex vitro* circumstances, 89.43% of plantlets were acclimatised. As a result, this approach will serve as the foundation for its mass production using *in vitro* methods.

2. Salt Tolerance

In many regions of the world, salinization of soils poses a serious threat to crop production. Climate change-related consequences, such as increasing seawater intrusion in coastal areas and the requirement for greater irrigation as a result of droughts, exacerbate it. Finding crops and crop varieties that can tolerate salt is one way to lessen the harmful effects on agriculture. Despite some scepticism about the ability of breeding programmes to counteract salinization (Plaut *et al*., 2013), there is widespread agreement that commercially interesting potato varieties that are more salt tolerant than others can be found or created and should be adopted in everyday practise (Pradel *et al*., 2019). Fast screening tests focusing on quantitative features that may be assessed *in vitro* within a brief amount of time are a frequent method for identifying potential crop varieties (Munns *et al*., 2002).

There are numerous opportunities to identify genotypes with the inherent capacity to produce significantly greater yields while withstanding moderate salt conditions. Using tissue culture technology, more dependable and time-saving procedures for selection have been devised (De-Filippis, 2014). Utilizing nodal cuttings of tissue culture grown (micropropagated) plants, *in vitro* assessment of salinity resistance enabled ranking of potato cultivars and wild species (Mawa *et al*., 2021). Similar approach was used in this thesis to screen *Solanum okadae* for

salt tolerance *in vitro* by evaluating morphological and biochemical parameters. Out of the four levels of salt treatments (50, 100, 150 and 200mM), *Solanum okadae* was able to withstand the 150mM salt stress. The performance of *Solanum okadae* to withstand higher salt concentration (150mM) was attributable to a robust antioxidant defence mechanism, as indicated by higher catalase (CAT) activity to counteract harmful H_2O_2 and improved osmotic adjustment (accumulation of proline). A plant's salt tolerance is sometimes characterised as the extent to which it can survive moderate or high amounts of salt in the water on its leaves or in the soil within reach of its roots without suffering severe harm. Therefore, *Solanum okadae* could be deemed salt tolerant and after a careful field trial, *Solanum okadae* can be suggested for growing under salinity stress and may be utilised in hybridization programmes to create new high-yielding and salt-tolerant breeding lines. Additionally, these plants can be utilised to comprehend the genetic and molecular pathways of potato salt tolerance.

In this thesis, the effect of exogenous melatonin application on severely salt damaged *Solanum okadae* plantlets *in vitro* was also examined. High salt concentration (200mM) harmfully affected plantlet growth, photosynthetic and antioxidant activity and ultimately killed the *Solanum okadae* plantlets. Interestingly, supplying 100µM of melatonin along with 200mM NaCl to the plantlets helped in maintaining the growth by enhancing the antioxidant and photosynthetic machinery as observed by higher catalase and total chlorophyll content as reported by Zhou *et al*., (2016) in tomato and by maintaining the K^+/Na^+ homeostasis as observed in sweet potato (Yu *et al*., 2018). There has been no research on application of exogenous melatonin in alleviation of stresses in potato apart from the results of Zhang *et al*., (2017) which contributed to a better knowledge of the direct functions of melatonin on *P. infestans*, as well as a potential eco-friendly biocontrol technique based on a melatonin-based model and bid to prevent potato late blight. Therefore, this study has successfully laid the foundation for future work by studying the effects of melatonin on salt stressed *Solanum okadae* plantlets for the first time.

3. Nutritional Analysis

People frequently think of potatoes as comfort food, whether they are mashed, baked, or roasted. It is a significant staple food and the top vegetable crop worldwide. Unfortunately, the majority of people consume potatoes as oily French fries or potato chips, and even baked potatoes frequently contain lipids like butter, sour cream, melted cheese, and bacon bits. Even roasted potatoes may be a potential cause of a heart attack as a result of such treatment. A baked

potato, on the other hand, is a very healthy low calorie, high fibre diet that gives great protection against cancer and cardiovascular disease when the excess oil and deep frying are removed.

Improving the nutritional and bioactive chemical content of crops that are mostly consumed by the local people, along with an efficient nutritional programme, is a key technique to eliminate malnutrition. Potato can feed and alleviate malnutrition in the globe, where 782 million out of 7.9 billion people are malnourished and an estimated 149 million children under the age of five were stunted in 2020 (2021, UNICEF, WHO, and World Bank). The potato tubers include starch as the most abundant component (Nauman *et al*., 2019) and are regarded as an attractive source of other nutrients like high-quality proteins and bioactive substances like phenolic compounds, carotenoids and dietary fibre.

Along with desirable and traditional breeding features of agronomic values like yield and resistance to biotic and abiotic stresses, it is vital for breeding programmes to create new potato cultivars with innovative traits like high nutritional and bioactive chemical contents (Navarre *et al*., 2019). Biofortification, the method of boosting the vitamin and mineral content of a crop by plant breeding, is a realistic and cost-effective method of distributing micro and macro nutrients to human populations with limited access to various nutrient-rich meals and other intrusions (Bouis & Saltzman, 2017). Several studies have demonstrated that Andean genotypes exhibit the greatest diversity in tuber shape, flesh and skin colour, texture, and flavour, which may lead to a substantial variation in nutritional and bioactive chemical content (Calliope *et al*., 2018).

One goal of the thesis was to understand more about the nutritional makeup of a wild Andean potato, *Solanum okadae*. A better understanding of this species' nutritional properties could aid in its conservation, however no data on its nutritional composition are currently available. This was accomplished using a variety of analytical techniques such as ion chromatography with pulsed amperometric detection (IC-PAD), gas chromatography-flame ionisation detector (GC-FID), liquid chromatography-diode array detection (LCUV-DAD), inductively coupled plasma mass spectrometry (ICP-MS), and liquid chromatography triple quadrupole-mass spectrometer (HPLC-QQQ-MS). In this thesis, a proximate composition analysis, vitamin C and B12 estimation, amino acid profiling, and macro and micro mineral evaluation of *Solanum okadae* was conducted and compared to commercially available *Solanum tuberosum 'Celandine'*. In terms of proximate composition, *Solanum okadae* had a larger content of proteins, carbohydrates, and energy contribution than *Solanum tuberosum 'Celandine.' Solanum okadae*

also had more vitamin C than *Celandine* and more vitamin B12, which was nearly non-existent in *Celandine*. The concentrations of all micro and macro minerals were slightly higher in *Solanum okadae*, with potassium, calcium, zinc, and manganese being significantly higher. Same was the case in terms of essential and non-essential amino acids, where isoleucine, leucine, lysine, phenylalanine, threonine, serine and glycine were almost more than double in concentration in *Solanum okadae* when compared to *Solanum tuberosum 'Celandine'*.

The International Potato Centre's (CIP) principal breeding goal has been the biofortification of commercial potato (*Solanum tuberosum L.*) with the important micronutrients iron and zinc (Amoros *et al*., 2020). This study not only assessed a wild diploid potato for iron and zinc content , but rather provided information on its full nutritional profile in comparison with its commercial tetraploid counterpart, *Solanum tuberosum*. This study adds to our understanding of the nutritional composition of the Andean diploid potato (which has received little attention thus far), which can be used in helping in the enhancement of current cultivated potato.

4. Self-Incompatibility

Despite potato's importance as a food crop, yield gains have been negligible over the past century, whereas maize yield has increased by 1% per year (Clot *et al*., 2020). This lack of advancement in breeding is exemplified by the widespread cultivation of century-old varieties such as 'Russet Burbank' and 'Bintje' (Lindhout *et al*. 2011). Potato sexual reproduction is characterised by polyploidy, tetrasomic inheritance of multiple alleles, and selfing-induced inbreeding depression. It is possible for tetraploids to self-pollinate, but this is uncommon in conventional breeding practises. As a result, conventional potato breeding involves crossing two highly heterozygous tetraploid clones, and the likelihood of producing offspring with a wide range of advantageous allele combinations is low. Destructive recessive alleles are rarely exposed, making selection against mutational load in the germplasm difficult to achieve. Furthermore, clonal selection requires 5–9 years (Gopal, 2015) because tubers are damaged during phenotyping and because of the slow rate of tuber reproduction. More significantly, compared to crops with annual sexual reproduction, the amount of allelic rearrangement over time is significantly lower with only one meiotic event per clonal selection cycle (Jansky and Spooner, 2018). Alternative breeding strategies have been proposed to get around the drawbacks of traditional breeding. Chase (1963) proposed analytical breeding, which entails ploidy reduction, breeding at the diploid level, and then re-synthesis of tetraploids. According to proposals by Jansky *et al*. (2016), F1 hybrid breeding at the diploid level allows for the

fixation of genetic gains and the elimination of undesirable alleles. To achieve this, diploid germplasm must be self-compatible and tolerant of inbreeding depression. While tetraploid potatoes are self-compatible, a gametophytic self-incompatibility system based on S-RNase prevents inbreeding in most diploid potatoes (McClure *et al*. 2011).

Due to outcrossing, the majority of wild potato relatives are highly heterozygous. Although most diploid *Solanum* species are self-incompatible due to a gametophytic self-incompatibility system, polyploid potato species are self-compatible (Pandey, 1962). However, recent genomic studies have found surprisingly high levels of homozygosity in wild relatives discovered through gene banks (Aversano *et al*., 2015). There are various plausible explanations. Small sample sizes during collection may have resulted in inbreeding occurring during gene bank maintenance, small wild populations may naturally be inbred, or self-incompatibility may not be as prevalent or effective as breeders previously thought. Relatives of the wild potato have significantly influenced the cultivated potato. As a result, it is believed that wild relatives have contributed significantly to the genetic improvement of the potato crop (Maxted *et al*., 2012). However, in general, the use of wild species relatives' germplasm and its integration into new varieties has been restricted. Although numerous programmes have attempted to introduce wild species germplasm into cultivars over the course of several decades, *S. tuberosum* still constitutes the majority of the potato genome. In fact, many wild species' potential value hasn't been considered (Castaeda-Alvarez *et al*., 2015).

In Solanaceae, S-RNase-based self-incompatibility (SI) is controlled by a single, highly polymorphic S-locus encoding for a style-specific S-RNase gene and numerous pollen-specific S-locus F-box (SLF) genes (Kao and Tsukamoto, 2004). The collaborative non-self-recognition hypothesis (Kubo *et al*. 2010) predicts that each SLF allelic variant can explicitly facilitate ubiquitination of certain of its non-self S-RNases, and that the combined action of the SLFs of one S haplotype promotes destruction of all except its own S-RNases (Sun *et al*. 2018). As a result, SI occurs when the pollen's S-haplotype matches either of the style's two S-haplotypes. According to Jacobs *et al*. (1995), the S-locus is found in potato on chromosome 1 in centromeric region, supporting the idea that SI can only be well-maintained in the absence of recombination between S-RNases and SLFs. Tetraploids are self-compatible due to their heteroallelic diploid pollen. According to Kubo *et al*. (2010), the expression of two different sets of SLFs allows for mutual weakening or competitive contact, which leads to the ubiquitination of all S-RNases. SC diploid clones can be created by adding an extra SLF gene

to generate competitive contact which has not been achieved in potato till date unlike *Petunia*, or by knocking out the S-RNase (Ye *et al*. 2018; Enciso-Rodriguez *et al*. 2019).

Previous research on tomato wild relatives showed that missense mutations and gene loss inhibit S-RNase ribonuclease activity in *S. peruvianum* and *S. pennellii*, resulting in self-compatibility (Li and Chetelat, 2015). Given that S-RNase is the gametophytic SI component directly involved in RNA degradation in self-pollen tubes, blocking S-RNase function is a simple technique for conferring self-compatibility in potato. The potato S-RNase gene, which governs self-incompatibility, possesses a high degree of allelic variability, with amino acid similarity ranging from 32.9% to 94.9% (Dzidzienyo *et al*., 2016). In the same year when the current project started, Ye *et al*. (2018) released their findings on generating self-compatible diploid potato by knocking out S-RNase with a justification that even though the majority of wild tomatoes are self-incompatible, S-RNase and other self-incompatibility-related genes had lost their function in cultivated tomatoes, making them self-compatible and therefore by using the CRISPR-Cas9 system to remove the S-RNase alleles, they could create a self-incompatible diploid potato. This thesis used a similar approach in knocking out S-RNase in *Solanum okadae* using S-RNase sequence data reported by Dzidzienyo *et al*. (2016). A CRISPR-Cas9 construct with single S_{o1} allele specific guide RNA that targeted the hypervariable B region of S-RNase to knock it out was created. Unfortunately, not a single transformant was obtained even after using two different CRISPR vectors (pRGEB31 and pKSE401) and two different *Agrobacterium tumefaciens* strains (LBA 4404 and EHA 105) and permutations and combinations of different factors like temperature, antibiotic treatment, co-culture and infection time which have been shown success in transformation of other crops including potatoes.

One of the main reasons that *Solanum okadae* could have been resistant to the *Agrobacterium* transformation could be due to pathogen-associated molecular pattern (PAMP) triggered immunity (PTI) which is facilitated by pattern recognition receptors (PRR). In plants, SERK3/BAK1, a leucine-rich repeat receptor that dimerizes with PRRs to support their activity, regulates PRRs. By inhibiting or degrading the receptors, pathogens can prevent immune responses from being triggered by PAMPs that are detected by PRRs in plant cells. Because plants have developed the ability to detect the presence of some of these effector proteins, they can respond quickly and hyper sensitively to stop and eliminate pathogen growth (Postel and Kemmerling, 2009).To detect the virulence-promoting effectors, many plant hosts have evolved a second type of immunity called effector triggered immunity (ETI), which uses

proteins encoded by R genes called polymorphic nucleotide-binding leucine-rich repeat (NB-LRR) or extracellular leucine-rich repeat (eLRR) proteins (Jones and Dangl, 2006). Some R proteins can also detect changes in host proteins that are targeted by pathogen effectors indirectly; this behaviour was first described as the "guard hypothesis" (Jones and Takemoto, 2004). To prevent the pathogen from spreading throughout the plant and causing disease, R gene-mediated resistance causes strong host defence activation, including a hypersensitive response, or apoptosis, at the infection site (He *et al*., 2007). In potatoes, (Du*et al.* 1994), the ability to regenerate and morph can result from a variety of causes. El-Kharbotly (1995), showed that even though potato genotypes such as J92-6400-A10 and J92-6400-A17 regenerated well, their transformation competence was low. Also, despite the good regeneration capacity of cv 'Saturna', a very low transformation efficiency was discovered (M. Goveia, personal communication) and it was demonstrated that the diploids of this variety were also resistant to transformation.

According to Ditt *et al.* (2001), *Agrobacterium* elicits reactions in tobacco that are often triggered by abiotic stress. The higher content of leucine in *Solanum okadae* compared to other essential amino acids, which was three times higher than in *Solanum tuberosum* as stated earlier, as well as *S. okadae's* high tolerance/resistance to salt and drought (abiotic stresses) as mentioned in previous chapters, supports the conclusion that unsuccessful *Agrobacterium* mediated transformation could be due to a strong defence mechanism, but more research on this topic is required. It is generally recognised that there is considerable heterogeneity in the ability of potatoes to regenerate and transform, and that both of these abilities are very protocol- and genotype-dependent (Visser, 1991). In several plant species, the impact of genotypes on regeneration has been studied. The influencing genetic elements can be recessive, as seen in the diploid domesticated potato *Solanum Phureja* (Taylor and Veilleux, 1992), or dominant, as characterised and localised in tomato (Koornneef *et al*., 1993).

On the basis of the molecular mechanism behind self-incompatibility in potatoes, two potential strategies may be utilised to overcome the reported self-incompatibility in diploid potatoes. The first approach involves modification of the S-locus, while the second is the transfer of *Sli*, the locus utilised to overcome SI by traditional breeding. Within S-locus modification, there are two options: the insertion of an additional SLF gene to degrade all S-RNases by mutual weakening or competitive interaction and the silencing of the S-RNase gene for its ribonuclease activity. Due to the failure of generating S-RNase knockouts in *Solanum okadae* in the current study owing to several possible reasons mentioned, three potential partial SLF sequences from

S_2 haplotype of *Solanum okadae*, which could be utilised in future for their possible insertions in SI genotypes were sequenced. The insertion of additional SLF to the SI genotypes ought to degrade all S-RNases, either by mutual dilution or competitive contact; nevertheless, this strategy has not been attempted yet and a transformable system such as *S. stenotomum* should be amenable to this approach. As it is now known that *Sli* encodes for products like SLF/SFB and works similarly to them in evoking the SC/SI response, this may become the preferred strategy in the future (Kardile *et al*., 2022). To identify the putative SLFs in *Solanum okadae*, three SLF sequences (SLF1, SLF2 and SLF3) from S_2 haplotype of *Petunia integrefolia subs. inflata* were chosen as reference sequences to design the primers. These reference sequences were subjected to BLAST alignment against *Solanum* taxid to find similar sequences in other *Solanum* species. Interestingly, all the initial hits with identity 90% and above belonged to cultivated and wild tomato species where PiS_2-SLF1 was most similar to SLF7s, PiS_2-SLF2 was most similar to SLF23s and PiS_2-SLF3 was most similar to SLF12, showing a strong orthologous relation. The primers were designed targeting the highly conserved regions after aligning the *Petunia* reference and ten corresponding SLFs from *Solanum* taxid. The PCR product was sequenced directly. All three of putative partial SLF sequences underwent phylogenetic analysis, and the results revealed that all putative SLFs grouped with the Solanaceae SLF clade which were classified into three kinds: SLF1 (Type 1), SLF12 (Type 12), and SLF23 (Type 23). This might suggest that all three forms of SLF developed before the division of tomato and potato.

5. Concluding Remarks

A handful of crop plants species, such as rice, corn, soybean, wheat, and potatoes, provide for the vast majority of human nutrition. Around 10 to 50 plant species produce around 95% of the world's calorie consumption. This reliance on a few species for the majority of food is a significant factor in the susceptibility of the global food supply to the effects of climate change and the emergence of major new plant diseases (von Wettberg et al., 2020). Crop wild relatives (CWRs) continue to be the greatest source of genetic variety for crop development and have been employed for significant gene disease and pest resistance, as well as abiotic stress tolerance (Coyne *et al*., 2020). There exists, however, a vast number of plant species from many plant families and genera that possess desirable characteristics but have not yet been domesticated.

Breeders of potatoes are lucky to have access to over 200 species within *Solanum* section *Petota* and the closely related outgroup section *Etuberosa*. These plants offer breeders a more diversified and accessible source of germplasm than any other crop (Hawkes, 1990). *Solanum* species are found in an astounding variety of habitats, including the dry Andean highlands, moist temperate mountain rain forests and cultivated fields (Hawkes, 1990). Wild species offer genes for tuber quality and resilience to biotic and abiotic stressors (Spooner and Bamberg, 1994; Jansky, 2000). Moreover, wild species contribute to the genetic variety of breeding programmes. Douches *et al.* (1989) discovered that wild *Solanum* species have an average of 5.7 isozyme alleles per locus, but North American cultivars have just 2.1 alleles. This allelic diversity is essential for optimising heterozygosity and epistasis, which are necessary for yield enhancements (Mendiburu and Peloquin, 1977). 10% of potato species have been investigated for use in breeding programs (Budin and Gavrilenko, 1994). Therefore, enormous prospects exist for breeders to improve the cultivated potato using germplasm from wild *Solanum*.

There are several strategies available for neo-domesticating a wild species or increase the genetic tractability of an existing crop. The most evident of the recently developed tools is gene editing techniques. Numerous orphan crops, which are agriculturally significant yet scientifically and economically neglected, have been modified using gene editing (Venezia and Krainer, 2021). Orphan crops include a variety of staple fruits, vegetables, cereals, roots, tubers, and pulses. Despite their importance for local and international agriculture, these crops have historically received little attention from scientists, funding etc., since the Green Revolution (Tadele, 2019). Despite being essential to smallholder farmers in less developed economies, orphan crops continue to produce and yield less than commercially significant crops grown in more industrialised nations (Tadele, 2014). This is mostly caused by the lack of better cultivars that are readily available (Tadele, 2019). In addition to providing smallholders with reliable food sources in a changing climate, these crops also serve as an important gene bank for future agricultural improvement (Mabhaudhi *et al.*, 2019).

It is essential to study the characteristics of native Andean potatoes in order to conserve crop genetic variety and assist local farmers in maintaining production diversity to prevent the extinction of species. *Solanum okadae*, a diploid Bolivian and Argentine potato, is one of these crop wild relatives that, according to the preceding description, can be categorised as an orphan crop. For the first time, this thesis not only investigated the nutritional content and evaluated *Solanum okadae* for salt tolerance, but also optimised a technique from its *in vitro* micropropagation and regeneration. SLF sequences were also discovered, which might be

employed in future research to break down the SI by competitive interaction, an alternative to S-RNase knockout. Even though there is abundant information on the *Solanum Phureja* (diploid) genome available in the database, the SLFs determined for *Solanum okadae* are the first to be identified in diploid potato, which might be attributed to a lack of SLF research and adequate gene annotations. *Solanum okadae* can be used as a potential donor candidate for desirable traits in improving the cultivated potatoes due to its superior nutritional value and high tolerance to abiotic stresses. This species should be able to cross easily with domesticated [https://www.cultivarable.com/product/potato/potato-wild-relatives/wild-potato-*Solanum-okadae*, accessed on 11/11/2022]; however, it does not appear to have been widely used in potato breeding to date.

BIBLIOGRAPHY

Abd El-Samad HM, Shaddad MAK (1997) Salt tolerance of soybean cultivars. Biol Plant 1997 392 39:263–269. https://doi.org/10.1023/A:1000309407275

Abdel Latef AAH, Chaoxing H (2011) Effect of arbuscular mycorrhizal fungi on growth, mineral nutrition, antioxidant enzymes activity and fruit yield of tomato grown under salinity stress. Sci Hortic (Amsterdam) 127:228–233. https://doi.org/10.1016/J.SCIENTA.2010.09.020

Abdelaleem KG (2015) Organogenesis of (*Solanum tuberosum L.*) Plant Cultivar Alpha through Tuber Segment Explants Callus. IntJCurrMicrobiolAppSci 4:267–276

Abderhalden, E. (1912). Experiment on the feeding with completely degraded nutrition substances. *Z Physiol Chem*, *77*, 22-58.

Abeuova LS, Kali BR, Rakhimzhanova AO, *et al* (2020) High frequency direct shoot regeneration from kazakh commercial potato cultivars. PeerJ 2020:.

Abrol DP (2011) Pollination Biology: Biodiversity conservation and agricultural production. Springer Dordrecht Heidelberg. London, 792. https://doi.org/10.1007/978-94-007-1942.

Abu Kawochar M (2017) Role of Explants and NAA on Callus Induction of Potato (*Solanum tuberosum L.*). Am J Life Sci 5:140.

Acosta-Motos JR, Ortuño MF, Bernal-Vicente A, *et al* (2017) Plant Responses to Salt Stress: Adaptive Mechanisms. Agron 2017, Vol 7, Page 18 7:18.

Adams E, Shin R (2014) Transport, signaling, and homeostasis of potassium and sodium in plants. J Integr Plant Biol 56:231–249.

Agarwal S, Pandey V (2004) Antioxidant enzyme responses to NaCl stress in Cassia angustifolia. Biol Plant 48:555–560.

Aghaei K, Ehsanpour AA, Komatsu S (2009) Potato Responds to Salt Stress by Increased Activity of Antioxidant Enzymes. J Integr Plant Biol 51:1095–1103.

Ahmad R, Kim YH, Kim MD, *et al* (2008) Development of selection marker-free transgenic potato plants with enhanced tolerance to oxidative stress. J Plant Biol 51:401–407.

Ahmed HA, Barpete S, Akdogan S (2018) Efficient regeneration and *Agrobacterium tumefaciens* mediated genetic transformation of potato. Feb 27:3020–3027

Ahmed HAA, Şahin NK, Akdoğan G, *et al* (2020) Variability in salinity stress tolerance of potato (*Solanum tuberosum* L.) varieties using *in vitro* screening. Cienc e Agrotecnologia 44:1–14. https://doi.org/10.1590/1413-7054202044004220

Akram MS, Ashraf M (2011) Exogenous application of potassium dihydrogen phosphate can alleviate the adverse effects of salt stress on sunflower. J Plant Nutr 34:1041–1057.

Al Mazrooei SS, Ghazala WS (2018) Characterization of the glyceraldehyde-3-phosphate dehydrogenase gene from the desert plant *Haloxylon salicornicum* using RT-PCR amplification and sequencing. J King Saud Univ - Sci 30:552–560.

Alam MK, Rana ZH, Islam SN (2016) Comparison of the Proximate Composition, Total Carotenoids and Total Polyphenol Content of Nine Orange-Fleshed Sweet Potato Varieties Grown in Bangladesh. Foods (Basel, Switzerland) 5:1–10.

Albiski F, Najla S, Sanoubar R, *et al* (2012) *In vitro* screening of potato lines for drought tolerance. Physiol Mol Biol Plants 18:315–321. https://doi.org/10.1007/S12298-012-0127-5

Alexander J (2015) Selenium. Handb Toxicol Met Fourth Ed 1:1175–1208. https://doi.org/10.1016/B978-0-444-59453-2.00052-4

Al-Hussaini, Z. A., Yousif, S. H. A., & Al-Ajeely, S. A. (2015) Effect of Different Medium on Callus Induction and Regeneration in Potato Cultivars. Int.J.Curr.Microbiol.App.Sci.,4(5): 856-865

Ali M, Kamran M, Abbasi GH, *et al* (2021) Melatonin-Induced Salinity Tolerance by Ameliorating Osmotic and Oxidative Stress in the Seedlings of Two Tomato (*Solanum lycopersicum L.*) Cultivars. J Plant Growth Regul 40:2236–2248. https://doi.org/10.1007/S00344-020-10273-3

Ali Soomro U, Zahoor-ul-Haq M, Gul S (2008) Effects of NaCl Salinity on Wheat (*Triticum aestivum L.*) Cultivars. World J Agric Sci 4:398–403

Aliche EB, Theeuwen TPJM, Oortwijn M, *et al* (2020) Carbon partitioning mechanisms in POTATO under drought stress. Plant Physiol Biochem PPB 146:211–219. https://doi.org/10.1016/J.PLAPHY.2019.11.019

Allen KGD, Klevay LM (1994) Copper: an antioxidant nutrient for cardiovascular health. Curr Opin Lipidol 5:22–28.

Almasia NI, Narhirñak V, Hopp Esteban H, Vazquez-Rovere C (2010) Isolation and characterization of the tissue and development-specific potato snakin-1 promoter inducible by temperature and wounding. Electron J Biotechnol 13

Altaf MA, Shahid R, Altaf MM, *et al* (2022) Melatonin: First-line soldier in tomato under abiotic stress current and future perspective. Plant Physiol Biochem 185:188–197. https://doi.org/10.1016/J.PLAPHY.2022.06.004

Altaf MA, Shahid R, Ren MX, *et al* (2021) Melatonin Mitigates Nickel Toxicity by Improving Nutrient Uptake Fluxes, Root Architecture System, Photosynthesis, and Antioxidant Potential in Tomato Seedling. J Soil Sci Plant Nutr 21:1842–1855. https://doi.org/10.1007/S42729-021-00484-2

Amoros W, Salas E, Hualla V, *et al* (2020) Heritability and genetic gains for iron and zinc concentration in diploid potato. Crop Sci 60:1884–1896. https://doi.org/10.1002/CSC2.20170

Amoros W, Salas E, Hualla V, *et al* (2020) Heritability and genetic gains for iron and zinc concentration in diploid potato. Crop Sci 60:1884–1896.

Anamul Hoque M, Okuma E, Nasrin Akhter Banu M, *et al* (2007) Exogenous proline mitigates the detrimental effects of salt stress more than exogenous betaine by increasing antioxidant enzyme activities. J Plant Physiol 164:553–561. https://doi.org/10.1016/J.JPLPH.2006.03.010

Anderson MA, Cornish EC, Mau SL, *et al* (1986) Cloning of cDNA for a stylar glycoprotein associated with expression of self-incompatibility in Nicotiana alata. Nat 321:38–44. https://doi.org/10.1038/321038a0

Andersson M, Turesson H, Nicolia A, *et al* (2017) Efficient targeted multiallelic mutagenesis in tetraploid potato (*Solanum tuberosum*) by transient CRISPR-Cas9 expression in protoplasts. Plant Cell Rep 36:117–128. https://doi.org/10.1007/S00299-016-2062-3

Andre CM, Ghislain M, Bertin P, *et al* (2007) Andean potato cultivars (*Solanum tuberosum L.*) as a source of antioxidant and mineral micronutrients. J Agric Food Chem 55:366–378.

AnjumSahair, R., Sneha, S., Raghu, N., T, G., Karthikeyan, M., Gnanasekaran, A., G, C., & Basalingappa, K.M. (2018). *Solanum tuberosum L*: Botanical, Phytochemical, Pharmacological and Nutritional Significance. *International Journal of Phytomedicine*, 10:115.

Anzalone A V., Randolph PB, Davis JR, *et al* (2019) Search-and-replace genome editing without double-strand breaks or donor DNA. Nature 576:149–157.

Arezki O, Boxus P, Kevers C, Gaspar T (2001) Changes in peroxidase activity, and level of phenolic compounds during light-induced plantlet regeneration from *Eucalyptus camaldulensis Dehn*. nodes *in vitro*. Plant Growth Regul 33:215–219. https://doi.org/10.1023/A:1017579623170

Arfan M, Athar HR, Ashraf M (2007) Does exogenous application of salicylic acid through the rooting medium modulate growth and photosynthetic capacity in two differently adapted spring wheat cultivars under salt stress? J Plant Physiol 164:685–694. https://doi.org/10.1016/J.JPLPH.2006.05.010

Arnao MB, Hernández-Ruiz J (2019) Melatonin and reactive oxygen and nitrogen species: a model for the plant redox network. Melatonin Res 2:152–168. https://doi.org/10.32794/11250036

Arnon DI (1949) COPPER ENZYMES IN ISOLATED CHLOROPLASTS. POLYPHENOLOXIDASE IN BETA VULGARIS. Plant Physiol 24:1. https://doi.org/10.1104/PP.24.1.1

Ashraf M, Foolad MR (2007) Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ Exp Bot 59:206–216. https://doi.org/10.1016/J.ENVEXPBOT.2005.12.006

Ashraf M, Foolad MR (2013) Crop breeding for salt tolerance in the era of molecular markers and marker-assisted selection. Plant Breed 132:10–20. https://doi.org/10.1111/PBR.12000

Ashraf M, Harris PJC (2004) Potential biochemical indicators of salinity tolerance in plants. Plant Sci 166:3–16. https://doi.org/10.1016/J.PLANTSCI.2003.10.024

Ashraf M, Sultana R (2000) Combination effect of NaCl salinity and nitrogen form on mineral composition of sunflower plants. Biol Plant 43:615–619. https://doi.org/10.1023/A:1002860202032

Ashraf M, Wu DL (2011) Breeding for Salinity Tolerance in Plants. CRC Crit Rev Plant Sci 13:17–42. https://doi.org/10.1080/07352689409701906

Ashrafzadeh S, Leung DWM (2017) Novel potato plants with enhanced cadmium resistance and antioxidative defence generated after *in vitro* cell line selection. PLoS One 12.

Assaha DVM, Liu L, Mekawy AMM, *et al* (2015) Effect of salt stress on Na accumulation, antioxidant enzyme activities and activity of cell wall peroxidase of huckleberry (*Solanum* scabrum) and eggplant (*Solanum* melongena). Int J Agric Biol 17:1149–1156. https://doi.org/10.17957/IJAB/15.0052

Aversano R, Contaldi F, Ercolano MR, *et al* (2015) The *Solanum commersonii* Genome Sequence Provides Insights into Adaptation to Stress Conditions and Genome Evolution of Wild Potato Relatives. Plant Cell 27:954–968.

Back K, Tan DX, Reiter RJ (2016) Melatonin biosynthesis in plants: multiple pathways catalyze tryptophan to melatonin in the cytoplasm or chloroplasts. J Pineal Res 61:426–437. https://doi.org/10.1111/JPI.12364

Badoni, A., and J. S. Chauhan. (2009) Effect of growth regulators on meristem-tip development and *in vitro* multiplication of potato cultivar 'Kufri Himalini'. Nature and Sci., 7 (9): 31-34.

Bae S, Park J, Kim JS (2014) Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. Bioinformatics 30:1473–1475.

Bakhsh A (2020) Development of efficient, reproducible, and stable *Agrobacterium*-mediated genetic transformation of five potato cultivars. Food Technol Biotechnol 58:57–63. https://doi.org/10.17113/ftb.58.01.20.6187

Bakhsh A, Anayol E, Ozcan SF (2014) Comparison of transformation efficiency of five agrobacterium tumefaciens strains in *Nicotiana tabacum L.* Emirates J Food Agric 26:259–264.

Balaško MK, Mikac KM, Bažok R, Lemic D (2020) Modern techniques in colorado potato beetle (*Leptinotarsa decemlineata*) control and resistance management: History review and future perspectives. Insects 11:1–17.

Banerjee AK, Prat S, Hannapel DJ (2006) Efficient production of transgenic potato (*S. tuberosum L. ssp. andigena*) plants via *Agrobacterium tumefaciens*-mediated transformation. Plant Sci 170:732–738. https://doi.org/10.1016/j.plantsci.2005.11.007

Banu MSA, Huda KMK, Sahoo RK, *et al* (2015) Pea p68 Imparts Salinity Stress Tolerance in Rice by Scavenging of ROS-Mediated H2O2 and Interacts with Argonaute. Plant Mol Biol Report 33:221–238. https://doi.org/10.1007/S11105-014-0748-7

Barrangou R, Fremaux C, Deveau H, *et al* (2007) CRISPR provides acquired resistance against viruses in prokaryotes. Science 315:1709–1712.

Barrell PJ, Meiyalaghan S, Jacobs JME, Conner AJ (2013) Applications of biotechnology and genomics in potato improvement. Plant Biotechnol J 11:907–920. https://doi.org/10.1111/pbi.12099

Barrell PJ, Yongjin S, Cooper PA, Conner AJ (2002) Alternative selectable markers for potato transformation using minimal T-DNA vectors. Plant Cell Tissue Organ Cult 70:61–68. https://doi.org/10.1023/A:1016013426923

Bártová V, Bárta J, Brabcová A, *et al* (2015) Amino acid composition and nutritional value of four cultivated South American potato species. J Food Compos Anal 40:78–85.

Basera M, Chandra A, Kumar A, *et al* (2018) Affect of brassinosteroids on *in vitro* proliferation and vegetative growth of potato. Pharma Innov J 7:04–09

Bashir I, Nicolao R, Heiden G (2021) Wild Potatoes: A Genetic Reservoir for Potato Breeding. Wild Germplasm Genet Improv Crop Plants 215–240.

Basu S, Kumar A, Benazir I, Kumar G (2021) Reassessing the role of ion homeostasis for improving salinity tolerance in crop plants. Physiol Plant 171:502–519. https://doi.org/10.1111/PPL.13112

Bateman AJ (1955) Self-incompatibility systems in angiosperms: III. Cruciferae. Hered 9:53–68. https://doi.org/10.1038/hdy.1955.2

Bates CJ (1997) Vitamin analysis. Ann Clin Biochem 34 (Pt 6):599–626. https://doi.org/10.1177/000456329703400604

Beaujean A, Sangwan RS, Lecardonnel A, Sangwan-Norreel BS (1998) *Agrobacterium*-mediated transformation of three economically important potato cultivars using sliced internodal explants: An efficient protocol of transformation. J Exp Bot 49:1589–1595.

Bedinger PA, Broz AK, Tovar-Mendez A, McClure B (2017) Pollen-Pistil Interactions and Their Role in Mate Selection. Plant Physiol 173:79–90.

Bernatzky R, Anderson MA, Clarke AE (1988) Molecular genetics of self-incompatibility in flowering plants. Dev Genet 9:1–12. https://doi.org/10.1002/DVG.1020090102

Bethke PC, Halterman DA, Jansky S (2017) Are we getting better at using wild potato species in light of new tools? Crop Sci 57:1241–1258.

Bethke PC, Jansky SH (2008) The effects of boiling and leaching on the content of potassium and other minerals in potatoes. J Food Sci 73.

Bhandal IS, Malik CP (1988) Potassium Estimation, Uptake, and Its Role in the Physiology and Metabolism of Flowering Plants. Int Rev Cytol 110:205–254. https://doi.org/10.1016/S0074-7696(08)61851-3

Biswas MS, Islam MR, Zakaria M (2017) Evaluation of Indigenous Potato Challisha (*Solanum tuberosum* L. Cv. Challisha) Somaclonals Tolerance to Salinity *In vitro*. J Trop Life Sci 7:77–82. https://doi.org/10.11594/JTLS.07.01.13

Blaha G, Stelzl U, Spahn CMT, *et al* (2000) Preparation of functional ribosomal complexes and effect of buffer conditions on tRNA positions observed by cryoelectron microscopy. Methods Enzymol 317:292–309. https://doi.org/10.1016/S0076-6879(00)17021-1

Blin K, Pedersen LE, Weber T, Lee SY (2016) CRISPy-web: An online resource to design sgRNAs for CRISPR applications. Synth Syst Biotechnol 1:118–121.

Blom-Zandstra M, Wolters W, Heinen M, *et al* (2014) Perspectives for the growth of salt tolerant cash crops : a case study with potato. Plant Res Int Bus Unit Agrosystems Res. https://doi.org/10.3/JQUERY-UI.JS

Bohs L (2007) Phylogeny of the Cyphomandra clade of the genus *Solanum* (Solanaceae) based on ITS sequence data. Taxon 56:1012–1026. https://doi.org/10.2307/25065901

Borsook H&, Huffman HM, James DB, *et al* (1958) The amino-acid composition of potato protein and of cooked potato. Int Congr Anim Husb VII Madrid 50:7. https://doi.org/10.1079/BJN19580026

Bortesi L, Fischer R (2015a) The CRISPR/Cas9 system for plant genome editing and beyond. Biotechnol Adv 33:41–52.

Bouis HE, Saltzman A (2017) Improving nutrition through biofortification: A review of evidence from HarvestPlus, 2003 through 2016. Glob Food Sec 12:49–58.

Brar Archana *et al* (2017) Biochemical and Phytochemical Properties of Potato : A Review. Chem Sci Rev Lett, 6(21), 117-129.

Broadley MR, White PJ, Hammond JP, *et al* (2007) Zinc in plants. New Phytol 173:677–702.

Brown CR (2005) Antioxidants in potato. Am J Potato Res 2005 822 82:163–172.

Brown P, Saa S (2015) Biostimulants in agriculture. Front Plant Sci 6:671. https://doi.org/10.3389/FPLS.2015.00671/BIBTEX

Bruce MA, Shoup Rupp JL (2019) *Agrobacterium*-mediated transformation of *Solanum tuberosum* L., Potato. Methods Mol Biol 1864:203–223. https://doi.org/10.1007/978-1-4939-8778-8_15

Budin KZ, Gavrilenko TA (1994) Genetic basis of distant hybridization in potato. Genet Moskva 30:1413–1422

Bündig C, Vu TH, Meise P, *et al* (2017) Variability in Osmotic Stress Tolerance of Starch Potato

Genotypes (*Solanum tuberosum* L.) as Revealed by an *In vitro* Screening: Role of Proline, Osmotic Adjustment and Drought Response in Pot Trials. J Agron Crop Sci 203:206–218. https://doi.org/10.1111/JAC.12186

Byun MO, Kwon H Bin, Park SC (2007) Recent advances in genetic engineering of potato crops for drought and saline stress tolerance. Adv Mol Breed Towar Drought Salt Toler Crop 713–737. https://doi.org/10.1007/978-1-4020-5578-2_29/COVER

Calliope SR, Lobo MO, Sammán NC (2018) Biodiversity of Andean potatoes: Morphological, nutritional and functional characterization. Food Chem 238:42–50.

Camadro EL, Saffarano SK, Espinillo JC, *et al* (2007) Cytological mechanisms of 2n pollen formation in the wild potato *Solanum okadae* and pollen-pistil relations with the cultivated potato, *Solanum tuberosum*. Genet Resour Crop Evol 2007 553 55:471–477. https://doi.org/10.1007/S10722-007-9254-1

Carrigan PE, Ballar P, Tuzmen S (2011) Site-directed mutagenesis. Methods Mol Biol 700:107–124.

Castañeda-Álvarez NP, De Haan S, Juárez H, *et al* (2015) Ex Situ Conservation Priorities for the Wild Relatives of Potato (*Solanum L.* Section *Petota*). PLoS One 10:e0122599.

Castillo AM, Egaña B, Sanz JM, Cistué L (1998) Somatic embryogenesis and plant regeneration from barley cultivars grown in Spain. Plant Cell Reports 1998 1711 17:902–906. https://doi.org/10.1007/S002990050506

Castric V, Vekemans X (2004) Plant self-incompatibility in natural populations: a critical assessment of recent theoretical and empirical advances. Mol Ecol 13:2873–2889.

Castric V, Vekemans X (2007) Evolution under strong balancing selection: how many codons determine specificity at the female self-incompatibility gene SRK in Brassicaceae? BMC Evol Biol 7. https://doi.org/10.1186/1471-2148-7-132

Chakraborty K, Mondal S, Ray S, *et al* (2020) Tissue Tolerance Coupled with Ionic Discrimination Can Potentially Minimize the Energy Cost of Salinity Tolerance in Rice. Front Plant Sci 11:265. https://doi.org/10.3389/FPLS.2020.00265/BIBTEX

Chakravarty B, Wang-Pruski G (2010) Rapid regeneration of stable transformants in cultures of potato by improving factors influencing *Agrobacterium*-mediated transformation. Adv Biosci Biotechnol 01:409–416.

Chakravarty B, Wang-Pruski G, Flinn B, *et al* (2007) Genetic transformation in potato: approaches and strategies. Am J Potato Res 2007 844 84:301–311. https://doi.org/10.1007/BF02986242

Chaparro JM, Holm DG, Broeckling CD, *et al* (2018) Metabolomics and Ionomics of Potato Tuber Reveals an Influence of Cultivar and Market Class on Human Nutrients and Bioactive Compounds. Front Nutr 5:

Charkowski A, Sharma K, Parker ML, Secor GA, Elphinstone J (2020). The Potato Crop: its

Agricultural, Nutritional and Social Contribution to Humankind. eds, Campos H, Ortiz O, Switzerland: Springer International Publishing, pp 351-388

Charlesworth D, Awadalla P (1998) Flowering plant self-incompatibility: the molecular population genetics of Brassica S-loci. Heredity (Edinb) 81:1–9. https://doi.org/10.1038/sj.hdy.6884000

Chase SS (2011) Analytic Breeding in *Solanum Tuberosum L.* – A Scheme Utilizing Parthenotes and Other Diploid Stocks. Can J Genet Cytol 5:359–363.

Chateau S, Sangwan RS, Sangwan-Norreel BS (2000) Competence of *Arabidopsis thaliana* genotypes and mutants for *Agrobacterium tumefaciens*-mediated gene transfer: Role of phytohormones. J Exp Bot 51:1961–1968. https://doi.org/10.1093/jexbot/51.353.1961

Chattopadhayay MK, Tiwari BS, Chattopadhyay G, *et al* (2002) Protective role of exogenous polyamines on salinity-stressed rice (Oryza sativa) plants. Physiol Plant 116:192–199. https://doi.org/10.1034/J.1399-3054.2002.1160208.X

Chaturvedi AK, Patel MK, Mishra A, *et al* (2014) The SbMT-2 gene from a halophyte confers abiotic stress tolerance and modulates ROS scavenging in transgenic tobacco. PLoS One 9:. https://doi.org/10.1371/JOURNAL.PONE.0111379

Chee PP (2019) High Frequency of Somatic Embryogenesis and Recover of Fertile Cucumber Plants. HortScience 25:792–793. https://doi.org/10.21273/HORTSCI.25.7.792

Chen CT, Chen LM, Lin CC, Kao CH (2001) Regulation of proline accumulation in detached rice leaves exposed to excess copper. Plant Sci 160:283–290. https://doi.org/10.1016/S0168-9452(00)00393-9

Chen L, Li W, Katin-Grazzini L, *et al* (2018) A method for the production and expedient screening of CRISPR/Cas9-mediated non-transgenic mutant plants. Hortic Res 5:.

Chen M, Yang Z, Liu J, *et al* (2018) Adaptation Mechanism of Salt Excluders under Saline Conditions and Its Applications. Int J Mol Sci 2018, Vol 19, Page 3668 19:3668. https://doi.org/10.3390/IJMS19113668

Chen P, Bornhorst J, Aschner M (2018) Manganese metabolism in humans. Front Biosci Landmark Ed 23:1655–1679. https://doi.org/10.2741/4665

Chen T, Zhang B (2016) Measurements of Proline and Malondialdehyde Content and Antioxidant Enzyme Activities in Leaves of Drought Stressed Cotton. BIO-PROTOCOL 6:. https://doi.org/10.21769/BIOPROTOC.1913

Chen W, Zhang J, Zheng S, *et al* (2021) Metabolite profiling and transcriptome analyses reveal novel regulatory mechanisms of melatonin biosynthesis in hickory. Hortic Res 2021 81 8:1–13. https://doi.org/10.1038/s41438-021-00631-x

Chen Z, Gu Q, Yu X, *et al* (2018) Hydrogen peroxide acts downstream of melatonin to induce lateral root formation. Ann Bot 121:1127–1136. https://doi.org/10.1093/AOB/MCX207

Chen Z, Newman I, Zhou M, *et al* (2005) Screening plants for salt tolerance by measuring K+ flux: A case study for barley. Plant, Cell Environ 28:1230–1246. https://doi.org/10.1111/J.1365-3040.2005.01364.X

Chen Z, Xie Y, Gu Q, *et al* (2017) The AtrbohF-dependent regulation of ROS signaling is required for melatonin-induced salinity tolerance in Arabidopsis. Free Radic Biol Med 108:465–477. https://doi.org/10.1016/J.FREERADBIOMED.2017.04.009

Chen ZC, Peng WT, Li J, Liao H (2018) Functional dissection and transport mechanism of magnesium in plants. Semin Cell Dev Biol 74:142–152. https://doi.org/10.1016/J.SEMCDB.2017.08.005

Cheng J, Veilleux RE (1991) Genetic analysis of protoplast culturability in *Solanum Phureja*. Plant Sci 75:257–265.

Cheng M, Lowe BA, Spencer TM, *et al* (2004) Invited review: Factors influencing *Agrobacterium*-mediated transformation of monocotyledonous species. Vitr Cell Dev Biol - Plant 40:31–45. https://doi.org/10.1079/IVP2003501

Cheng ZM, Schnurr JA, Kapaun JA (1998) Timentin as an alternative antibiotic for suppression of *Agrobacterium tumefaciens* in genetic transformation. Plant Cell Rep 17:646–649. https://doi.org/10.1007/s002990050458

Chi YH, Koo SS, Oh HT, *et al* (2019) The physiological functions of universal stress proteins and their molecular mechanism to protect plants from environmental stresses. Front Plant Sci 10:. https://doi.org/10.3389/FPLS.2019.00750/FULL

Chindi A, Woldegiorgis G, Solomon A, *et al* (2013) Enhancing potato seed production using rapid multiplication techniques. In: Proceedings of the National Workshop on Seed Potato Tuber Production and Dissemination. Bahir Dar, Ethiopia

Cho SW, Kim S, Kim JM, Kim JS (2013) Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nat Biotechnol 31:230–232. https://doi.org/10.1038/NBT.2507

Chourasia KN, Lal MK, Tiwari RK, *et al* (2021) Salinity Stress in Potato: Understanding Physiological, Biochemical and Molecular Responses. Life 2021, Vol 11, Page 545 11:545. https://doi.org/10.3390/LIFE11060545

Clausen AM, Colavita M, Butzonitch I, Carranza AV (2005) A potato collecting expedition in the province of Jujuy, Argentina and disease indexing of virus and fungus pathogens in Andean cultivars. Genet Resour Crop Evol 52:1099–1109. https://doi.org/10.1007/S10722-004-6131-Z

Clot CR, Polzer C, Prodhomme C, *et al* (2020) The origin and widespread occurrence of *Sli*-based self-compatibility in potato. Theor Appl Genet 133:2713–2728.

Coleman M, Davie p, Vessey J, Powell W (1991) Intraclonal Genetic Variation for Protoplast Regenerative Ability Within *Solanum tuberosum* cv. Record. Ann Bot 67:459–461.

Collins JF (2020) Copper. Present Knowl Nutr 409–427. https://doi.org/10.1016/B978-0-323-66162-1.00024-X

Compton ME (1999) Dark pretreatment improves adventitious shoot organogenesis from cotyledons of diploid watermelon. Plant Cell Tissue Organ Cult 58:185–188.

Concordet JP, Haeussler M (2018) CRISPOR: intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. Nucleic Acids Res 46:W242–W245.

Conde A, Chaves MM, Gerós H (2011) Membrane transport, sensing and signaling in plant adaptation to environmental stress. Plant Cell Physiol 52:1583–1602. https://doi.org/10.1093/PCP/PCR107

Conner A. J., Williams M. K., Deroles S. C., Deroles S. C., Shaw M. L., Lancaster J. E. (1992). *Agrobacterium*-mediated transformation of New Zealand potato cultivars. *New Zeal. J. Crop Hortic. Sci.* 19 1–8. 10.1080/01140671.1991.10418098

Conner, A. J. (2007). Chapter 31 - Field-Testing of Transgenic Potatoes. In D. Vreugdenhil, J. Bradshaw, C. Gebhardt, F. Govers, D. K. L. Mackerron, M. A. Taylor, and H. A. B. T.-P. B. and B. Ross (Eds.), Potato Biology and Biotechnology Advances and Perspectives (pp. 687–703). Amsterdam: Elsevier Science B.V.

Cook JD, Reddy MB (2001) Effect of ascorbic acid intake on nonheme-iron absorption from a complete diet. Am J Clin Nutr 73:93–98.

Coyne CJ, Kumar S, von Wettberg EJB, *et al* (2020) Potential and limits of exploitation of crop wild relatives for pea, lentil, and chickpea improvement. Legum Sci 2:e36.

Craig W, Gargano D, Scotti N, *et al* (2005) Direct gene transfer in potato: A comparison of particle bombardment of leaf explants and PEG-mediated transformation of protoplasts. Plant Cell Rep 24:603–611. https://doi.org/10.1007/s00299-005-0018-0

Crane MB, Brown AG (1937) Incompatibility and Sterility in the Sweet Cherry, *Prunus Avium L.* J Pomol Hortic Sci 15:2–116.

Crane MB, Lewis D (1942) Genetical studies in pears. J Genet 43:31–43. https://doi.org/10.1007/BF02982745

Craze M, Bates R, Bowden S, Wallington EJ (2018) Highly Efficient *Agrobacterium*-Mediated Transformation of Potato (*Solanum tuberosum*) and Production of Transgenic Microtubers. Curr Protoc plant Biol 3:33–41. https://doi.org/10.1002/cppb.20065

Cruzan MB (1993) Analysis of pollen-style interactions in *Petunia hybrida*; the determination of variance in male reproductive success. Sex Plant Reprod 1993 64 6:275–281. https://doi.org/10.1007/BF00231905

Cruz-Garcia F, Hancock CN, McClure B (2003) S-RNase complexes and pollen rejection. J Exp Bot 54:123–130. https://doi.org/10.1093/JXB/ERG045

Cuartero J, Bolarín MC, Asíns MJ, Moreno V (2006) Increasing salt tolerance in the tomato. J Exp Bot 57:1045–1058. https://doi.org/10.1093/JXB/ERJ102

Cuin TA, Betts SA, Chalmandrier R, Shabala S (2008) A root's ability to retain K+ correlates with salt tolerance in wheat. J Exp Bot 59:2697. https://doi.org/10.1093/JXB/ERN128

da Silva JAT, Fukai S (2003) Effect of aminoglycoside antibiotics on *in-vitro* morphogenesis from cultured cells of chrysanthemum and tobacco. J Plant Biol 46:71–82.

Dahal K, Li XQ, Tai H, *et al* (2019) Improving Potato Stress Tolerance and Tuber Yield Under a Climate Change Scenario – A Current Overview. Front Plant Sci 10:. https://doi.org/10.3389/FPLS.2019.00563

Daneshmand F, Arvin MJ, Kalantari KM (2010) Physiological responses to NaCl stress in three wild species of potato *in vitro*. Acta Physiol Plant 32:91–101. https://doi.org/10.1007/S11738-009-0384-2

Danku JMC, Lahner B, Yakubova E, Salt DE (2013) Large-scale plant ionomics. Methods Mol Biol 953:255–276. https://doi.org/10.1007/978-1-62703-152-3_17

Danku JMC, Lahner B, Yakubova E, Salt DE (2013) Large-scale plant ionomics. Methods Mol Biol 953:255–276. https://doi.org/10.1007/978-1-62703-152-3_17/COVER

Das Dangol S, Barakate A, Stephens J, *et al* (2019) Genome editing of potato using CRISPR technologies: current development and future prospective. Plant Cell Tissue Organ Cult 139:403–416. https://doi.org/10.1007/s11240-019-01662-y

Daudi A, O'brien JA, Author BP (2012) Detection of Hydrogen Peroxide by DAB Staining in Arabidopsis Leaves. Bio-protocol 2:. https://doi.org/10.21769/bioprotoc.263

Davidson MM, Takla MFG, Jacobs JME, *et al* (2010) Transformation of potato (*Solanum tuberosum*) cultivars with a cry1Ac9 gene confers resistance to potato tuber moth (*Phthorimaea operculella*). New Zeal J Crop Hortic Sci 32:39–50.

Davidson, R. D., and Xie, K. (2014). Seed potato production. In R. Navarre and M. Pavek (Eds.), The Potato Botany, Production and Uses (pp. 115–132). CABI.

Davies AMC (1977) The free amino acids of tubers of potato varieties grown in England and Ireland. Potato Res 20:9–21. https://doi.org/10.1007/BF02362297

Dawood MG, El-Awadi ME (2015) Disminución del estrés salino en plantas de Vicia faba L. a través de la activación de las semillas con melatonina. Acta Biol Colomb 20:223–235. https://doi.org/10.15446/ABC.V20N2.43291

De Azevedo Neto AD, Prisco JT, Enéas-Filho J, *et al* (2006) Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. Environ Exp Bot 56:87–94. https://doi.org/10.1016/J.ENVEXPBOT.2005.01.008

De Block M (1988) Genotype-independent leaf disc transformation of potato (*Solanum tuberosum*) using *Agrobacterium tumefaciens*. Theor Appl Genet 76:767–774.

De Clercq J, Zambre M, Van Montagu M, *et al* (2002) An optimized *Agrobacterium*-mediated transformation procedure for *Phaseolus acutifolius*. Plant Cell Rep 21:333–340. https://doi.org/10.1007/s00299-002-0518-0

De Fillipis LF (2014) Crop improvement through tissue culture. In:Ahmad P, Wani MR, Azooz MM, Tran LSP (eds) Improvement ofcrops in the era of climate changes, vol 1. Springer, New York, pp289–346

De Klerk GJ (2002) Rooting of microcuttings: Theory and practice. Vitr Cell Dev Biol - Plant 38:415–422. https://doi.org/10.1079/IVP2002335

De Lacerda CF, Cambraia J, Cano MAO, Ruiz HA (2001) Plant growth and solute accumulation and distribution in two sorghum genotypes, under NaCl stress. Rev Bras Fisiol Veg 13:270–284. https://doi.org/10.1590/S0103-31312001000300003

de Nettancourt D (2001) Incompatibility and Incongruity in Wild and Cultivated Plants. Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-662-04502-2.

De Vetten N, Wolters AM, Raemakers K, *et al* (2003) A transformation method for obtaining marker-free plants of a cross-pollinating and vegetatively propagated crop. Nat Biotechnol 21:439–442. https://doi.org/10.1038/nbt801

Décima Oneto C, Coronel J, Storani L, *et al* (2020) Transformación eficiente de *Solanum tuberosum* cv. Spunta mediada por *Agrobacterium tumefaciens* utilizando higromicina como agente selectivo. Rev Investig Agropecu 46:248–257

del Mar Martínez-Prada M, Curtin SJ, Gutiérrez-González JJ (2021) Potato improvement through genetic engineering. GM Crop Food 12:479–496.

Dereje B, Chibuzo N (2021a) Nutritional Composition and Biochemical Properties of *Solanum tuberosum* - A Promis Crop Starvation Probl.

Dereje B, Chibuzo N (2021b) Nutritional Composition and Biochemical Properties of *Solanum tuberosum* . *Solanum tuberosum* - A Promis Crop Starvation Probl. https://doi.org/10.5772/INTECHOPEN.98179

Dessoky E, Attia A, Ismail I, and El-Hallous E (2016). “*In vitro* Propagation of Potato under Different Hormonal Combinations,” Int. J. Adv. Res., vol. 4, no. 1, pp. 684-689, 2016.

Devaux A, Goffart JP, Kromann P, *et al* (2021) The Potato of the Future: Opportunities and Challenges in Sustainable Agri-food Systems. Potato Res 64:681–720. https://doi.org/10.1007/S11540-021-09501-4

Devaux A, Kromann P, Ortiz O (2014) Potatoes for Sustainable Global Food Security. Potato Res 57:185–199. https://doi.org/10.1007/S11540-014-9265-1

Dhital SP, Lim HT, Manandhar HK (2011) Direct and Efficient Plant Regeneration from Different Explants Sources of Potato Cultivars as Influenced by Plant Growth Regulators. Nepal J Sci Technol 12:1–6. https://doi.org/10.3126/NJST.V12I0.6471

Dhole AM, Shelat HN (2018) Phytomelatonin A plant hormone for management of stress. J Anal Pharm Res 7:. https://doi.org/10.15406/japlr.2018.07.00224

Dillen W, De Clercq J, Kapila J, *et al* (1997) The effect of temperature on *Agrobacterium tumefaciens*-mediated gene transfer to plants. Plant J 12:1459–1463.

Dimkpa CO, Bindraban PS (2016) Fortification of micronutrients for efficient agronomic production: a review. Agron Sustain Dev 36:7. https://doi.org/10.1007/S13593-015-0346-6

Ding F, Wang G, Wang M, Zhang S (2018) Exogenous Melatonin Improves Tolerance to Water Deficit by Promoting Cuticle Formation in Tomato Plants. Molecules 23:. https://doi.org/10.3390/MOLECULES23071605

Ditt RF, Nester EW, Comai L (2001) Plant gene expression response *to Agrobacterium tumefaciens*. Proc Natl Acad Sci U S A 98:10954–10959.

Dobnik D, Lazar A, Stare T, *et al* (2016) *Solanum venturii*, a suitable model system for *vir*us-induced gene silencing studies in potato reveals *StMKK6* as an important player in plant immunity. Plant Methods 12: https://doi.org/10.1186/s13007-016-0129-3

Dobránszki J, Magyar-Tábori K, Hudák I (2008) *In vitro* Tuberization in Hormone-Free Systems on Solidified Medium and Dormancy of Potato Microtubers. Fruit, Veg Cereal Sci Biotechnol 2:82–94

Douches DS, Maas D, Jastrzebski K, Chase RW (1996) Assessment of Potato Breeding Progress in the USA over the Last Century. Crop Sci 36:1544–1552. https://doi.org/10.2135/CROPSCI1996.0011183X003600060024X

Doudna JA, Charpentier E (2014) The new frontier of genome engineering with CRISPR-Cas9. Science (80-) 346:. https://doi.org/10.1126/SCIENCE.1258096

Drenkard E, Richter BG, Rozen S, *et al* (2000) A Simple Procedure for the Analysis of Single Nucleotide Polymorphisms Facilitates Map-Based Cloning in *Arabidopsis*. Plant Physiol 124:1483–1492.

Drewnowski A (2013) New metrics of affordable nutrition: which vegetables provide most nutrients for least cost? J Acad Nutr Diet 113:1182–1187.

Drewnowski A, Rehm CD (2013) Vegetable Cost Metrics Show That Potatoes and Beans Provide Most Nutrients Per Penny. PLoS One 8.

Dreyer I, Uozumi N (2011) Potassium channels in plant cells. FEBS J 278:4293–4303. https://doi.org/10.1111/J.1742-4658.2011.08371.X

du Jardin P (2015) Plant biostimulants: Definition, concept, main categories and regulation. Sci Hortic (Amsterdam) 196:3–14. https://doi.org/10.1016/J.SCIENTA.2015.09.021

Du M, Wang T, Lian Q, *et al* (2021) Developing a new model system for potato genetics by androgenesis. J Integr Plant Biol 63:628–633.

Durai S, Mani M, Kandavelou K, *et al* (2005) Zinc finger nucleases: custom-designed molecular scissors for genome engineering of plant and mammalian cells. Nucleic Acids Res 33:5978–5990. https://doi.org/10.1093/NAR/GKI912

Duvick DN (2005) The Contribution of Breeding to Yield Advances in maize (Zea mays L.). Adv Agron 86:83–145. https://doi.org/10.1016/S0065-2113(05)86002-X

Dzidzienyo DK, Bryan GJ, Wilde G, Robbins TP (2016) Allelic diversity of S-RNase alleles in diploid potato species. Theor Appl Genet 129:1985–2001.

East EM, Park JB (1917) Studies on Self-Sterility I. the Behavior of Self-Sterile Plants. Genetics 2:505. https://doi.org/10.1093/GENETICS/2.6.505

East EM, Yarnell SH (1929) Studies on Self-Sterility. VIII. Self-Sterility Allelomorphs. Genetics 14:455. https://doi.org/10.1093/GENETICS/14.5.455

Ebert PR, Anderson MA, Bernatzky R, *et al* (1989) Genetic polymorphism of self-incompatibility in flowering plants. Cell 56:255–262. https://doi.org/10.1016/0092-8674(89)90899-4

Eggers EJ, van der Burgt A, van Heusden SAW, *et al* (2021) Neofunctionalisation of the *Sli* gene leads to self-compatibility and facilitates precision breeding in potato. Nat Commun 12. https://doi.org/10.1038/S41467-021-24267-6

Eliazer Nelson ARL, Ravichandran K, Antony U (2019) The impact of the Green Revolution on indigenous crops of India. J Ethn Foods 6:1–10. https://doi.org/10.1186/S42779-019-0011-9/TABLES/2

El-Kharbotly A, Jacobsen E, Stiekema WJ, Pereira A (1995) Genetic localisation of transformation competence in diploid potato. Theor Appl Genet 91:557–562.

El-Samad HMA, Shaddad MAK (1997) Salt tolerance of soybean cultivars. Biol Plant 1997 392 39:263–269. https://doi.org/10.1023/A:1000309407275

Embaby HE, Rayan AM (2016) Chemical composition and nutritional evaluation of the seeds of *Acacia tortilis* (Forssk.) *Hayne ssp. raddiana*. Food Chem 200:62–68. https://doi.org/10.1016/J.FOODCHEM.2016.01.019

Enciso-Rodriguez F, Manrique-Carpintero NC, Nadakuduti SS, *et al* (2019) Overcoming self-incompatibility in diploid potato using CRISPR-cas9. Front Plant Sci 10:.

Endelman JB, Jansky SH (2016) Genetic mapping with an inbred line derived F2 population in potato. Theor Appl Genet 129:935–943. https://doi.org/10.1007/s00122-016-2673-7

Entani T, Iwano M, Shiba H, *et al* (1999) Centromeric localization of an S-RNase gene in *Petunia* hybrida Vilm. Theor Appl Genet 99:391–397. https://doi.org/10.1007/S001220051249

Entani T, Iwano M, Shiba H, *et al* (2003) Comparative analysis of the self-incompatibility (S-) locus region of Prunus mume: identification of a pollen-expressed F-box gene with allelic diversity. Genes Cells 8:203–213. https://doi.org/10.1046/J.1365-2443.2003.00626.X

Entani T, Kubo KI, Isogai S, *et al* (2014) Ubiquitin-proteasome-mediated degradation of S-RNase in a solanaceous cross-compatibility reaction. Plant J 78:1014–1021. https://doi.org/10.1111/TPJ.12528

Epstein E, Rains DW (1987) Advances in salt tolerance. Genet Asp Plant Miner Nutr 113–125. https://doi.org/10.1007/978-94-009-3581-5_10

Escudero J, Hohn B (1997) Transfer and integration of T-DNA without cell injury in the host plant. Plant Cell 9:2135–2142. https://doi.org/10.1105/tpc.9.12.2135

Evers D, Overney S, Simon P, *et al* (1999) Salt Tolerance of *Solanum tuberosum* L. Overexpressing an Heterologous Osmotin-like Protein. Biol Plant 1999 421 42:105–112. https://doi.org/10.1023/A:1002131812340

Ezekiel P, Williams T, Nuhu P, *et al* (2020) Phytochemical, Elemental, Proximate and anti-nutrients composition cf Irish potato (*Solanum tuberosum*) obtained in Kwaja, Mubi Sourth Local government area of Adamawa State. IJISET-International J Innov Sci Eng Technol 7:

FAO (2021) "Seed Potato Multiplication to Improve Food Security of the People of Paekam County, Ryanggang Province, and Democratic People's Republic Of Korea," Rome, Italy.

Faried HN, Ayyub CM, Amjad M, Ahmed R (2016) Salinity impairs ionic, physiological and biochemical attributes in potato. Pakistan J Agric Sci 53:17–25. https://doi.org/10.21162/PAKJAS/16.4766

Faried HN, Ayyub CM, Amjad M, Ahmed R (2016) Salinity impairs ionic, physiological and biochemical attributes in potato. Pakistan J Agric Sci 53:17–25. https://doi.org/10.21162/PAKJAS/16.4766

Fehér A, Felföldi K, Preiszner J, Dudits D (1991) PEG-mediated transformation of leaf protoplasts of *Solanum tuberosum L.* cultivars. Plant Cell Tissue Organ Cult 27:105–114. https://doi.org/10.1007/BF00048214

Feng C, Su H, Bai H, *et al* (2018) High-efficiency genome editing using a dmc1 promoter-controlled CRISPR/Cas9 system in maize. Plant Biotechnol J 16:1848–1857.

Fernandez Bidondo L, Almasia N, Bazzini A, *et al* (2019) The overexpression of antifungal genes enhances resistance to *Rhizoctonia solani* in transgenic potato plants without affecting arbuscular mycorrhizal symbiosis. Crop Prot 124:104837.

Fernandez GCJ (1993) Effective Selection Criteria for Assessing Plant Stress Tolerance, 1st edn. AVRDC, Taipei, Taiwan

Fernie AR, Alseekh S, Liu J, Yan J (2021) Using precision phenotyping to inform *de novo* domestication. Plant Physiol 186:1397–1411.

Fernie AR, Willmitzer L, Trethewey RN (2002) Sucrose to starch: a transition in molecular plant physiology. Trends Plant Sci 7:35–41. https://doi.org/10.1016/S1360-1385(01)02183-5

Finlay M, Dale B, Griffiths DW, Todd DT (2003) Effects of genotype, environment, and postharvest storage on the total ascorbate content of potato (*Solanum tuberosum*) tubers. J Agric Food Chem 51:244–248. https://doi.org/10.1021/JF020547S

Fletcher JD (2012) A virus survey of New Zealand fresh process and seed potato crops during 201011. New Zeal Plant Prot 65:197–203.

Flowers TJ, Andreas M. Lauchli (1983) Sodium versus potassium: substitution and compartmentation. Encycl plant Physiol 651–681

Fornkwa I, Harahagazwe V, Ngwa D, Adamu L (2021) "Production of Early Generation Seed (EGS) Potato."

Foxe JP, Slotte T, Stahl EA, *et al* (2009) Recent speciation associated with the evolution of selfing in *Capsella*. Proc Natl Acad Sci U S A 106:5241–5245. https://doi.org/10.1073/PNAS.0807679106

Frame BR, Shou H, Chikwamba RK, *et al* (2002) *Agrobacterium tumefaciens*-mediated transformation of maize embryos using a standard binary vector system. Plant Physiol 129:13–22. https://doi.org/10.1104/pp.000653

Franklin-Tong N, Franklin FCH (2003) Gametophytic self-incompatibility inhibits pollen tube growth using different mechanisms. Trends Plant Sci 8:598–605. https://doi.org/10.1016/J.TPLANTS.2003.10.008

Frey PA (2010) Cobalamin Coenzymes in Enzymology. Compr Nat Prod II Chem Biol 7:501–546.

Fricke W, Akhiyarova G, Veselov D, Kudoyarova G (2004) Rapid and tissue-specific changes in ABA and in growth rate in response to salinity in barley leaves. J Exp Bot 55:1115–1123. https://doi.org/10.1093/JXB/ERH117

Fujii S, Kubo KI, Takayama S (2016) Non-self- and self-recognition models in plant self-incompatibility. Nat Plants 2:1–9. https://doi.org/10.1038/nplants.2016.130

Fullner KJ, Nester EW (1996) Temperature affects the T-DNA transfer machinery of *Agrobacterium tumefaciens*. J Bacteriol 178:1498–1504.

Gagne JM, Downes BP, Shiu SH, *et al* (2002) The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in Arabidopsis. Proc Natl Acad Sci U S A 99:11519–11524. https://doi.org/10.1073/PNAS.162339999

Galdón BR, Rodríguez LH, Mesa DR, *et al* (2012) Differentiation of potato cultivars experimentally cultivated based on their chemical composition and by applying linear discriminant analysis. Food Chem 133:1241–1248.

Gao S, Ouyang C, Wang S, *et al* (2008) Effects of salt stress on growth, antioxidant enzyme and phenylalanine ammonia-lyase activities in Jatropha curcas L. seedlings. Plant, Soil Environ 54:374–381. https://doi.org/10.17221/410-PSE

Gaude T, Fobis-Loisy I, Miége C (2006) Control of fertilization by self-incompatibility mechanisms. Mol Biol Biotechnol Flower Second Ed 269–297. https://doi.org/10.1079/9781845930424.0269

Gavrilenko TA, Yermishin AP (2017) Interspecific hybridization of potato: Theoretical and applied aspects. Vavilov J Genet Breed 21:16–29.

Gelvin SB (2017) Integration of *Agrobacterium* T-DNA into the Plant Genome. Annu Rev Genet 51:195–217. https://doi.org/10.1146/annurev-genet-120215-035320

George EF, Hall MA, Klerk GJD (2008) Plant propagation by tissue culture 3rd edition. Plant Propag by Tissue Cult 3rd Ed 1:1–501.

Gerashchenkov GA, Rozhnova NA, Kuluev BR, *et al* (2020) Design of Guide RNA for CRISPR/Cas Plant Genome Editing. Mol Biol 54:24–42.

Gerendás J, Führs H (2013) The significance of magnesium for crop quality. Plant Soil 368:101–128. https://doi.org/10.1007/S11104-012-1555-2

Gerstel DU, Riner ME (1950) Self-incompatibility studies in guayule: I. Pollen-tube behavior. J Hered 41:49–55. https://doi.org/10.1093/OXFORDJOURNALS.JHERED.A106087/2/41-2-49.PDF.GIF

Gharsallah C, Fakhfakh H, Grubb D, Gorsane F (2016) Effect of salt stress on ion concentration, proline content, antioxidant enzyme activities and gene expression in tomato cultivars. AoB Plants 8:. https://doi.org/10.1093/AOBPLA/PLW055

Ghifari AMA (2021) Acceptability And Nutrients Content (Vitamin B12 And Folic Acid) Of substituted Snack Bar With Purple Sweet Potato (*Ipomoea Batatas L.*) enriched With Nutritional Yeast As Healthy Vegan Food. Media Gizi Indones 16:79. https://doi.org/10.20473/MGI.V16I1.79-85

Ghosh SC, Asanuma KI, Kusutani A, Toyota M (2001) Effect of salt stress on some chemical components and yield of potato. Soil Sci Plant Nutr 47:467–475. https://doi.org/10.1080/00380768.2001.10408411

Gibson RS, Raboy V, King JC (2018) Implications of phytate in plant-based foods for iron and zinc bioavailability, setting dietary requirements, and formulating programs and policies. Nutr Rev 76:793–804. https://doi.org/10.1093/nutrit/nuy028

Gilbert LA, Horlbeck MA, Adamson B, *et al* (2014) Genome-Scale CRISPR-Mediated Control of Gene Repression and Activation. Cell 159:647–661.

Goldraij A, Kondo K, Lee CB, *et al* (2006) Compartmentalization of S-RNase and HT-B degradation in self-incompatible *Nicotiana*. Nature 439:805–810. https://doi.org/10.1038/nature04491

Gomathi R, Rakkiyapan P (2011) Comparative lipid peroxidation, leaf membrane thermostability, and antioxidant system in four sugarcane genotypes differing in salt tolerance. Int J Plant Physiol Biochem 3:67–74

González-Montaña JR, Escalera-Valente F, Alonso AJ, *et al* (2020) Relationship between Vitamin B12 and Cobalt Metabolism in Domestic Ruminant: An Update. Anim an Open Access J from MDPI 10:1–36. https://doi.org/10.3390/ANI10101855

Gopal J (2015) Challenges and Way-forward in Selection of Superior Parents, Crosses and Clones in Potato Breeding. Potato Res 58:165–188.

Gordon-Kamm W, Dilkes BP, Lowe K, *et al* (2002) Stimulation of the cell cycle and maize transformation by disruption of the plant retinoblastoma pathway. Proc Natl Acad Sci U S A 99:11975–11980. https://doi.org/10.1073/pnas.142409899

Graham DB, Root DE (2015) Resources for the design of CRISPR gene editing experiments. Genome Biol 2015 161 16:1–21. https://doi.org/10.1186/S13059-015-0823-X

Gray-Mitsumune M, Yoo BY, Charest PJ (1996) Chloroplast-dependent and light-independent expression of the tobacco rbcS promoter GUS chimeric gene in black spruce. Can J For Res 26:909–917. https://doi.org/10.1139/x26-100

Griffith HW (2000) Vitamins, Herbs, Minerals & Supplements: The Complete Guide. In: Revis. Ed. MJF Books. http://www.ndhealthfacts.org/wiki/Cobalt#cite_ref-griffith_1-1. Accessed 12 Jul 2022

Gugała M, -Mystkowska I, * -Rogóż-Matyszczak, *et al* (2019) Content of the selected macro-elements in potato tubers (*Solanum tuberosum* l.) treated with biostimulators. Appl. Ecol. Environ. Res.,17, 11925–11933.

Gumi AM, Aliero AA, K. Shehu, A. Danbaba (2013) Salinity Stress: Effects on Growth, Biochemical Parameters and Ion Homeostasis in *Solanum* lycospersicum L. (Cv. Dan eka). Sch Res Libr Cent Eur J Exp Biol 2:20–25

Guo YL, Bechsgaard JS, Slotte T, *et al* (2009) Recent speciation of *Capsella rubella* from *Capsella grandiflora*, associated with loss of self-incompatibility and an extreme bottleneck. Proc Natl Acad Sci U S A 106:5246–5251. https://doi.org/10.1073/PNAS.0808012106

Hajare ST, Chauhan NM, Kassa G (2021) Effect of Growth Regulators on *in vitro* Micropropagation of Potato (*Solanum tuberosum* L.) Gudiene and Belete Varieties from Ethiopia. Sci World J 2021. https://doi.org/10.1155/2021/5928769

Halterman D, Guenthner J, Collinge S, *et al* (2016) Biotech Potatoes in the 21st Century: 20 Years Since the First Biotech Potato. Am J Potato Res 93:1–20.

Hameed A, Ali Zaidi SS, Shakir S, Mansoor S (2018) Applications of New Breeding Technologies for Potato Improvement. Front Plant Sci 9:925.

Hameed A, Mehmood MA, Shahid M, *et al* (2020) Prospects for potato genome editing to engineer resistance against viruses and cold-induced sweetening. GM Crops Food 11:185–205.

Hamouz K, Bečka D, Capouchová I (2018) Ascorbic acid content in potato tubers with coloured flesh as affected by genotype, environment, and storage. Plant, Soil Environ 64 (2018):605–611. https://doi.org/10.17221/542/2018-PSE

Han EH, Goo YM, Lee MK, Lee SW (2015) An efficient transformation method for a potato (*Solanum tuberosum L. var. Atlantic*). J Plant Biotechnol 42:77–82.

Hanna RE, Doench JG (2020) Design and analysis of CRISPR–Cas experiments. Nat Biotechnol 38:813–823.

Haque A, Samad MAE, Shapla T (2009) *In vitro* callus initiation and regeneration of potato. Bangladesh J Agric Res 34:449–456. https://doi.org/10.3329/BJAR.V34I3.3971

Hartmann HT, Kester DE, Davies FT, Geneve RL (1997) Plant propagation: principles and practices. Prentice-Hall Inc.

Harushima Y, Yano M, Shomura A, *et al* (1998) A high-density rice genetic linkage map with 2275 markers using a single F2 population. Genetics 148:479. https://doi.org/10.1093/GENETICS/148.1.479

Hassan MM, Zhang Y, Yuan G, *et al* (2021) Construct design for CRISPR/Cas-based genome editing in plants. Trends Plant Sci 26:1133–1152.

Hauser F, Horie T (2010) A conserved primary salt tolerance mechanism mediated by HKT transporters: a mechanism for sodium exclusion and maintenance of high K+/Na+ ratio in leaves during salinity stress. Plant Cell Environ 33:552–565. https://doi.org/10.1111/J.1365-3040.2009.02056.X

Hawkes JG (1990) The potato: evolution, biodiversity, and genetic resources. potato Evol Biodivers Genet Resour

Hawkes JG, Hjerting JP (1985) New *Solanum* taxa from Bolivia: a brief note. Bot J Linn Soc 91:445–446. https://doi.org/10.1111/J.1095-8339.1985.TB01013.X

Hawkes JGA (1990) The potato: evolution, biodiversity and genetic resources and genetic resources., 1st edn. Belhaven Press, London

He P, Shan L, Sheen J (2007) Elicitation and suppression of microbe-associated molecular pattern-triggered immunity in plant–microbe interactions. Cell Microbiol 9:1385–1396.

Heeres P, Schippers-Rozenboom M, Jacobsen E, Visser RGF (2002) Transformation of a large number of potato varieties: Genotype-dependent variation in efficiency and somaclonal variability. Euphytica 124:13–22.

Hellens R, Mullineaux P, Klee H (2000) A guide to *Agrobacterium* binary Ti vectors. Trends Plant Sci 5:446–451. https://doi.org/10.1016/S1360-1385(00)01740-4

Herman AC, Busch JW, Schoen DJ (2012) Phylogeny of *Leavenworthia* S-alleles suggests unidirectional mating system evolution and enhanced positive selection following an ancient population bottleneck. Evolution 66:1849–1861. https://doi.org/10.1111/J.1558-5646.2011.01564.X

Heřmanová V, Bárta J, Čurn V (2018) Wild potato species: characterization and biological potential for potato breeding – a revoew. Czech J Genet Plant Breed 43:73–81. https://doi.org/10.17221/2069-CJGPB

Hernández JA, Jiménez A, Mullineaux P, Sevilla F (2000) Tolerance of pea (Pisum sativum L.) to long-term salt stress is associated with induction of antioxidant defences. Plant Cell Environ 23:853–862. https://doi.org/10.1046/J.1365-3040.2000.00602.X

Heuer B (2003) Influence of exogenous application of proline and glycinebetaine on growth of salt-stressed tomato plants. Plant Sci 165:693–699. https://doi.org/10.1016/S0168-9452(03)00222-X

Heuer B, Nadler A (1998) Physiological response of potato plants to soil salinity and water deficit. Plant Sci 137:43–51. https://doi.org/10.1016/S0168-9452(98)00133-2

Hijmans RJ (2003) The effect of climate change on global potato production. Am J Potato Res 2003 804 80:271–279. https://doi.org/10.1007/BF02855363

Hirasawa T, Sato K, Yamaguchi M, *et al* (2017) Differences in dry matter production, grain production, and photosynthetic rate in barley cultivars under long-term salinity. Plant Prod Sci 20:288–299. https://doi.org/10.1080/1343943X.2017.1343647

Hisano H, Sato K (2016) Genomic regions responsible for amenability to *Agrobacterium*-mediated transformation in barley. Sci Rep 6:1–11. https://doi.org/10.1038/srep37505

Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983) A binary plant vector strategy based on separation of vir- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303:179–180.

Hofvander P, Andreasson E, Andersson M (2022) Potato trait development going fast-forward with genome editing. Trends Genet 38:218–221.

Holford P, Newbury HJ (1992) The effects of antibiotics and their breakdown products on the *in vitro* growth of *Antirrhinum majus*. Plant Cell Rep 11:93–96. https://doi.org/10.1007/BF00235261

Hood EE, Gelvin SB, Melchers LS, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res 2:208–218.

Hoque MA, Okuma E, Banu MNA, *et al* (2007) Exogenous proline mitigates the detrimental effects of salt stress more than exogenous betaine by increasing antioxidant enzyme activities. J Plant Physiol 164:553–561. https://doi.org/10.1016/J.JPLPH.2006.03.010

Hosaka K, Hanneman RE (1998) Genetics of self-compatibility in a self-incompatible wild diploid potato species *Solanum chacoense*. 1. Detection of an S locus inhibitor (*Sli*) gene. Euphytica 99:191–197.

Hou Y, Yin Y, Wu G (2015) Dietary essentiality of “nutritionally non-essential amino acids” for animals and humans. Exp Biol Med 240:997.

Hrabovská D, Heldák J, Volnová B, Hrabovská ID (2013) Changes in the content of vitamin C in potato tubers depending on variety. J Microbiol 1:2052–2058

Hu X, Meng X, Liu Q, *et al* (2018) Increasing the efficiency of CRISPR-Cas9-VQR precise genome editing in rice. Plant Biotechnol J 16:292–297.

Hu, T., Banzhaf, W. (2008). Nonsynonymous to Synonymous Substitution Ratio k_a/k_s: Measurement for Rate of Evolution in Evolutionary Computation. *In*: Rudolph, G., Jansen, T., Beume, N., Lucas, S., Poloni, C. (*eds*) Parallel Problem Solving from Nature – PPSN X. PPSN 2008. Lecture Notes in Computer Science, vol 5199. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-540-87700-4_45

Hua Z, Meng X, Kao TH (2007) Comparison of *Petunia inflata* S-Locus F-box protein (Pi SLF) with Pi SLF like proteins reveals its unique function in S-RNase based self-incompatibility. Plant Cell 19:3593–3609. https://doi.org/10.1105/TPC.107.055426

Huang Holger Puchta T-K, Schillberg S, Huang Á Puchta TH, Orzaez D (2021) Novel CRISPR/Cas applications in plants: from prime editing to chromosome engineering. Transgenic Res 30:529–549.

Huang S, Lee H-S, Karunanandaa B, Kao T-H (1994) Ribonuclease activity of *Petunia inflata* S proteins is essential for rejection of self-pollen. Plant Cell 6:1021. https://doi.org/10.1105/TPC.6.7.1021

Huang X, Wei Z (2005) Successful *Agrobacterium*-mediated genetic transformation of maize elite inbred lines. Plant Cell Tissue Organ Cult 83:187–200. https://doi.org/10.1007/s11240-005-5772-8

Huda, K. M. K., Bhuiyan, M. S. R., Kabir, M. H., Jamal Uddin, A. F. M., & Khatun, A. (2007) *In vitro* plant regeneration protocol in tossa jute (*Corchorus olitorius*). Internatinal Journal of Integrative Biology, 1, 96-101.

HUGHES MB, BABCOCK EB (1950) Self-Incompatibility in *Crepis Foetida (L.)* Subsp. *Rhoeadifolia* (Bieb.) Schinz Et Keller. Genetics 35:570. https://doi.org/10.1093/genetics/35.5.570

Hurrell RF (1997) Preventing Iron Deficiency through Food Fortification. Nutr Rev 55:210–222. https://doi.org/10.1111/J.1753-4887.1997.TB01608.X

Hussain I, Muhammad A, Chaudhry Z, *et al* (2005) Morphogenic potential of three potato (*Solanum tuberosum*) cultivars from diverse explants, a prerequisite in genetic manipulation. Pak J Bot 37:889–898

Ida K, Norioka S, Yamamoto M, *et al* (2001) The 1.55 A resolution structure of *Nicotiana alata* S(F11)-RNase associated with gametophytic self-incompatibility. J Mol Biol 314:103–112. https://doi.org/10.1006/JMBI.2001.5127

Ioerger TR, Gohlke JR, Xu B, Kao TH (1991) Primary structural features of the self-incompatibility protein in solanaceae. Sex Plant Reprod 1991 42 4:81–87. https://doi.org/10.1007/BF00196492

Isayenkov S V., Maathuis FJM (2019) Plant salinity stress: Many unanswered questions remain. Front Plant Sci 10:80. https://doi.org/10.3389/FPLS.2019.00080/BIBTEX

Ishfaq M, Zhong Y, Wang Y, Li X (2021) Magnesium Limitation Leads to Transcriptional Down-Tuning of Auxin Synthesis, Transport, and Signaling in the Tomato Root. Front Plant Sci 12:. https://doi.org/10.3389/FPLS.2021.802399

Ishida BK, Snyder GW, Belknap WR (1989) The use of *in vitro*-grown microtuber discs in *Agrobacterium*-mediated transformation of Russet Burbank and Lemhi Russet potatoes. Plant Cell Rep 8:325–328. https://doi.org/10.1007/BF00716665

Ishikawa T, Shabala S (2019) Control of xylem Na + loading and transport to the shoot in rice and barley as a determinant of differential salinity stress tolerance. Physiol Plant 165:619–631. https://doi.org/10.1111/PPL.12758

Iwano M, Takayama S (2012) Self/non-self-discrimination in angiosperm self-incompatibility. Curr Opin Plant Biol 15:78–83. https://doi.org/10.1016/J.PBI.2011.09.003

Jaarsma R, de Boer AH (2018) Salinity tolerance of two potato cultivars (*Solanum tuberosum*) correlates with differences in vacuolar transport activity. Front Plant Sci 9:. https://doi.org/10.3389/FPLS.2018.00737

Jaarsma R, de Vries RSM, de Boer AH (2013) Effect of Salt Stress on Growth, Na+ Accumulation and Proline Metabolism in Potato (*Solanum tuberosum*) Cultivars. PLoS One 8:e60183. https://doi.org/10.1371/JOURNAL.PONE.0060183

Jacobs JME, Van Eck HJ, Arens P, *et al* (1995) A genetic map of potato (*Solanum tuberosum*) integrating molecular markers, including transposons, and classical markers.

Jaganathan D, Ramasamy K, Sellamuthu G, *et al* (2018) CRISPR for crop improvement: An update review. Front Plant Sci 9:985.

Jaiswal VS, Narayan P (1985) Regeneration of plantlets from the callus of stem segments of adult plants of *Ficus religiosa L.* Plant Cell Rep 4:256–258. https://doi.org/10.1007/BF00269371

Jansky S (2000) Plant Breeding Reviews. Plant Breed Rev 19:69–155.

Jansky SH, Charkowski AO, Douches DS, *et al* (2016) Reinventing potato as a diploid inbred line-based crop. Crop Sci 56:1412–1422.

Jansky SH, Chung YS, Kittipadukal P (2014) M6: A Diploid Potato Inbred Line for use in Breeding and Genetics Research. J Plant Regist 8:195–199. https://doi.org/10.3198/jpr2013.05.0024crg

Jansky SH, Spooner DM (2017) The Evolution of Potato Breeding. Plant Breed Rev 41:169–214. https://doi.org/10.1002/9781119414735.CH4

Jansky, S.H., De Jong, W.S., Douches, D.S., Haynes, K.G., Holm, D.G. (2021). Cultivar Improvement with Exotic Germplasm: An Example from Potato. In: Carputo, D., Aversano,

R., Ercolano, M.R. (eds) The Wild *Solanum* Genomes. Compendium of Plant Genomes. Springer, Cham.

Jarret RL, Hasegawa PM, Erickson HT (1980) Effects of Medium Components on Shoot Formation from Cultured Tuber Discs of Potato1. J Am Soc Hortic Sci 105:238–242.

Jbir-Koubaa R, Charfeddine S, Ellouz W, *et al* (2014) Investigation of the response to salinity and to oxidative stress of interspecific potato somatic hybrids grown in a greenhouse. Plant Cell, Tissue Organ Cult 2014 1203 120:933–947. https://doi.org/10.1007/S11240-014-0648-4

Ježek P, Hlušek J, Lošák T, *et al* (2011) Effect of foliar application of selenium on the content of selected amino acids in potato tubers (*Solanum tuberosum* L.). Plant, Soil Environ 57:315–320. https://doi.org/10.17221/57/2011-PSE

Jha G, Choudhary OP, Sharda R (2017) Comparative effects of saline water on yield and quality of potato under drip and furrow irrigation. Cogent Food & Agriculture, 3:1, DOI: 10.1080/23311932.2017.1369345

Jiang C, Cui Q, Feng K, *et al* (2016) Melatonin improves antioxidant capacity and ion homeostasis and enhances salt tolerance in maize seedlings. Acta Physiol Plant 2016 384 38:1–9. https://doi.org/10.1007/S11738-016-2101-2

Jiang D, Lu B, Liu L, *et al* (2021) Exogenous melatonin improves the salt tolerance of cotton by removing active oxygen and protecting photosynthetic organs. BMC Plant Biol 21:1–19. https://doi.org/10.1186/S12870-021-03082-7/FIGURES/12

Jiang S, Carroll L, Mariotti M, *et al* (2021) Formation of protein cross-links by singlet oxygen-mediated disulfide oxidation. Redox Biol 41:. https://doi.org/10.1016/J.REDOX.2021.101874

Jiménez ME, Rossi AM, Sammán NC (2009) Phenotypic, agronomic and nutritional characteristics of seven varieties of Andean potatoes. J Food Compos Anal 22:613–616.

Jin S, Song YN, Deng WY, *et al* (1993) The regulatory *Vir*A protein of *Agrobacterium tumefaciens* does not function at elevated temperatures. J Bacteriol 175:6830–6835.

Jin S, Zhang X, Liang S, *et al* (2005) Factors affecting transformation efficiency of embryogenic callus of Upland cotton (*Gossypium hirsutum*) with *Agrobacterium tumefaciens*. Plant Cell Tissue Organ Cult 81:229–237.

Jinek M, Chylinski K, Fonfara I, *et al* (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337:816–821. https://doi.org/10.1126/SCIENCE.1225829

Joersbo M, Jørgensen K, Brunstedt J (2003) A selection system for transgenic plants based on galactose as selective agent and a UDP-glucose: galactose-1-phosphate uridyltransferase gene as selective gene. Mol Breed 11:315–323.

Johnson MA, Harper JF, Palanivelu R (2019) A Fruitful Journey: Pollen Tube Navigation from Germination to Fertilization. Annu Rev Plant Biol 70:809–837. https://doi.org/10.1146/ANNUREV-ARPLANT-050718-100133

Jones DA, Takemoto D (2004) Plant innate immunity – direct and indirect recognition of general and specific pathogen-associated molecules. Curr Opin Immunol 16:48–62.

Jones DT, Taylor WR, Thornton JM (1992) The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci 8:275–282. https://doi.org/10.1093/BIOINFORMATICS/8.3.275

Jones JDG, Dangl JL (2006) The plant immune system. Nature 444:323–329. https://doi.org/10.1038/NATURE05286

Juan M, Rivero RM, Romero L, Ruiz JM (2005) Evaluation of some nutritional and biochemical indicators in selecting salt-resistant tomato cultivars. Environ Exp Bot 54:193–201.

Kahlaoui B, Hachicha M, Rejeb S, *et al* (2015) Response of two tomato cultivars to field-applied proline under irrigation with saline water: Growth, chlorophyll fluorescence and nutritional aspects. Photosynth 2014 523 52:421–429. https://doi.org/10.1007/S11099-014-0053-6

Kaiser NR, Jansky S, Coombs JJ, *et al* (2021) Assessing the Contribution of *Sli* to Self-Compatibility in North American Diploid Potato Germplasm Using KASP^{TM} Markers. Am J Potato Res 98:104–113. https://doi.org/10.1007/S12230-021-09821-8

Kámán-Tóth E, Pogány M, Dankó T, *et al* (2018) A simplified and efficient *Agrobacterium tumefaciens* electroporation method. 3 Biotech 8:148.

Kao TH, Mccubbin AG (1996) How flowering plants discriminate between self and non-self-pollen to prevent inbreeding. Proc Natl Acad Sci U S A 93:12059. https://doi.org/10.1073/PNAS.93.22.12059

Kao TH, Tsukamoto T (2004) The molecular and genetic bases of S-RNase-based self-incompatibility. Plant Cell 16 Suppl: https://doi.org/10.1105/TPC.016154

Kaothien-Nakayama P, Isogai A, Takayama S (2010) Self-Incompatibility Systems in Flowering Plants. Plant Dev Biol 1:459–485. https://doi.org/10.1007/978-3-642-02301-9_22

Karami O (2008) Factors Affecting *Agrobacterium*-mediated Transformation of Plants. Transgenic Plant Journal 2 (2):127-137.

Kardile HB, Yilma S, Sathuvalli V (2022) Molecular Approaches to Overcome Self-Incompatibility in Diploid Potatoes. Plants 11. https://doi.org/10.3390/PLANTS11101328

Karimi G, Ghorbanli M, Heidari H, *et al* (2005) The effects of NaCl on growth, water relations, osmolytes and ion content in Kochia prostrata. Biol Plant 2005 492 49:301–304. https://doi.org/10.1007/S10535-005-1304-Y

Karki HS, Jansky SH, Halterman DA (2021) Screening of wild potatoes identifies new sources of late blight resistance. Plant Dis 105:368–376.

Karkute SG, Singh AK, Gupta OP, *et al* (2017) CRISPR/Cas9 mediated genome engineering for improvement of horticultural crops. Front Plant Sci 8:1635. https://doi.org/10.3389/FPLS.2017.01635/BIBTEX

Karp A, Jones MGK, Ooms G, Bright SWJ (2013) Potato Protoplasts and Tissue Culture in Crop Improvement. Biotechnol Genet Eng Rev 5:1–32. https://doi.org/10.1080/02648725.1987.10647833

Kashyap SP, Prasanna HC, Kumari N, *et al* (2020) Understanding salt tolerance mechanism using transcriptome profiling and de novo assembly of wild tomato *Solanum* chilense. Sci Rep 10:. https://doi.org/10.1038/S41598-020-72474-W

Kaur A, Guleria S, Reddy MS, Kumar A (2020) A robust genetic transformation protocol to obtain transgenic shoots of *Solanum tuberosum* L. cultivar '*Kufri Chipsona 1*.' Physiol Mol Biol Plants 26:367–377.

Kaur RP, Devi S (2019) In Planta Transformation in Plants: A Review. Agric Rev 40:159–174. https://doi.org/10.18805/ag.r-1597

Kavi Kishor PB, Hong Z, Miao GH, *et al* (1995) Overexpression of [delta]-Pyrroline-5-Carboxylate Synthetase Increases Proline Production and Confers Osmotolerance in Transgenic Plants. Plant Physiol 108:1387–1394. https://doi.org/10.1104/PP.108.4.1387

Kaya C, Higgs D, Ashraf M, *et al* (2020) Integrative roles of nitric oxide and hydrogen sulfide in melatonin-induced tolerance of pepper (Capsicum annuum L.) plants to iron deficiency and salt stress alone or in combination. Physiol Plant 168:256–277.

Kaya C, Okant M, Ugurlar F, *et al* (2019) Melatonin-mediated nitric oxide improves tolerance to cadmium toxicity by reducing oxidative stress in wheat plants. Chemosphere 225:627–638.

Kaźmierczak-Barańska J, Boguszewska K, Adamus-Grabicka A, Karwowski BT (2020) Two Faces of Vitamin C—Antioxidative and Pro-Oxidative Agent. Nutrients 12:.

Ke J, Khan R, Johnson T, *et al* (2001) High-efficiency gene transfer to recalcitrant plants by *Agrobacterium tumefaciens*. Plant Cell Rep 20:150–156.

Ke Q, Ye J, Wang B, *et al* (2018) Melatonin Mitigates Salt Stress in Wheat Seedlings by Modulating Polyamine Metabolism. Front Plant Sci 9:. https://doi.org/10.3389/FPLS.2018.00914

Keutgen AJ, Wszelaczyńska E, Pobereżny J, *et al* (2019) Antioxidant properties of potato tubers (*Solanum tuberosum L.*) as a consequence of genetic potential and growing conditions. PLoS One 14. https://doi.org/10.1371/JOURNAL.PONE.0222976

Khalafalla M, Sulieman Modawi R, Khalafalla MM, *et al* (2010) Callus formation and organogenesis of potato (*Solanum tuberosum L.*) cultivar Almera. J Phytol 2010:40–46

Khan MN, Zhang J, Luo T, *et al* (2019) Seed priming with melatonin coping drought stress in rapeseed by regulating reactive oxygen species detoxification: Antioxidant defense system, osmotic adjustment, stomatal traits and chloroplast ultrastructure perseveration. Ind Crops Prod 140:. https://doi.org/10.1016/J.INDCROP.2019.111597

Khan S, Ahmad F, Ali F, *et al* (2011) Callus induction via different growth regulators from cotyledon explants of indigenous chickpea (*Cicer arietinum L.*) cultivars KK-1 and Hassan-2K. African J Biotechnol 10:7825–7830. https://doi.org/10.5897/ajb10.2378

Khatun N, Bari M.A, Islam R, Huda S, Siddique N.A , Rahman M.H. and Mollah M.U. (2003) Callus Induction and Regeneration from Nodal Segment of Potato Cultivar Diamant. J Biol Sci 3:1101–1106. https://doi.org/10.3923/JBS.2003.1101.1106

Khenifi ML, Boudjeniba M, Kameli A (2011) Effects of salt stress on micropropagation of potato (*Solanum tuberosum* L.). African J Biotechnol 10:7840–7845. https://doi.org/10.5897/AJB10.982

Kikuta Y, Okazawa Y (1984) Control of root and shoot-bud formation from potato tuber tissue cultured *in vitro*. Physiol Plant 61:8–12.

Kim GB, Nam YW (2013) A novel Δ(1)-pyrroline-5-carboxylate synthetase gene of Medicago truncatula plays a predominant role in stress-induced proline accumulation during symbiotic nitrogen fixation. J Plant Physiol 170:291–302. https://doi.org/10.1016/J.JPLPH.2012.10.004

Kim SH, Kim DY, Yacoubi I, Seo YW (2018) Physiological and Genetic Responses of Salt-stressed Tunisian Durum (Triticum turgidum ssp. durum) Cultivars. KOREAN J Crop Sci 63:314–321. https://doi.org/10.7740/KJCS.2018.63.4.314

Kim WS, Bhatia S, Elliott DA, *et al* (2010) Increased ATP-binding cassette transporter A1 expression in Alzheimer's disease hippocampal neurons. J Alzheimers Dis 21:193–205. https://doi.org/10.3233/JAD-2010-100324

King JC, Slavin JL (2013) White Potatoes, Human Health, and Dietary Guidance. Adv Nutr 4:393S. https://doi.org/10.3945/AN.112.003525

Kirk WW, Wharton PS (2014) The potato: botany, production and uses.eds, Navarre R, Pavek M. Oxon: CABI: pp 167-201.

Kishor PBK, Hong Z, Miao GH, *et al* (1995) Overexpression of [delta]-Pyrroline-5-Carboxylate Synthetase Increases Proline Production and Confers Osmotolerance in Transgenic Plants. Plant Physiol 108:1387–1394. https://doi.org/10.1104/PP.108.4.1387

Kitajima K, Hogan KP (2003) Increases of chlorophyll a/b ratios during acclimation of tropical woody seedlings to nitrogen limitation and high light. Plant Cell Environ 26:857–865. https://doi.org/10.1046/J.1365-3040.2003.01017.X

Klein S, Cohn SM, Alpers DH (2006) Modern nutrition in health and disease, 10th ed. Lippincott Williams & Wilkins, Philadelphia

Ko TS, Korban SS (2004) Enhancing the frequency of somatic embryogenesis following *Agrobacterium*-mediated transformation of immature cotyledons of soybean [*Glycine max (L.) Merrill.*]. Vitr Cell Dev Biol - Plant 40:552–558.

Koca H, Bor M, Özdemir F, Türkan I (2007) The effect of salt stress on lipid peroxidation, antioxidative enzymes and proline content of sesame cultivars. Environ Exp Bot 60:344–351.

Koleva GL, Trajkova F, Gudeva LK *et al* (2012) Micropropagation of Potato (*Solanum tuberosum L*). Electron J Biol 8:45-49.

Kolomeichuk L V., Efimova M V., Zlobin IE, *et al* (2020) 24-Epibrassinolide alleviates the toxic effects of NaCl on photosynthetic processes in potato plants. Photosynth Res 146:151–163. https://doi.org/10.1007/S11120-020-00708-Z

Kondo K, Yamamoto M, Itahashi R, *et al* (2002a) Insights into the evolution of self-compatibility in *Lycopersicon* from a study of stylar factors. Plant J 30:143–153. https://doi.org/10.1046/J.1365-313X.2002.01275.X

Kondo K, Yamamoto M, Matton DP, *et al* (2002b) Cultivated tomato has defects in both S-RNase and HT genes required for stylar function of self-incompatibility. Plant J 29:627–636. https://doi.org/10.1046/J.0960-7412.2001.01245.X

Kondo T, Hasegawa H, Suzuki M (2000) Transformation and regeneration of garlic (*Allium sativum L.*) by *Agrobacterium*-mediated gene transfer. Plant Cell Rep 19:989–993. https://doi.org/10.1007/s002990000222

Konieczny A, Ausubel FM (1993) A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. Plant J 4:403–410. https://doi.org/10.1046/J.1365-313X.1993.04020403.X

Koornneef M, Bade J, Hanhart C, *et al* (1993) Characterization and mapping of a gene controlling shoot regeneration in tomato. Plant J 3:131–141. https://doi.org/10.1111/J.1365-313X.1993.TB00016.X

Kowalczewski PŁ, Olejnik A, Białas W, *et al* (2019) The Nutritional Value and Biological Activity of Concentrated Protein Fraction of Potato Juice. Nutrients 11:. https://doi.org/10.3390/NU11071523

Koyro HW (2006) Effect of salinity on growth, photosynthesis, water relations and solute composition of the potential cash crop halophyte Plantago coronopus (L.). Environ Exp Bot 56:136–146. https://doi.org/10.1016/J.ENVEXPBOT.2005.02.001

Kreuze JF, Souza-Dias JAC, Jeevalatha A, Figueira AR, Valkonen JPT, Jones RAC (2020). The Potato Crop: its Agricultural, Nutritional and Social Contribution to Humankind. eds, Campos H, Ortiz O, Switzerland: Springer International Publishing, pp 389–430

Kubo KI, Entani T, Takara A, *et al* (2010) Collaborative non-self recognition system in S-RNase-based self-incompatibility. Science (80-) 330:796–799. https://doi.org/10.1126/SCIENCE.1195243/SUPPL_FILE/KUBO.SOM.PDF

Kubo KI, Paape T, Hatakeyama M, *et al* (2015) Gene duplication and genetic exchange drive the evolution of S-RNase-based self-incompatibility in *Petunia*. Nat Plants 2014 11 1:1–9. https://doi.org/10.1038/nplants.2014.5

Kudirka DT, Colburn SM, Hinchee MA, Wright MS (1986) Interactions of *Agrobacterium tumefaciens* with soybean (*Glycine max (L.) Merr.*) leaf explants in tissue culture. Can J Genet Cytol 28:808–817.

Kumar A, Kumar S, Bains S, *et al* (2016) De novo Transcriptome Analysis Revealed Genes Involved in Flavonoid and Vitamin C Biosynthesis in *Phyllanthus emblica (L.)*. Front Plant Sci 7:1610. https://doi.org/10.3389/fpls.2016.01610

Kumar A, Miller M, Whitty P, *et al* (1995) *Agrobacterium*-mediated transformation of five wild *Solanum* species using *in vitro* microtubers. Plant Cell Rep 14:324–328.

Kumar D, Ezekiel R (2006) Developmental changes in sugars and dry matter content of potato tuber under sub-tropical climates. Sci Hortic (Amsterdam) 110:129–134. https://doi.org/10.1016/J.SCIENTA.2006.06.022

Kumar D, Singh BP, Kumar P (2004) An overview of the factors affecting sugar content of potatoes. Ann Appl Biol 145:247–256. https://doi.org/10.1111/J.1744-7348.2004.TB00380.X

Kumlay A.M., Ercisli S. (2015) Callus Induction, Shoot Proliferation and Root Regeneration of Potato (*Solanum Tuberosum L.*) Stem Node and Leaf Explants under Long-Day Conditions. Biotechnol. Biotechnol. Equip.;29:1075–1084.

Labun K, Montague TG, Krause M, *et al* (2019) CHOPCHOP v3: expanding the CRISPR web toolbox beyond genome editing. Nucleic Acids Res 47:W171–W174.

Lacerda CF De, Cambraia J, Cano MAO, Ruiz HA (2001) Plant growth and solute accumulation and distribution in two sorghum genotypes, under NaCl stress. Rev Bras Fisiol Veg 13:270–284. https://doi.org/10.1590/S0103-31312001000300003

Lai Z, Ma W, Han B, *et al* (2002) An F-box gene linked to the self-incompatibility (S) locus of Antirrhinum is expressed specifically in pollen and tapetum. Plant Mol Biol 50:29–41. https://doi.org/10.1023/A:1016050018779

Lal MK, Kumar A, Raigond P, *et al* (2021) Impact of Starch Storage Condition on Glycemic Index and Resistant Starch of Cooked Potato (*Solanum tuberosum*) Tubers. Starch - Stärke 73:1900281. https://doi.org/10.1002/STAR.201900281

Latef AAHA, Chaoxing H (2011) Effect of arbuscular mycorrhizal fungi on growth, mineral nutrition, antioxidant enzymes activity and fruit yield of tomato grown under salinity stress. Sci Hortic (Amsterdam) 127:228–233. https://doi.org/10.1016/J.SCIENTA.2010.09.020

Laurie S, Feeney KA, Maathuis FJM, *et al* (2002) A role for HKT1 in sodium uptake by wheat roots. Plant J 32:139–149. https://doi.org/10.1046/J.1365-313X.2002.01410.X

Lazo GR, Stein PA, Ludwig RA (1991) A DNA Transformation–Competent *Arabidopsis* Genomic Library in Agrobacterium. Nat Biotechnol 9:963–967.

Lee HS, Huang S, Kao TH (1994) S proteins control rejection of incompatible pollen in *Petunia inflata*. Nature 367:560–563. https://doi.org/10.1038/367560A0

Leisner CP, Hamilton JP, Crisovan E, *et al* (2018) Genome sequence of M6, a diploid inbred clone of the high-glycoalkaloid-producing tuber-bearing potato species *Solanum chacoense*, reveals residual heterozygosity. Plant J 94:562–570.

Levy D (1992) The response of potatoes (*Solunum tuberosum* L.) to salinity: plant growth and tuber yields in the arid desert of Israel. Ann Appl Biol 120:547–555. https://doi.org/10.1111/J.1744-7348.1992.TB04914.X

Levy D, Veilleux RE (2007) Adaptation of potato to high temperatures and salinity-a review. Am J Potato Res 2007 846 84:487–506. https://doi.org/10.1007/BF02987885

Lewis D (1947) Competition and dominance of incompatibility alleles in diploid pollen. Heredity (Edinb) 1:85–108. https://doi.org/10.1038/hdy.1947.5

Lewis D, Crowe LK (1954) The Induction of Self-Fertility in Tree Fruits. J Hortic Sci 29:220–225. https://doi.org/10.1080/00221589.1954.11513813

Lewis D, Crowe LK (1958) Unilateral interspecific incompatibility in flowering plants. Heredity (Edinb) 12:233–256. https://doi.org/10.1038/HDY.1958.26

Li C, Hao M, Wang W, Wang H, Chen F, Chu W, Zhang B, Mei D, Cheng H and Hu Q (2018) An Efficient CRISPR/Cas9 Platform for Rapidly Generating Simultaneous Mutagenesis of Multiple Gene Homoeologs in Allotetraploid Oilseed Rape. Front. Plant Sci. 9:442. doi: 10.3389/fpls.2018.00442

Li C, Wang P, Wei Z, *et al* (2012) The mitigation effects of exogenous melatonin on salinity-induced stress in Malus hupehensis. J Pineal Res 53:298–306. https://doi.org/10.1111/J.1600-079X.2012.00999.X

Li F, Vallabhaneni R, Wurtzel ET (2008) PSY3, a new member of the phytoene synthase gene family conserved in the Poaceae and regulator of abiotic stress-induced root carotenogenesis. Plant Physiol 146:1333–1345. https://doi.org/10.1104/PP.107.111120

Li H, Chang J, Chen H, *et al* (2017) Exogenous Melatonin Confers Salt Stress Tolerance to Watermelon by Improving Photosynthesis and Redox Homeostasis. Front Plant Sci 8:. https://doi.org/10.3389/FPLS.2017.00295

Li L, Yang X (2018) The essential element manganese, oxidative stress, and metabolic diseases: Links and interactions. Oxid Med Cell Longev 2018:. https://doi.org/10.1155/2018/7580707

Li M, Li Y, Li H, Wu G (2011) Overexpression of AtNHX5 improves tolerance to both salt and drought stress in *Broussonetia papyrifera (L.)* Vent. Tree Physiol 31:349–357.

Li M, Xie C, Song B, *et al* (2015) Construction of efficient, tuber-specific, and cold-inducible promoters in potato. Plant Sci 235:14–24.

Li M, Zhang D, Gao Q, *et al* (2019) Genome structure and evolution of *Antirrhinum majus L.* Nat plants 5:174–183. https://doi.org/10.1038/S41477-018-0349-9

Li S, Sun P, Williams JS, Kao T hui (2014) Identification of the self-incompatibility locus F-box protein-containing complex in *Petunia inflata*. Plant Reprod 27:31–45. https://doi.org/10.1007/S00497-013-0238-3

Li W, Chetelat RT (2014) The role of a pollen expressed *Cullin1* protein in gametophytic self-incompatibility in *Solanum*. Genetics 196:439–442. https://doi.org/10.1534/GENETICS.113.158279/-/DC1/GENETICS.113.158279-5.PDF

Li W, Chetelat RT (2015) Unilateral incompatibility gene ui1.1 encodes an S-locus F-box protein expressed in pollen of *Solanum* species. Proc Natl Acad Sci U S A 112:4417–4422.

Li W, Royer S, Chetelat RT (2010) Fine Mapping of ui6.1, a Gametophytic Factor Controlling Pollen-Side Unilateral Incompatibility in Interspecific *Solanum* Hybrids. Genetics 185:1069. https://doi.org/10.1534/GENETICS.110.116343

Li Y, Zhang T, Zhang Z, He K (2019) The physiological and biochemical photosynthetic properties of Lycium ruthenicum Murr in response to salinity and drought. Sci Hortic (Amsterdam) 256:108530. https://doi.org/10.1016/J.SCIENTA.2019.05.057

Li Y, Zhang Y, Feng F, *et al* (2010) Overexpression of a Malus vacuolar Na+/H+ antiporter gene (MdNHX1) in apple rootstock M.26 and its influence on salt tolerance. Plant Cell Tissue Organ Cult 102:337–345. https://doi.org/10.1007/S11240-010-9738-0

Liang C, Li A, Yu H, *et al* (2017) Melatonin Regulates Root Architecture by Modulating Auxin Response in Rice. Front Plant Sci 8:. https://doi.org/10.3389/FPLS.2017.00134

Librado P, Rozas J (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452.

Lindhout P, Meijer D, Schotte T, *et al* (2011) Towards F 1 Hybrid Seed Potato Breeding. Potato Res 54:301–312. https://doi.org/10.1007/S11540-011-9196-Z

Ling HQ, Kriseleit D, Ganal MW (1998) Effect of ticarcillin/potassium clavulanate on callus growth and shoot regeneration in *Agrobacterium*-mediated transformation of tomato (*Lycopersicon esculentum Mill*.). Plant Cell Rep 17:843–847.

Lino CA, Harper JC, Carney JP, Timlin JA (2018) Delivering CRISPR: a review of the challenges and approaches. Drug Deliv 25:1234–1257.

Lisch D (2012) How important are transposons for plant evolution? Nat Rev Genet 14:49–61. https://doi.org/10.1038/nrg3374

Liu H, Ding Y, Zhou Y, *et al* (2017) CRISPR-P 2.0: An Improved CRISPR-Cas9 Tool for Genome Editing in Plants. Mol Plant 10:530–532.

Liu, T.H.A., Stephens, L.C. & Hannapel, D.J (1995). Transformation of *Solanum* brevidens using *Agrobacterium tumefaciens* . Plant Cell Reports 15, 196–199.

Londhe S (2016) Cultivation of potato on abiotic stress-affected soils of India | World Agroforestry | Transforming Lives and Landscapes with Trees. Curr Sci 111:21–22

Lopez SJ, Kumar RR, Pius PK, Muraleedharan N (2004) *Agrobacterium tumefacien*s-Mediated genetic transformation in tea (*Camellia sinensis [L.] O. Kuntze*). Plant Mol Biol Report 2004 222 22:201–202.

Love SL, Salaiz T, Mosley AR, *et al* (2003) Ascorbic acid concentration and stability in North American potato germplasm. Acta Hortic 619:87–93.

Lowe BA, Krul WR (1991) Physical, chemical, developmental, and genetic factors that modulate the *Agrobacterium*-vitis interaction. Plant Physiol 96:121–129. https://doi.org/10.1104/pp.96.1.121

Loyola-Vargas VM, Vázquez-Flota F (2006) Plant Cell Culture Protocols. In: Plant Cell Culture Protocols. Humana Press.

Lutaladio NB, Castaldi L (2009) Potato: The hidden treasure. J Food Compos Anal 22:491–493. https://doi.org/10.1016/J.JFCA.2009.05.002

Luu DT, Qin X, Laublin G, *et al* (2001) Rejection of S-heteroallelic pollen by a dual-specific s-RNase in *Solanum* chacoense predicts a multimeric SI pollen component. Genetics 159:329–335. https://doi.org/10.1093/GENETICS/159.1.329

Ma L, Zhang C, Zhang B, *et al* (2021) A non-S-locus F-box gene breaks self-incompatibility in diploid potatoes. Nat Commun 12. https://doi.org/10.1038/S41467-021-24266-7

Ma X, Zhang Q, Zhu Q, *et al* (2015) A Robust CRISPR/Cas9 System for Convenient, High-Efficiency Multiplex Genome Editing in Monocot and Dicot Plants. Mol Plant 8:1274–1284. https://doi.org/10.1016/J.MOLP.2015.04.007

Ma Y, Dias MC, Freitas H (2020) Drought and Salinity Stress Responses and Microbe-Induced Tolerance in Plants. Front Plant Sci 11:1750.

Maas E V., Hoffman GJ (1977) Crop Salt Tolerance—Current Assessment. J Irrig Drain Div 103:115–134. https://doi.org/10.1061/JRCEA4.0001137

Maathuis FJM, Amtmann A (1999) K + Nutrition and Na + Toxicity: The Basis of Cellular K + /Na + Ratios. Ann Bot 84:123–133. https://doi.org/10.1006/ANBO.1999.0912

Mabhaudhi T, Chimonyo VGP, Hlahla S, *et al* (2019) Prospects of orphan crops in climate change. Planta 250:695–708.

Machado, R., & Serralheiro, R. (2017). Soil Salinity: Effect on Vegetable Crop Growth. Management Practices to Prevent and Mitigate Soil Salinization. *Horticulturae*, *3*(2), 30. https://doi.org/10.3390/horticulturae3020030

Malla D, Qi Q, Robbins T, Sims TL (2016) Characterization of SI Loci in *P* . *inflata* and *P* . *axillaris* identifies multiple linked S-locus F-box genes. Nat Plants 2:

Manai J, Kalai T, Gouia H, Corpas FJ (2014) Exogenous nitric oxide (NO) ameliorates salinity-induced oxidative stress in tomato (*Solanum* lycopersicum) plants. J soil Sci plant Nutr 14:433–446. https://doi.org/10.4067/S0718-95162014005000034

Manuel R, Machado A, Serralheiro RP, *et al* (2017) Soil Salinity: Effect on Vegetable Crop Growth. Management Practices to Prevent and Mitigate Soil Salinization. Hortic 2017, Vol 3, Page 30 3:30. https://doi.org/10.3390/HORTICULTURAE3020030

Marco F, Bitrián M, Carrasco P, *et al* (2015) Genetic engineering strategies for abiotic stress tolerance in plants. Plant Biol Biotechnol Vol II Plant Genomics Biotechnol 579–609. https://doi.org/10.1007/978-81-322-2283-5_29/COVER

Marin JA, Andreu ; P, Carrasco ; A, *et al* (2009) Determination of proline concentration, an abiotic stress marker, in root exudates ofexcised root cultures of fruit tree rootstocks under salt stress. Rev Biol Trop 57:1271–1281

Markova DN, Petersen JJ, Yam SE, *et al* (2017) Evolutionary history of two pollen self-incompatibility factors reveals alternate routes to self-compatibility within *Solanum*. Am J Bot 104:1904–1919. https://doi.org/10.3732/AJB.1700196

Marriott B, Birt D, Stalling V, Yates A (2020) Present Knowledge in Nutrition: Basic Nutrition and Metabolism, volume 1, London, United Kingdom; San Diego, CA : Academic Press, an imprint of Elsevier : ILSI

Marschner P (2011) Marschner's Mineral Nutrition of Higher Plants: Third Edition. Marschner's Miner Nutr High Plants Third Ed 1–651. https://doi.org/10.1016/C2009-0-63043-9

Martinez V, Nieves-Cordones M, Lopez-Delacalle M, *et al* (2018) Tolerance to Stress Combination in Tomato Plants: New Insights in the Protective Role of Melatonin. Molecules 23. https://doi.org/10.3390/MOLECULES23030535

Massaretto IL, Albaladejo I, Purgatto E, *et al* (2018) Recovering tomato landraces to simultaneously improve fruit yield and nutritional quality against salt stress. Front Plant Sci 871:1778. https://doi.org/10.3389/FPLS.2018.01778/BIBTEX

Matsuura T, Sakai H, Unno M, *et al* (2001) Crystal structure at 1.5-A resolution of Pyrus pyrifolia pistil ribonuclease responsible for gametophytic self-incompatibility. J Biol Chem 276:45261–45269. https://doi.org/10.1074/JBC.M107617200

Matton DP, Luu DT, Xike Q, *et al* (1999) Production of an S RNase with Dual Specificity Suggests a Novel Hypothesis for the Generation of New S Alleles. Plant Cell 11:2087–2097. https://doi.org/10.1105/TPC.11.11.2087

Matzk A, Mantell S, Schiemann J (1996) Localization of persisting agrobacteria in transgenic tobacco plants. Mol Plant-Microbe Interact 9:373–381.

Maune JF, Camadro EL, Erazzú LE (2018) Cross-incompatibility and self-incompatibility: unrelated phenomena in wild and cultivated potatoes? Botany 96:33–45.

Mawa M, Haque M, Saikat M & Islam S (2021) Screening of Salt Tolerant Potato Genotypes using Salt Stress and Molecular Markers. Int. j. plant soil sci. 49-56 https://doi.org/10.9734/ijpss/2021/v33i1630522

Maxted N, Kell S, Ford-Lloyd B, *et al* (2012) Toward the Systematic Conservation of Global Crop Wild Relative Diversity. Crop Sci 52:774–785.

McClure B (2004) S-RNase and SLF determine S-haplotype-specific pollen recognition and rejection. *The Plant cell*, *16*(11), 2840–2847.

Mcclure B (2009) Darwin's foundation for investigating self-incompatibility and the progress toward a physiological model for S-RNase-based SI. J Exp Bot 60:1069–1081. https://doi.org/10.1093/JXB/ERP024

McClure B, Cruz-García F, Romero C (2011) Compatibility and incompatibility in S-RNase-based systems. Ann Bot 108:647–658.

McClure B, Mou B, Canevascini S, Bernatzky R (1999) A small asparagine-rich protein required for S-allele-specific pollen rejection in *Nicotiana*. Proc Natl Acad Sci U S A 96:13548–13553. https://doi.org/10.1073/PNAS.96.23.13548

McClure BA, Gray JE, Anderson MA, Clarke AE (1990) Self-incompatibility in Nicotiana alata involves degradation of pollen rRNA. Nat 1990 3476295 347:757–760. https://doi.org/10.1038/347757a0

McClure BA, Haring V, Ebert PR, *et al* (1989) Style self-incompatibility gene products of Nicotiana alata are ribonucleases. Nature 342:955–957. https://doi.org/10.1038/342955A0

McCormac AC, Wu H, Bao M, *et al* (1998) The use of visual marker genes as cell-specific reporters of *Agrobacterium*-mediated T-DNA delivery to wheat (*Triticum aestivum L.*) and barley (*Hordeum vulgare L.*). Euphytica 99:17–25.

McCormick S (1998) Self-incompatibility and other pollen-pistil interactions. Curr Opin Plant Biol 1:18–25. https://doi.org/10.1016/S1369-5266(98)80122-2

McCouch SR, Teytelman L, Xu Y, *et al* (2002) Development and Mapping of 2240 New SSR Markers for Rice (*Oryza sativa L.*). DNA Res 9:199–207. https://doi.org/10.1093/DNARES/9.6.199

McCubbin A (2008) Heteromorphic self-incompatibility in Primula: Twenty-first century tools promise to unravel a classic nineteenth century model system. Self-Incompatibility Flower Plants Evol Divers Mech 289–308. https://doi.org/10.1007/978-3-540-68486-2_14/COVER

McCubbin AG, Kao TH (2000) Molecular recognition and response in pollen and pistil interactions. Annu Rev Cell Dev Biol 16:333–364.

McDowell LR. 2nd ed. Amsterdam: Elsevier Science; 2003. Minerals in Animal And Human Nutrition; p. 660.

Mellor F and Stace-Smith R (1987) "Virus-Free Potatoes Through Meristem Culture," in Biotechnology in Agriculture and Forestry, Vol. 3., B. Y. P. S. (eds), Ed. Berlin: Springer, 1987, pp. 30-39.

Mendiburu AO, Peloquin SJ (1977) Bilateral sexual polyploidization in potatoes. Euphytica 26:573–583. https://doi.org/10.1007/BF00021683

Mendoza HA, Mihovilovich EJ, Saguma F (1996) Identification of triplex (YYYy) potato *vir*us Y (PVY) immune progenitors derived from *Solanum tuberosum* ssp. *andigena*. Am Potato J 73:13–19.

Meng JF, Xu TF, Wang ZZ, *et al* (2014) The ameliorative effects of exogenous melatonin on grape cuttings under water-deficient stress: Antioxidant metabolites, leaf anatomy, and chloroplast morphology. J Pineal Res 57:200–212. https://doi.org/10.1111/JPI.12159

Millam S (2006) Potato (*Solanum tuberosum* L.). Methods Mol Biol 344:25–36. https://doi.org/10.1385/1-59745-131-2:25

Mishra T, Raigond P, Thakur N, *et al* (2020) Recent Updates on Healthy Phytoconstituents in Potato: a Nutritional Depository. Potato Res 63:323–343.

Misra AN, Sahu SM, Misra M, *et al* (1997) Sodium chloride induced changes in leaf growth, and pigment and protein contents in two rice cultivars. Biol Plant 1997 392 39:257–262. https://doi.org/10.1023/A:1000357323205

Mohapatra PP, Batra VK (2017) Tissue Culture of Potato (*Solanum tuberosum L.*): A Review. IntJCurrMicrobiolAppSci 6:489–495.

Mohr SE, Hu Y, Ewen-Campen B, *et al* (2016) CRISPR guide RNA design for research applications. Febs J 283:3232.

Molassiotis A, Sotiropoulos T, Tanou G, *et al* (2006) Boron-induced oxidative damage and antioxidant and nucleolytic responses in shoot tips culture of the apple rootstock EM 9 (Malus domestica Borkh). Environ Exp Bot 56:54–62. https://doi.org/10.1016/J.ENVEXPBOT.2005.01.002

Mollika SR, Sarker R, Hoque M (2020) *In vitro* Regeneration and *Agrobacterium*-mediated Genetic Transformation of a Cultivated Potato Variety Using Marker Genes. Plant Tissue Cult Biotechnol 30:149–160. https://doi.org/10.3329/ptcb.v30i1.47800

Morales-Fernández SD, Mora-Aguilar R, Salinas-Moreno Y, *et al* (2018) Growth and sugar content of potato tubers in four maturity stages under greenhouse conditions. Rev Chapingo, Ser Hortic 24:53–67.

Morris WL, Taylor MA (2017) The Solanaceous vegetable crops: potato, tomato, pepper and egg plant. Life sciences: Encylopedia of applied plant sciences; 2(3): 55-58.

Morton MJL, Awlia M, Al-Tamimi N, *et al* (2019) Salt stress under the scalpel – dissecting the genetics of salt tolerance. Plant J 97:148–163. https://doi.org/10.1111/TPJ.14189

Munns R (2002) Comparative physiology of salt and water stress. Plant, Cell Environ 25:239–250. https://doi.org/10.1046/J.0016-8025.2001.00808.X

Munns R, Husain S, Rivelli AR, *et al* (2002) Avenues for increasing salt tolerance of crops, and the role of physiologically based selection traits. Prog Plant Nutr Plenary Lect XIV Int Plant Nutr Colloq 93–105.

Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annu Rev Plant Biol 59:651–681. https://doi.org/10.1146/ANNUREV.ARPLANT.59.032607.092911

Murashige T, Skoog F (1962) A Revised Medium for Rapid Growth and Bioassays with Tobacco

Tissue Cultures. Physiol Plant 15:473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Muratova OA, Beketova MP, Kuznetsova MA, *et al* (2020) South American species *Solanum* alandiae Card. And S. *Okadae* Hawkes et Hjerting as potential sources of genes for potato late blight resistance. Proc Appl Bot Genet Breed 181:73–83. https://doi.org/10.30901/2227-8834-2020-1-73-83

Murch SJ, Erland LAE (2021) A Systematic Review of Melatonin in Plants: An Example of Evolution of Literature. Front Plant Sci 12:1060. https://doi.org/10.3389/FPLS.2021.683047/BIBTEX

Murshed R, Najla S, Albiski F, *et al* (2015) Using Growth Parameters for In-vitro Screening of Potato Varieties Tolerant to Salt Stress. J Agric Sci Technol 17:483–494

Mushinskiy AA, Aminova E V., Fedotova LS, Dergileva TT (2021) Evaluation of potato tubers of Nevsky variety and selection hybrids by amino acid composition. IOP Conf Ser Earth Environ Sci 624:012155.

Muthoni J, Kabira J (2014) Multiplication of seed potatoes in a conventional potato breeding programme: A case of Kenya's national potato programme. AJCS 8:1195–1199

Nadakuduti SS, Buell CR, Voytas DF, *et al* (2018) Genome Editing for Crop Improvement - Applications in Clonally Propagated Polyploids With a Focus on Potato (*Solanum tuberosum L.*). Front Plant Sci 9.

Nadakuduti SS, Starker CG, Voytas DF, *et al* (2019) Genome editing in potato with CRISPR/Cas9. Methods Mol Biol 1917:183–201.

Nadolska-Orczyk A, Pietrusinska A, Binka-Wyrwa A, *et al* (2007) Diploid potato (*Solanum tuberosum L.*) as a model crop to study transgene expression. Cell Mol Biol Lett 12:206–219.

Nadolska-Orczyk A, Pietrusinska A, Binka-Wyrwa A, *et al* (2007) Diploid potato (*Solanum tuberosum* L.) as a model crop to study transgene expression. Cell Mol Biol Lett 12:206–219.

Nahirñak V, Almasia NI, González MN, *et al* (2022) State of the Art of Genetic Engineering in Potato: From the First Report to Its Future Potential. Front Plant Sci 12:3181.

Nahirñak V, Rivarola M, Almasia NI, *et al* (2019) Snakin-1 affects reactive oxygen species and ascorbic acid levels and hormone balance in potato. PLoS One 14.

Naik P, Sarkar D (2000) *In vitro* approaches to propagation and conservation of genetic resources in potato. Malhotra Publishing House

Naik PS, Widholm JM (1993) Comparison of tissue culture and whole plant responses to salinity in potato. Plant Cell, Tissue Organ Cult 1993 333 33:273–280. https://doi.org/10.1007/BF02319012

Nam J, Matthysse AG, Gelvin SB (1997) Differences in susceptibility of arabidopsis ecotypes to crown gall disease may result from a deficiency in T-DNA integration. Plant Cell 9:317–333.

Nan GL, Tang CS, Kuehnle AR, Kado CI (1997) *Dendrobium* orchids contain an inducer of *Agrobacterium vir*ulence genes. Physiol Mol Plant Pathol 51:391–399.

Nandakumar R, Chen L, Rogers SMD (2005) *Agrobacterium*-mediated transformation of the wetland monocot *Typha latifolia L.* (*Broadleaf cattail*). Plant Cell Rep 23:744–750.

Nandy S, Pathak B, Zhao S, Srivastava V (2019) Heat-shock-inducible CRISPR/Cas9 system generates heritable mutations in rice. Plant Direct 3.

Narváez-Cuenca CE, Peña C, Restrepo-Sánchez LP, *et al* (2018) Macronutrient contents of potato genotype collections in the *Solanum tuberosum Group Phureja*. J Food Compos Anal 66:179–184. https://doi.org/10.1016/J.JFCA.2017.12.019

Nasrin S, Monzur M, Khatun A , Firoz Alam M and Mondal M. R. K. (2003) Induction and Evaluation of Somaclonal Variation in Potato (*Solanum tuberosum* L.). J Biol Sci 3:183–190. https://doi.org/10.3923/JBS.2003.183.190

Naumann M, Koch M, Thiel H, *et al* (2020) The Importance of Nutrient Management for Potato Production Part II: Plant Nutrition and Tuber Quality. Potato Res 63:121–137.

Naumkina EM, Bolyakina YP, Romanov GA (2007) Organ-specificity and inducibility of patatin class I promoter from potato in transgenic *Arabidopsis* plants. Russ J Plant Physiol 54:350–359.

Navarre DA, Brown CR, Sathuvalli VR (2019) Potato Vitamins, Minerals and Phytonutrients from a Plant Biology Perspective. Am J Potato Res 96:111–126.

Naveed M, Sajid H, Mustafa A, *et al* (2020a) Alleviation of salinity-induced oxidative stress, improvement in growth, physiology and mineral nutrition of canola (Brassica napus L.) through calcium-fortified composted animal manure. Sustain 12:. https://doi.org/10.3390/SU12030846

Nei M, Gojobori T (1986) Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol Biol Evol 3:418–426. https://doi.org/10.1093/OXFORDJOURNALS.MOLBEV.A040410

Nemudryi AA, Valetdinova KR, Medvedev SP, Zakian SM (2014) TALEN and CRISPR/Cas genome editing systems: Tools of discovery. Acta Naturae 6:19–40. https://doi.org/10.32607/20758251-2014-6-3-19-40

Neto ADDA, Prisco JT, Enéas-Filho J, *et al* (2006) Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. Environ Exp Bot 56:87–94.

Newell CA, Rozman R, Hinchee MA, *et al* (1991) *Agrobacterium*-mediated transformation of *Solanum tuberosum* L. cv. “Russet Burbank.” Plant Cell Rep 10:30–34.

Nielsen R, Yang Z (1998) Likelihood Models for Detecting Positively Selected Amino Acid Sites and Applications to the HIV-1 Envelope Gene. Genetics 148:929–936. https://doi.org/10.1093/GENETICS/148.3.929

Nikitin MM, Statsyuk N V., Frantsuzov PA, *et al* (2018) Matrix approach to the simultaneous detection of multiple potato pathogens by real-time PCR. J Appl Microbiol 124:797–809. https://doi.org/10.1111/JAM.13686

Novy, R. (2014). Traditional Breeding and Cultivar Development. In R. Navarre and M. Pavek (Eds.), The Potato Botany, Production and Uses (pp. 272–289). CABI.

Nuwagira F, Mukasa S, Wagoire W, *et al* (2016) Determination of hormonal combination for increased multiplication of tissue culture potato plantlets. Uganda J Agric Sci 16:129. https://doi.org/10.4314/UJAS.V16I1.11

O'Brien M, Kapfer C, Major G, *et al* (2002) Molecular analysis of the stylar-expressed *Solanum* chacoense small asparagine-rich protein family related to the HT modifier of gametophytic self-incompatibility in Nicotiana. Plant J 32:985–996. https://doi.org/10.1046/J.1365-313X.2002.01486.X

Ochoa CM, International Potato Center. (1990) The potatoes of South America. Bolivia. Cambridge University Press

ÖDEMİŞ B, ÇALIŞKAN ME (2014) Photosynthetic Response of Potato Plants to Soil Salinity. Turkish J Agric Nat Sci 1:1429–1439

Ohara-Takada A, Matsuura-Endo C, Chuda Y, *et al* (2005) Change in content of sugars and free amino acids in potato tubers under short-term storage at low temperature and the effect on acrylamide level after frying. Biosci Biotechnol Biochem 69:1232–1238.

Olmstead RG, Sweere A, Spangler R, *et al* (1999) Phylogeny and provisional classification of the Solanaceae based on chloroplast DNA. Adv Biol Util 111–137

Omidi M, Shahpiri A (2003) Callus induction and plant regeneration *in vitro* in potato. Acta Hortic 619:315–322. https://doi.org/10.17660/ACTAHORTIC.2003.619.36

Ooms G, Bossen ME, Burrell MM, Karp A (1986) Genetic manipulation in potato with *Agrobacterium rhizogenes*. Potato Res 29:367–379.

Ordon J, Bressan M, Kretschmer C, *et al* (2019) Optimized Cas9 expression systems for highly efficient *Arabidopsis* genome editing facilitate isolation of complex alleles in a single generation. Funct Integr Genomics 2019 201 20:151–162.

Othman MHA, Abido AIA, Jabal AAA (2016) *In vitro* Propagation and *Ex vitro* Acclimatization of Potato (*Solanum tuberosum L.*) Using Nodal Cutting Explants. J Adv Agric Res 21:108–137. https://doi.org/10.21608/JALEXU.2016.188577

Otroshy M. (2006) Utilization of tissue culture techniques in a seed potato tuber production scheme.

Owens LD, Cress DE (1985) Genotypic Variability of Soybean Response to *Agrobacterium* Strains harbouring the Ti or Ri Plasmids. Plant Physiol 77:87–94.

Paape T, Igic B, Smith SD, *et al* (2008) A 15-Myr-Old Genetic Bottleneck. Mol Biol Evol 25:655–663. https://doi.org/10.1093/MOLBEV/MSN016

Paape T, Kohn JR (2011) Differential strengths of selection on S-RNases from *Physalis* and *Solanum* (Solanaceae). BMC Evol Biol 11. https://doi.org/10.1186/1471-2148-11-243

Pandey KK (1962) Interspecific Incompatibility In *Solanum* Species. Am J Bot 49:874–882. https://doi.org/10.1002/J.1537-2197.1962.TB15023.X

Parida AK, Das AB (2005) Salt tolerance and salinity effects on plants: A review. Ecotoxicol Environ Saf 60:324–349. https://doi.org/10.1016/J.ECOENV.2004.06.010

Park HS, Kazerooni EA, Kang SM, *et al* (2021) Melatonin Enhances the Tolerance and Recovery Mechanisms in Brassica juncea (L.) Czern. Under Saline Conditions. Front Plant Sci 12:. https://doi.org/10.3389/FPLS.2021.593717

Park J, Bae S, Kim JS (2015) Cas-Designer: A web-based tool for choice of CRISPR-Cas9 target sites. Bioinformatics 31:4014–4016.

Parvin K, Hasanuzzaman M, Bhuyan MHMB, *et al* (2019) Comparative Physiological and Biochemical Changes in Tomato (*Solanum lycopersicum L.*) under Salt Stress and Recovery: Role of Antioxidant Defense and Glyoxalase Systems. Antioxidants 8:. https://doi.org/10.3390/ANTIOX8090350

Pawar KR, Wagh SG, Daspute AA, Avhad GB, Choudharee PD and Harke SN (2019) *In vitro* regeneration of potato (Kufri Pukhraj). J Pharmacogn Phytochem; 8(5):515–518

Peethambaran PK, Glenz R, Hoeninger S, *et al* (2018) Salt-inducible expression of OsJAZ8 improves resilience against salt-stress. BMC Plant Biol 18:. https://doi.org/10.1186/s12870-018-1521-0ï

Peloquin S, Werner EJ and GL Yerk (1991) The use of potato haploids in genetics and breeding. In: Chromosome Engineering in Plants: Genetics, Breeding, Evolution, Part B, Gupta PK and Tsuchiya T (Eds.), Elsevier, Amsterdam. p. 79.

Peters JM, Koo BM, Patino R, *et al* (2019) Enabling genetic analysis of diverse bacteria with Mobile-CRISPRi. Nat Microbiol 4:244–250. https://doi.org/10.1038/S41564-018-0327-Z

Pino MT, Skinner JS, Park EJ, *et al* (2007) Use of a stress inducible promoter to drive ectopic *AtCBF* expression improves potato freezing tolerance while minimizing negative effects on tuber yield. Plant Biotechnol J 5:591–604.

Plaut Z, Edelstein M, Ben-Hur M (2013) Overcoming Salinity Barriers to Crop Production Using Traditional Methods. Crit Rev Plant Sci 32:250–291.

Popp MW, Maquat LE (2016) Leveraging Rules of Nonsense-Mediated mRNA Decay for Genome Engineering and Personalized Medicine. Cell 165:1319–1322.

Postel S, Kemmerling B (2009) Plant systems for recognition of pathogen-associated molecular patterns. Semin Cell Dev Biol 20:1025–1031.

Pradel W, Gatto M, Hareau G, *et al* (2019) Adoption of potato varieties and their role for climate change adaptation in India. Clim Risk Manag 23:114–123.

Pradhan K, Rout S, Tripathy B, *et al* (2021) Role of Biotechnology in Vegetable Breeding. Turkish Online J Qual Inq 12:5092–5102

Prado de MORAIS T, Abreu ASMAR S, Fernando de Jesus SILVA H, *et al* (2007) Application Of Tissue Culture Techniques in Potato Aplicação Da Cultura De Tecidos Vegetais Em Batata. Rev Artic Biosci J 4:952–969

Puchta T-KHH, Schillberg S, Puchta THHÁ, Orzaez D (2021) Novel CRISPR/Cas applications in plants: from prime editing to chromosome engineering. Transgenic Res 30:529–549.

Purohit SD, Silva J, Habibi N (2011) Current Approaches for Cheaper and Better Micropropagation Technologies. Int J Plant Dev Biol 5:1–36

Qadir M, Quillérou E, Nangia V, *et al* (2014) Economics of salt-induced land degradation and restoration. Nat Resour Forum 38:282–295. https://doi.org/10.1111/1477-8947.12054

Qiao H, Wang H, Zhao L, *et al* (2004) The F-Box Protein AhSLF-S2 Physically Interacts with S-RNases That May Be Inhibited by the Ubiquitin/26S Proteasome Pathway of Protein Degradation during Compatible Pollination in *Antirrhinum*. Plant Cell 16:582–595. https://doi.org/10.1105/TPC.017673

Qiu QS, Guo Y, Dietrich MA, *et al* (2002) Regulation of SOS1, a plasma membrane Na+/H+ exchanger in Arabidopsis thaliana, by SOS2 and SOS3. Proc Natl Acad Sci U S A 99:8436–8441. https://doi.org/10.1073/PNAS.122224699/ASSET/3687C739-6630-4E8E-B2EB-63918CF8733B/ASSETS/GRAPHIC/PQ1222246005.JPEG

Qiu QS, Guo Y, Quintero FJ, *et al* (2004) Regulation of Vacuolar Na+/H+ Exchange in Arabidopsis thaliana by the Salt-Overly-Sensitive (SOS) Pathway. J Biol Chem 279:207–215. https://doi.org/10.1074/JBC.M307982200

Quan R, Lin H, Mendoza I, *et al* (2007) SCABP8/CBL10, a putative calcium sensor, interacts with the protein kinase SOS2 to protect Arabidopsis shoots from salt stress. Plant Cell 19:1415–1431. https://doi.org/10.1105/TPC.106.042291

Queirós F, Fontes N, Silva P, *et al* (2009) Activity of tonoplast proton pumps and Na^+/H^+ exchange in potato cell cultures is modulated by salt. J Exp Bot 60:1363–1374.

Ragavendran C, Natarajan D (2017) Role of plant tissue culture for improving the food security in India: A review update. Sustain Agric Towar Food Secur 231–262. https://doi.org/10.1007/978-981-10-6647-4_13/COVER

Rahnama H, Ebrahimzadeh H (2004) The effect of NaCl on proline accumulation in potato seedlings and calli. Acta Physiol Plant 26:263–270. https://doi.org/10.1007/S11738-004-0016-9

Rahnama H, Ebrahimzadeh H (2005) The effect of NaCl on antioxidant enzyme activities in potato seedlings. Biol Plant 49:93–97. https://doi.org/10.1007/S10535-005-3097-4

Rahnama H, Vakilian H, Fahimi H, Ghareyazie B (2011) Enhanced salt stress tolerance in transgenic potato plants (*Solanum tuberosum* L.) expressing a bacterial mtlD gene. Acta Physiol Plant 2011 334 33:1521–1532. https://doi.org/10.1007/S11738-010-0690-8

Raigond P, Atkinson FS, Lal MK, *et al* (2020) Potato Carbohydrates. Potato 13–36. https://doi.org/10.1007/978-981-15-7662-1_2

Rakosy-Tican E, Aurori CM, Dijkstra C, *et al* (2007) The usefulness of the *gfp* reporter gene for monitoring *Agrobacterium*- mediated transformation of potato dihaploid and tetraploid genotypes. Plant Cell Rep 26:661–671.

Rana MM, Takamatsu T, Baslam M, *et al* (2019) Salt Tolerance Improvement in Rice through Efficient SNP Marker-Assisted Selection Coupled with Speed-Breeding. Int J Mol Sci 20:2585. https://doi.org/10.3390/IJMS20102585

Rangani J, Parida AK, Panda A, Kumari A (2016) Coordinated Changes in Antioxidative Enzymes Protect the Photosynthetic Machinery from Salinity Induced Oxidative Damage and Confer Salt Tolerance in an Extreme Halophyte Salvadora persica L. Front Plant Sci 7:. https://doi.org/10.3389/FPLS.2016.00050

Rashid MHO, Islam S, Bari M (2019) *In vitro* screening for salt stress tolerance of native and exotic potato genotypes by morphological and physiological parameters. J Bio-Science 28:21–32. https://doi.org/10.3329/JBS.V28I0.44707

Raspor M, Motyka V, Ninković S, *et al* (2020) Endogenous levels of cytokinins, indole-3-acetic acid and abscisic acid in *in vitro* grown potato: A contribution to potato hormonomics. Sci Rep 10:1–13.

Reguera M, Bassil E, Blumwald E (2014) Intracellular NHX-Type Cation/H + Antiporters in Plants Na + /H + ANTIPORTERS ARE REGULATORS OF pH AND ION HOMEOSTASIS. https://doi.org/10.1093/mp/sst091

Richardson KVA, Wetten AC, Caligari PDS (2001) Cell and nuclear degradation in root meristems following exposure of potatoes (*Solanum tuberosum* L.) to salinity. Potato Res 2001 444 44:389–399. https://doi.org/10.1007/BF02358598

Richman AD, Kohn JR (2000) Evolutionary genetics of self-incompatibility in the Solanaceae. Plant Mol Biol 42:169–179. https://doi.org/10.1023/A:1006336206637

Ricroch A, Clairand P, Harwood W (2017) Use of CRISPR systems in plant genome editing: Toward new opportunities in agriculture. Emerg Top Life Sci 1:169–182. https://doi.org/10.1042/ETLS20170085

Ritchie SW, Lui CN, Sellmer JC, *et al* (1993) *Agrobacterium tumefaciens*-mediated expression of gusA in maize tissues. Transgenic Res 2:252–265.

Ritter E, Barandalla L, López R, De Galarreta JIR (2008) Exploitation of exotic, cultivated *Solanum* germplasm for breeding and commercial purposes. Potato Res 51:301–311. https://doi.org/10.1007/S11540-008-9109-Y

Rivero M, Furman N, Mencacci N, *et al* (2012) Stacking of antimicrobial genes in potato transgenic plants confers increased resistance to bacterial and fungal pathogens. J Biotechnol 157:334–343.

Robbins TP, Harbord RM, Sonneveld T, Clarke K (2000) The Molecular Genetics of Self-incompatibility in *Petunia* hybrida. Ann Bot 85:105–112.

Rohela GK, Jogam P, Shabnam AA, *et al* (2018) *In vitro* regeneration and assessment of genetic fidelity of acclimated plantlets by using ISSR markers in PPR-1 (Morus sp.): An economically important plant. Sci Hortic (Amsterdam) 241:313–321.

Romano A, Raemakers K, Visser R, Mooibroek H (2001) Transformation of potato (*Solanum tuberosum*) using particle bombardment. Plant Cell Rep 20:198–204.

Rosell G, De Bertoldi FG, Tizio R (1987) *In vitro* mass tuberisation as a contribution to potato micropropagation. Potato Res 30:111–116. https://doi.org/10.1007/BF02357689

Ross H (1986) Potato breeding - problems and perspectives. Verlag Paul Parey, Berlin and Hamburg, Germany.

Rout GR, Palai SK, Samantaray S, Das P (2001) Effect of growth regulator and culture conditions on shoot multiplication and rhizome formation in ginger (*Zingiber officinale Rosc.*) *in vitro*. Vitr Cell Dev Biol - Plant 2001 376 37:814–819.

Ruchir Raman (2017) The impact of Genetically Modified (GM) crops in modern agriculture: A review, GM Crops & Food, 8:4, 195-208, DOI: 10.1080/21645698.2017.1413522

Saar-Reismaa P, Kotkas K, Rosenberg V, *et al* (2020) Analysis of Total Phenols, Sugars, and Mineral Elements in Colored Tubers of *Solanum tuberosum* L. Foods 9:. https://doi.org/10.3390/FOODS9121862

Sabeena Farvin KH, Grejsen HD, Jacobsen C (2012) Potato peel extract as a natural antioxidant in chilled storage of minced horse mackerel (*Trachurus trachurus*): Effect on lipid and protein oxidation. Food Chem 131:843–851.

Sabir P, Ashraf M, Hussain M, Jamil A (2009) Relationship Of Photosynthetic Pigments And Water Relations With Salt Tolerance Of Proso Millet (*Panicum miliaceum L.)* accessions. Pak J Bot 41:2957–2964

Sack L, John GP, Buckley TN (2018) ABA Accumulation in Dehydrating Leaves Is Associated with Decline in Cell Volume, Not Turgor Pressure. Plant Physiol 176:489. https://doi.org/10.1104/PP.17.01097

Sakai S (2016) How Have Self-Incompatibility Haplotypes Diversified? Generation of New Haplotypes during the Evolution of Self-Incompatibility from Self-Compatibility. Am Nat 188:163–174. https://doi.org/10.1086/687110

Salas M, Park S, Srivatanakul M, Smith R (2001) Temperature influence on stable T-DNA integration in plant cells. Plant Cell Rep 20:701–705.

Sanavy M & Moeini JM (2003) PTC Effects of Different pH Levels of Medium on Growth and Rooting of Single Nodes Resulted from Potato Meristem Culture. Plant Tissue Cult 13:151–154

Sanwal SK, Kumar P, Kesh H, *et al* (2022) Salinity Stress Tolerance in Potato Cultivars: Evidence from Physiological and Biochemical Traits. Plants 11:1842. https://doi.org/10.3390/PLANTS11141842

Sarker U, Islam MT, Oba S (2019) Salinity stress accelerates nutrients, dietary fiber, minerals, phytochemicals and antioxidant activity in Amaranthus tricolor leaves. PLoS One 13:. https://doi.org/10.1371/JOURNAL.PONE.0206388

Sarker U, Oba S (2018) Drought Stress Effects on Growth, ROS Markers, Compatible Solutes, Phenolics, Flavonoids, and Antioxidant Activity in Amaranthus tricolor. Appl Biochem Biotechnol 186:999–1016. https://doi.org/10.1007/S12010-018-2784-5

Sarker, R. H., & Mustafa, B. M. (2002) Regeneration and *Agrobacterium*-mediated genetic transformation of two indigenous potato varieties of Bangladesh. Plant Tissue Cult, 12(1), 69-77.

Sassa H, Nishio T, Kowyama Y, *et al* (1996) Self-incompatibility (S) alleles of the Rosaceae encode members of a distinct class of the T2/S ribonuclease superfamily. Mol Gen Genet 250:547–557. https://doi.org/10.1007/BF02174443

Sawahel WA (2002) The production of transgenic potato plants expressing human α-interferon using lipofectin-mediated transformation. Cell Mol Biol Lett 7:19–29

Schafleitner R, Gutierrez R, Espino R, *et al* (2007) Field Screening for Variation of Drought Tolerance in *Solanum tuberosum* L. by Agronomical, Physiological and Genetic Analysis. Potato Res 2007 501 50:71–85. https://doi.org/10.1007/S11540-007-9030-9

Sehgal N, Singh S (2018) Progress on deciphering the molecular aspects of cell-to-cell communication in Brassica self-incompatibility response. 3 Biotech 8:1–17. https://doi.org/10.1007/S13205-018-1372-2

Shah Z, Azra Q, Ghulam H, *et al* (2001) Meristem Culture of Potato (*Solanum tuberosum* L.) for Production of Virus-Free Plantlets. J Biol Sci 1:898–899.

Shan Q, Wang Y, Li J, *et al* (2013) Targeted genome modification of crop plants using a CRISPR-Cas system. Nat Biotechnol 2013 318 31:686–688. https://doi.org/10.1038/nbt.2650

Shao H, Chu L, Lu H, *et al* (2019) Towards sustainable agriculture for the salt-affected soil. L Degrad Dev 30:574–579. https://doi.org/10.1002/LDR.3218

Shao Q, Han N, Ding T, *et al* (2014) SsHKT1;1 is a potassium transporter of the C3 halophyte Suaeda salsa that is involved in salt tolerance. Funct Plant Biol 41:790–802. https://doi.org/10.1071/FP13265

Sharma DK, Singh A (2015) Salinity Research in India- Achievements, Challenges and Future Prospects. WATER Resour Sect WATER Resour Sect

Sharma S, Villamor JG, Verslues PE (2011) Essential Role of Tissue-Specific Proline Synthesis and Catabolism in Growth and Redox Balance at Low Water Potential. Plant Physiol 157:292. https://doi.org/10.1104/PP.111.183210

Shaw J. M. H (2007) A new hybrid genus for Calibrachoa × *Petunia*(Solanaceae). HANBURYANA

Shi J, Wang E, Milazzo JP, *et al* (2015) Discovery of cancer drug targets by CRISPR-Cas9 screening of protein domains. Nat Biotechnol 2015 336 33:661–667.

Shibata M, Nishimasu H, Kodera N, *et al* (2017) Real-space and real-Time dynamics of CRISPR-Cas9 visualized by high-speed atomic force microscopy. Nat Commun 8:.

Shirin, F., Hossain, M., Kabir, M. F., Roy, M., & Sarker, S. R. (2007) Callus induction and plant regeneration from internodal and leaf explants of four potato (*Solanum tuberosum L.*) cultivars. World J. Agric. Sci, 3(1), 01-06.

Shouzheng Lv, Xin Qiao, Wei Zhang, *et al* (2022) The Origin and Evolution of RNase T2 Family and Gametophytic Self-incompatibility System in Plants, *Genome Biology and Evolution*, 14(7).

Shuji Y, Bressan RA, Paul H (2002) Salt Stress Tolerance of Plants. JIRCAS Work. Rep. 25–33

Siddiqui MH, Al-Whaibi MH, Faisal M, Al Sahli AA (2014) Nano-silicon dioxide mitigates the adverse effects of salt stress on Cucurbita pepo L. Environ Toxicol Chem 33:2429–2437. https://doi.org/10.1002/ETC.2697

Sievers F, Higgins DG (2014) Clustal Omega. Curr Protoc Bioinforma 48:3.13.1-3.13.16. https://doi.org/10.1002/0471250953.BI0313S48

Sijacic P, Wang X, Skirpan AL, *et al* (2004) Identification of the pollen determinant of S-RNase-mediated self-incompatibility. Nature 429:302–305. https://doi.org/10.1038/NATURE02523

Sijacic P, Wang X, Skirpan AL, *et al* (2004) Identification of the pollen determinant of S-RNase-mediated self-incompatibility. Nature 429:302–305. https://doi.org/10.1038/NATURE02523

Silva NF, Goring DR (2001) Mechanisms of self-incompatibility in flowering plants. Cell Mol Life Sci 58:1988–2007. https://doi.org/10.1007/PL00000832

Silva-Ortega CO, Ochoa-Alfaro AE, Reyes-Agüero JA, *et al* (2008) Salt stress increases the expression of p5cs gene and induces proline accumulation in cactus pear. Plant Physiol Biochem PPB 46:82–92. https://doi.org/10.1016/J.PLAPHY.2007.10.011

Simonian MH, Smith JA (2006) Spectrophotometric and Colorimetric Determination of Protein Concentration. Curr Protoc Mol Biol 76:10.1.1-10.1A.9. https://doi.org/10.1002/0471142727.MB1001AS76

Sims TL, Ordanic M (2001) Identification of a S-ribonuclease-binding protein in *Petunia* hybrida. Plant Mol Biol 47:771–783. https://doi.org/10.1023/A:1013639528858

Singh P (2012) Comparative study of potato cultivation through micropropagation and conventional farming methods. AFRICAN J Biotechnol 11.

Sivakumar P, Sharmila P, Pardha Saradhi P (2000) Proline Alleviates Salt-Stress-Induced Enhancement in Ribulose-1,5-Bisphosphate Oxygenase Activity. Biochem Biophys Res Commun 279:512–515. https://doi.org/10.1006/BBRC.2000.4005

Skoog, F., and Miller, C.O. (1957) Chemical regulation of growth and organ formation in plant tissue cultures *in vitro*. Symp. Soc. Exp. Biol. 11, 118–131.

Slama I, Abdelly C, Bouchereau A, *et al* (2015) Diversity, distribution and roles of osmoprotective compounds accumulated in halophytes under abiotic stress. Ann Bot 115:433–447. https://doi.org/10.1093/AOB/MCU239

Smith MAL, Spomer LA, Shibli RA, Knight SL (2008) Effects of NaCl salinity on miniature dwarf tomato 'Micro-Tom': II. Shoot and root growth responses, fruit production, and osmotic adjustment 1. http://dx.doi.org/101080/01904169209364477 15:2329–2341. https://doi.org/10.1080/01904169209364477

Sonneveld T, Robbins TP, Bošković R, Tobutt KR (2001) Cloning of six cherry self-incompatibility alleles and development of allele-specific PCR detection. Theor Appl Genet 2001 1026 102:1046–1055. https://doi.org/10.1007/S001220000525

Sonnino A, Tanaka S, Iwanaga M, Schilde-Rentschler L (1989) Genetic control of embryo formation in anther culture of diploid potatoes. Plant Cell Rep 8:105–107.

Soomro UA, Zahoor-ul-Haq M, Gul S (2008) Effects of NaCl Salinity on Wheat (Triticum aestivum L.) Cultivars. World J Agric Sci 4:398–403

Soshinkova TN, Radyukina NL, Korolkova D V., Nosov A V. (2012) Proline and functioning of the antioxidant system in Thellungiella salsuginea plants and cultured cells subjected to oxidative stress. Russ J Plant Physiol 2013 601 60:41–54. https://doi.org/10.1134/S1021443713010093

Spooner DM, Bamberg JB (2008) Potato genetic resources: Sources of resistance and systematics. Am Potato J 71:325–337. https://doi.org/10.1007/BF02849059

Spooner DM, Ghislain M, Simon R, *et al* (2014) Systematics, Diversity, Genetics, and Evolution of Wild and Cultivated Potatoes. Bot Rev 80:283–383. https://doi.org/10.1007/S12229-014-9146-Y

Spooner DM, Salas A (2006) Chapter 1 Structure, Biosystematics, and Genetic Resources Structure, Biosystematics, and Genetic Resources. Handbook of Potato Production, Improvement, and Postharvest Management,. The Haworth Press, Inc

Srivastava AK, Diengdoh LC, Rai R, *et al* (2012) *In vitro* Micropropagation and Micro-tuberization Potential of Selected Potato Varieties. Indian J Hill Farming 25:

Srivatanakul M, Park SH, Salas MG, Smith RH (2001) Transformation parameters enhancing T-DNA expression in kenaf (*Hibiscus cannabinus*). J Plant Physiol 158:255–260.

Steinger T, Gilliand H, Hebeisen T (2014) Epidemiological analysis of risk factors for the spread of potato viruses in Switzerland. Ann Appl Biol 164:200–207. https://doi.org/10.1111/AAB.12096

Struik S, Wiersema P (1999) Seed Potato Technology. The Netherlands: Wageningen Academic Publishers.

Subramanian NK, White PJ, Broadley MR, Ramsay G (2011) The three-dimensional distribution of minerals in potato tubers. Ann Bot 107:681.

Subramanian NK, White PJ, Broadley MR, Ramsay G (2017) Variation in tuber mineral concentrations among accessions of *Solanum* species held in the Commonwealth Potato Collection. Genet Resour Crop Evol 2017 648 64:1927–1935. https://doi.org/10.1007/S10722-016-0483-Z

Sudhersan C, Jibi Manuel S, Ashkanani J, Al- Ajeel A (2012) *In vitro* screening of potato cultivars for salinity tolerance. Am J Sustain Agric 344

Sulli M, Mandolino G, Sturaro M, *et al* (2017) Molecular and biochemical characterization of a potato collection with contrasting tuber carotenoid content. PLoS One 12.

Sultana N, Ikeda T, Itoh R (1999) Effect of NaCl salinity on photosynthesis and dry matter accumulation in developing rice grains. Environ Exp Bot 42:211–220. https://doi.org/10.1016/S0098-8472(99)00035-0

Sun L, Williams JS, Li S, *et al* (2019) S-Locus F-Box Proteins Are Solely Responsible for S-RNase-Based Self-Incompatibility of *Petunia* Pollen. Plant Cell 30:2959–2972. https://doi.org/10.1105/TPC.18.00615

Sun P, Kao T hui (2013) Self-Incompatibility in *Petunia inflata*: The Relationship between a Self-Incompatibility Locus F-Box Protein and Its Non-Self S-RNases. Plant Cell 25:470. https://doi.org/10.1105/TPC.112.106294

Sun X, Hu Z, Chen R, *et al* (2015) Targeted mutagenesis in soybean using the CRISPR-Cas9 system. Sci Rep 5:. https://doi.org/10.1038/SREP10342

Sutherland BG, Tobutt KR, Robbins TP (2008) Trans-specific S-RNase and SFB alleles in *Prunus* self-incompatibility haplotypes. Mol Genet Genomics 279:95–106. https://doi.org/10.1007/S00438-007-0300-7

Szafrańska K, Reiter RJ, Posmyk MM (2016) Melatonin Application to Pisum sativum L. Seeds Positively Influences the Function of the Photosynthetic Apparatus in Growing Seedlings during Paraquat-Induced Oxidative Stress. Front Plant Sci 7:. https://doi.org/10.3389/FPLS.2016.01663

Tadele Z (2019) Orphan crops: their importance and the urgency of improvement. Planta 250:677–694. https://doi.org/10.1007/S00425-019-03210-6

Takahashi-Iñiguez T, García-Hernandez E, Arreguín-Espinosa R, Flores ME (2012) Role of vitamin B12 on methylmalonyl-CoA mutase activity. J Zhejiang Univ Sci B 13:423.

Takayama S, Isogai A (2005) Self-incompatibility in plants. Annu Rev Plant Biol 56:467–489. https://doi.org/10.1146/ANNUREV.ARPLANT.56.032604.144249

Tamura K, Stecher G, Kumar S (2021) MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Mol Biol Evol 38:3022–3027. https://doi.org/10.1093/MOLBEV/MSAB120

Tan DX, Manchester LC, Liu X, *et al* (2013) Mitochondria and chloroplasts as the original sites of melatonin synthesis: a hypothesis related to melatonin's primary function and evolution in eukaryotes. J Pineal Res 54:127–138.

Tan DX, Reiter RJ (2020) An evolutionary view of melatonin synthesis and metabolism related to its biological functions in plants. J Exp Bot 71:4677–4689. https://doi.org/10.1093/JXB/ERAA235

Tang H, Ren Z, Krczal G, Glimelius K (2000) An evaluation of antibiotics for the elimination of *Agrobacterium tumefaciens* from walnut somatic embryos and for the effects on the proliferation of somatic embryos and regeneration of transgenic plants. Plant Cell Rep 19:881–887.

Tavakkoli E, Rengasamy P, McDonald GK (2010) High concentrations of Na+ and Cl- ions in soil solution have simultaneous detrimental effects on growth of faba bean under salinity stress. J Exp Bot 61:4449–4459. https://doi.org/10.1093/JXB/ERQ251

Taylor TE, Veilleux RE (1992) Inheritance of competencies for leaf disc regeneration, anther culture, and protoplast culture in *Solanum Phureja* and corrleations among them. Plant Cell, Tissue Organ Cult 31:95–103.

Tedone L, Hancock RD, Alberino S, *et al* (2004) Long-distance transport of L-ascorbic acid in potato. BMC Plant Biol 4:1–32. https://doi.org/10.1186/1471-2229-4-16

Tegen H, Mohammed W (2016) The Role of Plant Tissue Culture to Supply Disease Free Planting Materials of Major Horticultural Crops in Ethiopia. J Biol Agric Healthc 6:122–129

Terao M, Romão MJ, Leimkühler S, *et al* (2016) Structure and function of mammalian aldehyde oxidases. Arch Toxicol 90:753–780. https://doi.org/10.1007/S00204-016-1683-1

The Potato Genome Sequencing Consortium. Genome sequence and analysis of the tuber crop potato. Nature 475, 189–195 (2011). https://doi.org/10.1038/nature10158

Thompson KF, Taylor JP (1966) Non-linear dominance relationships between S alleles. Hered 21:345–362. https://doi.org/10.1038/hdy.1966.36

Tittal M, Mir RA, Jatav KS, Agarwal RM (2021) Supplementation of potassium alleviates water stress-induced changes in Sorghum bicolor L. Physiol Plant 172:1149–1161. https://doi.org/10.1111/PPL.13306

Tiwari RK, Lal MK, Naga KC, *et al* (2020) Emerging roles of melatonin in mitigating abiotic and biotic stresses of horticultural crops. Sci Hortic (Amsterdam) 272:109592.

Toevs EA, Guenthner JF (Joseph F 1951-, Johnson AJ, *et al* (2011) An Industry Perspective of All-native and Transgenic Potatoes. AgBioForum 14:14–19

Tomato Genome Consortium (2012). The tomato genome sequence provides insights into fleshy fruit evolution. *Nature*, *485*(7400), 635–641. https://doi.org/10.1038/nature11119

Tsoka O, Demo P, Ngamau (2012) Potato seed tuber production from *in vitro* and apical stem cutting under aeroponic system. African J Biotechnol 11:12612–12618. https://doi.org/10.5897/AJB10.1048

Tudela JA, Espín JC, Gil MI (2002) Vitamin C retention in fresh-cut potatoes. Postharvest Biol Technol 26:75–84. https://doi.org/10.1016/S0925-5214(02)00002-9

Tuteja N (2007) Mechanisms of high salinity tolerance in plants. Methods Enzymol 428:419–438. https://doi.org/10.1016/S0076-6879(07)28024-3

Tyree MT, Engelbrecht BMJ, Vargas G, Kursar TA (2003) Desiccation tolerance of five tropical seedlings in Panama. Relationship to a field assessment of drought performance. Plant Physiol 132:1439–1447. https://doi.org/10.1104/PP.102.018937

Ushijima K, Sassa H, Dandekar AM, *et al* (2003) Structural and transcriptional analysis of the self-incompatibility locus of almond: identification of a pollen-expressed F-box gene with haplotype-specific polymorphism. Plant Cell 15:771–781. https://doi.org/10.1105/TPC.009290

Ushijima K, Yamane H, Watari A, *et al* (2004) The S haplotype-specific F-box protein gene, SFB, is defective in self-compatible haplotypes of Prunus avium and P. mume. Plant J 39:573–586. https://doi.org/10.1111/J.1365-313X.2004.02154.X

Vafadar F, Amooaghaie R, Ehsanzadeh P, *et al* (2020) Crosstalk between melatonin and Ca 2+/CaM evokes systemic salt tolerance in Dracocephalum kotschyi. J Plant Physiol 252:. https://doi.org/10.1016/J.JPLPH.2020.153237

Vaitkevičienė N (2019) A comparative study on proximate and mineral composition of coloured potato peel and flesh. J Sci Food Agric 99:6227–6233.

Valkov VT, Gargano D, Manna C, *et al* (2011) High efficiency plastid transformation in potato and regulation of transgene expression in leaves and tubers by alternative 5′ and 3′ regulatory sequences. Transgenic Res 20:137–151.

Van Der Fits L, Deakin EA, Hoge JHC, Memelink J (2000) The ternary transformation system: Constitutive *vir*G on a compatible plasmid dramatically increases *Agrobacterium*-mediated plant transformation. Plant Mol Biol 43:495–502.

Van Eck J (2018) Genome editing and plant transformation of solanaceous food crops. Curr Opin Biotechnol 49:35–41.

van Roekel JSC, Damm B, Melchers LS, Hoekema A (1993) Factors influencing transformation frequency of tomato (*Lycopersicon esculentum*). Plant Cell Rep 12:644–647.

Vandenkoornhuyse P, Quaiser A, Duhamel M, *et al* (2015) The importance of the microbiome of the plant holobiont. New Phytol 206:1196–1206. https://doi.org/10.1111/NPH.13312

Veeranagamallaiah G, Chandraobulreddy P, Jyothsnakumari G, Sudhakar C (2007) Glutamine synthetase expression and pyrroline-5-carboxylate reductase activity influence proline accumulation in two cultivars of foxtail millet (*Setaria italica L.*) with differential salt sensitivity. Environ Exp Bot 60:239–244.

Veillet F, Kermarrec M-P, Chauvin L, *et al* (2020) Prime editing is achievable in the tetraploid potato, but needs improvement. bioRxiv 2020.06.18.159111.

Veillet F, Perrot L, Chauvin L, *et al* (2019) Transgene-Free Genome Editing in Tomato and Potato Plants Using Agrobacterium-Mediated Delivery of a CRISPR/Cas9 Cytidine Base Editor. Int J Mol Sci 20:. https://doi.org/10.3390/IJMS20020402

Velásquez B, Balzarini M, Taleisnik E (2005) Salt tolerance variability amongst Argentine Andean potatoes (*Solanum tuberosum L. subsp.andigena*). Potato Res 2005 481 48:59–67. https://doi.org/10.1007/BF02733682

Venezia M, Creasey Krainer KM (2021) Current Advancements and Limitations of Gene Editing in Orphan Crops. Front Plant Sci 12:1987.

Verhees J, Van der Krol AR, Vreugdenhil D, Van Der Plas LHW (2002) Characterization of gene expression during potato tuber development in individuals and populations using the luciferase reporter system. Plant Mol Biol 50:653–665.

Vieira J, Morales-Hojas R, Santos RAM, Vieira CP (2007) Different positively selected sites at the gametophytic self-incompatibility pistil S-RNase gene in the Solanaceae and Rosaceae (*Prunus, Pyrus,* and *Malus*). J Mol Evol 65:175–185. https://doi.org/10.1007/S00239-006-0285-6

Villemont E, Dubois F, Sangwan RS, *et al* (1997) Role of the host cell cycle in the *Agrobacterium*-mediated genetic transformation of *Petunia*: Evidence of an S-phase control mechanism for T-DNA transfer. Planta 201:160–172.

Visser RGF (1991) Regeneration and transformation of potato by *Agrobacterium tumefaciens*. In K Lindsey, ed, Plant Tissue Culture Manual B5. Dordrecht, The Netherlands, pp 1–9.

Vitart V, Baxter I, Doerner P, Harper JF (2001) Evidence for a role in growth and salt resistance of a plasma membrane H+-ATPase in the root endodermis. Plant J 27:191–201. https://doi.org/10.1046/J.1365-313X.2001.01081.X

von Wettberg E, Davis TM, Smýkal P (2020) Editorial: Wild Plants as Source of New Crops. Front Plant Sci 11. https://doi.org/10.3389/FPLS.2020.591554

Wahid A, Perveen M, Gelani S, Basra SMA (2007) Pretreatment of seed with H_2O_2 improves salt tolerance of wheat seedlings by alleviation of oxidative damage and expression of stress proteins. J Plant Physiol 164:283–294.

Waltz E (2012) Tiptoeing around transgenics. Nat Biotechnol 30:215–217. https://doi.org/10.1038/NBT.2143

Wang L, Lin Z, Triviño M, *et al* (2019) Self-incompatibility in *Papaver* pollen: programmed cell death in an acidic environment. J Exp Bot 70:2113–2123.

Wang L, Wang L, Tan Q, *et al* (2016) Efficient Inactivation of Symbiotic Nitrogen Fixation Related Genes in *Lotus japonicus* Using CRISPR-Cas9. Front Plant Sci 7.

Wang LY, Liu JL, Wang WX, Sun Y (2015) Exogenous melatonin improves growth and photosynthetic capacity of cucumber under salinity-induced stress. Photosynth 2016 541 54:19–27. https://doi.org/10.1007/S11099-015-0140-3

Wang P, Yin L, Liang D, *et al* (2012) Delayed senescence of apple leaves by exogenous melatonin treatment: toward regulating the ascorbate–glutathione cycle. J Pineal Res 53:11–20.

Wang T, Wei JJ, Sabatini DM, Lander ES (2014) Genetic screens in human cells using the CRISPR-Cas9 system. Science 343:80–84.

Wang W, Vinocur B, Altman A (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta 2003 2181 218:1–14. https://doi.org/10.1007/S00425-003-1105-5

Wang W, Xu Y, Chen TX, *et al* (2019) Regulatory mechanisms underlying the maintenance of homeostasis in Pyropia haitanensis under hypersaline stress conditions. Sci Total Environ 662:168–179. https://doi.org/10.1016/J.SCITOTENV.2019.01.214

Wang X, Hughes AL, Tsukamoto T, *et al* (2001) Evidence that intragenic recombination contributes to allelic diversity of the S-RNase gene at the self-incompatibility (S) locus in *Petunia inflata*. Plant Physiol 125:1012–1022. https://doi.org/10.1104/PP.125.2.1012

Wang Y, Branicky R, Noë A, Hekimi S (2018) Superoxide dismutases: Dual roles in controlling ROS damage and regulating ROS signaling. J Cell Biol 217:1915. https://doi.org/10.1083/JCB.201708007

Wang Y, Nii N (2015) Changes in chlorophyll, ribulose bisphosphate carboxylase-oxygenase, glycine betaine content, photosynthesis and transpiration in Amaranthus tricolor leaves during salt stress. http://dx.doi.org/101080/14620316200011511297 75:623–627. https://doi.org/10.1080/14620316.2000.11511297

Wang Y, Tsukamoto T, Yi KW, *et al* (2004) Chromosome walking in the *Petunia inflata* self-incompatibility (S-) locus and gene identification in an 881-kb contig containing S2-RNase. Plant Mol Biol 54:727–742.

Wang Y, Tsukamoto T, Yi KW, *et al* (2004) Chromosome walking in the *Petunia inflata* self-incompatibility (S-) locus and gene identification in an 881-kb contig containing S2-RNase. Plant Mol Biol 54:727–742.

Wang Y, Wang X, McCubbin AG, Kao TH (2003) Genetic mapping and molecular characterization of the self-incompatibility (S) locus in *Petunia inflata*. Plant Mol Biol 53:565–580. https://doi.org/10.1023/B:PLAN.0000019068.00034.09

Wang ZP, Xing HL, Dong L, *et al* (2015) Egg cell-specific promoter-controlled CRISPR/Cas9 efficiently generates homozygous mutants for multiple target genes in *Arabidopsis* in a single generation. Genome Biol 16:1–12.

Watanabe F, Yabuta Y, Bito T, Teng F (2014) Vitamin B_{12}-containing plant food sources for vegetarians. Nutrients 6:1861–1873.

Watanabe K, Oda-Yamamizo C, Sage-Ono K, *et al* (2018) Alteration of flower colour in Ipomoea nil through CRISPR/Cas9-mediated mutagenesis of carotenoid cleavage dioxygenase 4. Transgenic Res 27:25–38.

Watanabe KN, Kikuchi A, Shimazaki T, Asahina M (2011) Salt and Drought Stress Tolerances in Transgenic Potatoes and Wild Species. Potato Res 54:319–324.

Watanabe M, Suzuki G, Takayama S (2008) Milestones identifying self-incompatibility genes in *Brassica* species: From old stories to new findings. Self-Incompatibility Flower Plants Evol Divers Mech 151–172.

Wei B, Jing R, Wang C, *et al* (2008) Dreb1 genes in wheat (*Triticum aestivum L.*): development of functional markers and gene mapping based on SNPs. Mol Breed 23:13–22. https://doi.org/10.1007/S11032-008-9209-Z

Wei W, Li QT, Chu YN, *et al* (2015) Melatonin enhances plant growth and abiotic stress tolerance in soybean plants. J Exp Bot 66:695. https://doi.org/10.1093/JXB/ERU392

Weiland JJ (2003) Transformation of *Pythium aphanidermatum* to geneticin resistance. Curr Genet 42:344–352.

Weiss E (2015) "'Beginnings of Fruit Growing in the Old World'" two generations later. Isr J Plant Sci 62:75–85. https://doi.org/10.1080/07929978.2015.1007718

Westcott RJ, Henshaw GG, Roca WM (1977) Tissue culture storage of potato germplasm: Culture initiation and plant regeneration. Plant Sci Lett 9:309–315. https://doi.org/10.1016/0304-4211(77)90101-8

White PJ, Bradshaw JE, Dale MFB, *et al* (2009) Relationships Between Yield and Mineral Concentrations in Potato Tubers. HortScience 44:6–11.

White PJ, Broadley MR (2009) Biofortification of crops with seven mineral elements often lacking in human diets – iron, zinc, copper, calcium, magnesium, selenium and iodine. New Phytol 182:49–84.

Wichrowska D, Szczepanek M (2020) Possibility of limiting mineral fertilization in potato cultivation by using bio-fertilizer and its influence on protein content in potato tubers. Agric 10:1–16.

Wilf P, Carvalho MR, Gandolfo MA, Cúneo NR (2017) Eocene lantern fruits from Gondwanan Patagonia and the early origins of Solanaceae. Science (80-) 355.

Williams JS, Der JP, dePamphilis CW, Kao TH (2014) Transcriptome Analysis Reveals the Same 17 S-Locus F-Box Genes in Two Haplotypes of the Self-Incompatibility Locus of *Petunia inflata*. Plant Cell 26:2873–2888.

Wong N, Liu W, Wang X (2015) WU-CRISPR: Characteristics of functional guide RNAs for the CRISPR/Cas9 system. Genome Biol 16:. https://doi.org/10.1186/S13059-015-0784-0

Wszelaki AL, Delwiche JF, Walker SD, *et al* (2005) Sensory quality and mineral and glycoalkaloid concentrations in organically and conventionally grown redskin potatoes (*Solanum tuberosum*). J Sci Food Agric 85:720–726.

Wu G (2017) Principles of Animal Nutrition. Princ Anim Nutr. https://doi.org/10.1201/9781315120065/PRINCIPLES-ANIMAL-NUTRITION-GUOYAO-WU

Wu H, Shabala L, Zhou M, Shabala S (2015) Chloroplast-generated ROS dominate NaCl(-) induced K(+) efflux in wheat leaf mesophyll. Plant Signal Behav 10:1–4. https://doi.org/10.1080/15592324.2015.1013793

Wu H, Zhang X, Giraldo JP, Shabala S (2018) It is not all about sodium: revealing tissue specificity and signalling roles of potassium in plant responses to salt stress. Plant Soil 431:1–17. https://doi.org/10.1007/S11104-018-3770-Y/TABLES/3

Wu L, Williams JS, Sun L, Kao TH (2020) Sequence analysis of the *Petunia inflata* S-locus region containing 17 S-Locus F-Box genes and the S-RNase gene involved in self-incompatibility. Plant J 104:1348–1368. https://doi.org/10.1111/TPJ.15005

Wu Y, Hu H, Dai X, *et al* (2020) Comparative Study of the Nutritional Properties of 67 Potato Cultivars (*Solanum tuberosum L.*) Grown in China Using the Nutrient-Rich Foods (NRF 11.3) Index. Plant Foods Hum Nutr 75:169–176.

Xie K, Minkenberg B, Yang Y (2014) Targeted Gene Mutation in Rice Using a CRISPR-Cas9 System. BIO-PROTOCOL 4:. https://doi.org/10.21769/BIOPROTOC.1225

Xing HL, Dong L, Wang ZP, *et al* (2014) A CRISPR/Cas9 toolkit for multiplex genome editing in plants. BMC Plant Biol 14:. https://doi.org/10.1186/S12870-014-0327-Y

Xu C, Li M, Wu J, *et al* (2013) Identification of a canonical SCF(SLF) complex involved in S-RNase-based self-incompatibility of *Pyrus* (Rosaceae). Plant Mol Biol 81:245–257.

Xu X, Pan S, Cheng S, *et al* (2011) Genome sequence and analysis of the tuber crop potato. Nature 475:189–195.

Xue Y, Carpenter R, Dickinson HG, Coen ES (1996) Origin of allelic diversity in *Antirrhinum* S locus RNases. Plant Cell 8:805–814.

Yang Y, Achaerandio I, Pujolà M (2016) Classification of potato cultivars to establish their

processing aptitude. J Sci Food Agric 96:413–421.

Yang Y, Shi R, Wei X, *et al* (2010) Effect of salinity on antioxidant enzymes in calli of the halophyte *Nitraria tangutorum Bobr*. Plant Cell, Tissue Organ Cult 2010 1023 102:387–395. https://doi.org/10.1007/S11240-010-9745-1

Yang Z, Bielawski JR (2000) Statistical methods for detecting molecular adaptation. Trends Ecol Evol 15:496–503

Yasmin S, Nasiruddin KM, Begum R, Talukder SK (2003) Regeneration and Establishment of Potato Plantlets through Callus Formation with BAP and NAA. Asian J Plant Sci 2:936–940.

Yazici I, Türkan I, Sekmen AH, Demiral T (2007) Salinity tolerance of purslane (*Portulaca oleracea L.*) is achieved by enhanced antioxidative system, lower level of lipid peroxidation and proline accumulation. Environ Exp Bot 61:49–57.

Ye M, Peng Z, Tang D, *et al* (2018) Generation of self-compatible diploid potato by knockout of S-RNase. Nat Plants 4:651–654. https://doi.org/10.1038/S41477-018-0218-6

Yermishin, A.P.; Voronkova, E.V.; Kozlov, V.A.; Polyuhovich, Y.V.; Luksha, V.I.; Levy, A.V.; Yakovleva, G.A.; Semanyuk, T.V.; Dubinich, V.L.; Rodkina, I.A. *Interspecific Hybridization in Potato Breeding*; Yermishin, A.P., Ed.; Institute of Genetics and Cytology NAS of Belarus: Minsk, Belarus, 2021; pp. 1–396. (In Belarus)

Yevtushenko DP, Sidorov VA, Romero R, *et al* (2004) Wound-inducible promoter from poplar is responsive to fungal infection in transgenic potato. Plant Sci 167:715–724.

Yokoi Shuji, Ray A. Bressan, Hasegawa Paul (2002) Salt Stress Tolerance of Plants. In: JIRCAS Work. Rep. https://www.academia.edu/27236817/Salt_Stress_Tolerance_of_Plants. Accessed 23 Jul 2022

Yousef, A. A. R., Suwwan, M. A., Al-Musa, A. M., & Abu-Qaoud, H. A. (1997) *In vitro* Culture and Microtuberization of 'Spunta' Potato (*Solanum tuberosum L.*). Dirasat Agricultural Sciences, 24, 173-181.

Yu Y, Wang A, Li X, *et al* (2018) Melatonin-stimulated triacylglycerol breakdown and energy turnover under salinity stress contributes to the maintenance of plasma membrane H+ –ATPase activity and K+/Na+ homeostasis in sweet potato. Front Plant Sci 9:256. https://doi.org/10.3389/FPLS.2018.00256/BIBTEX

Yuan F, Leng B, Wang B (2016) Progress in Studying Salt Secretion from the Salt Glands in Recretohalophytes: How Do Plants Secrete Salt? Front Plant Sci 7:. https://doi.org/10.3389/FPLS.2016.00977

Yuan H, Meng D, Gu Z, *et al* (2014) A novel gene, MdSSK1, as a component of the SCF complex rather than MdSBP1 can mediate the ubiquitination of S-RNase in apple. J Exp Bot 65:3121–3131. https://doi.org/10.1093/JXB/ERU164

Zaki HEM, Radwan KSA (2022) The use of osmoregulators and antioxidants to mitigate the adverse impacts of salinity stress in diploid and tetraploid potato genotypes (*Solanum* spp.).

Chem Biol Technol Agric 9:1–21. https://doi.org/10.1186/S40538-022-00286-3/TABLES/6

Zaki HEM, Yokoi S (2016) A comparative *in vitro* study of salt tolerance in cultivated tomato and related wild species. Plant Biotechnol (Tokyo, Japan) 33:361–372. https://doi.org/10.5511/PLANTBIOTECHNOLOGY.16.1006A

Zaman MM, Mannan MA, Samanta SC (2015) Salt Induced Changes in Dry Matter Accumulation and Yield of Mustard *(Brassica juncea*). Bangladesh Agron J 18:65–70. https://doi.org/10.3329/BAJ.V18I1.25569

Zaman MS, Ali GM, Muhammad A, *et al* (2015) *In vitro* Screening of Salt Tolerance in Potato (*Solanum tuberosum L.*) Varieties. Sarhad J Agric 31:106–113.

Zaman QU, Li C, Cheng H, Hu Q (2019) Genome editing opens a new era of genetic improvement in polyploid crops. Crop J 7:141–150.

Zamani Z, Amiri H, Ismaili A (2020) Improving drought stress tolerance in fenugreek *(Trigonella foenum-graecum*) by exogenous melatonin. Plant Biosyst 154:643–655.

Zambre M, Terryn N, De Clercq J, *et al* (2003) Light strongly promotes gene transfer from *Agrobacterium tumefaciens* to plant cells. Planta 216:580–586.

Zarzecka K, Gugała M, Baranowska A, *et al* (2016) Concentrations of copper, zinc and manganese in potato tubers under the influence of herbicides. J Elem 21:259–267.

Zeng L, Cai J song, Li J jing, *et al* (2018) Exogenous application of a low concentration of melatonin enhances salt tolerance in rapeseed (*Brassica napus L.*) seedlings. J Integr Agric 17:328–335.

Zhang C, Wang D, Yang Y, Chen Q (2020) Construction of Potato DM1-3-516-R44 (DM) Transgenic System Based on *Agrobacterium* Transformation. Res Sq [Preprint] 44:

Zhang C, Wang P, Tang D, *et al* (2019) The genetic basis of inbreeding depression in potato. Nat Genet 51:374–378. https://doi.org/10.1038/s41588-018-0319-1

Zhang D, Hurst T, Duan D, Chen SJ (2019) Unified energetics analysis unravels SpCas9 cleavage activity for optimal gRNA design. Proc Natl Acad Sci U S A 116:8693–8698.

Zhang J, Boone L, Kocz R, *et al* (2000) At the maize/*Agrobacterium* interface: Natural factors limiting host transformation. Chem Biol 7:611–621.

Zhang J, Li D, Wei J, *et al* (2019) Melatonin alleviates aluminum-induced root growth inhibition by interfering with nitric oxide production in Arabidopsis. Environ Exp Bot 161:157–165. https://doi.org/10.1016/J.ENVEXPBOT.2018.08.014

Zhang LQ, Niu YD, Huridu H, *et al* (2014) Salicornia europaea L. Na^+/H^+ antiporter gene improves salt tolerance in transgenic alfalfa (Medicago sativa L.). Genet Mol Res 13:5350–5360. https://doi.org/10.4238/2014.JULY.24.14

Zhang N, Zhang HJ, Sun QQ, *et al* (2017) Proteomic analysis reveals a role of melatonin in promoting cucumber seed germination under high salinity by regulating energy production. Sci Rep 7. https://doi.org/10.1038/S41598-017-00566-1

Zhang S, Zheng X, Reiter RJ, *et al* (2017) Melatonin attenuates potato late blight by disrupting cell growth, stress tolerance, fungicide susceptibility and homeostasis of gene expression in *Phytophthora infestans*. Front Plant Sci 8:1993.

Zhang Y, Donnelly DJ (1997) *In vitro* bioassays for salinity tolerance screening of potato. Potato Res 1997 403 40:285–295. https://doi.org/10.1007/BF02358010

Zhang Y, Fan Y, Rui C, *et al* (2021) Melatonin Improves Cotton Salt Tolerance by Regulating ROS Scavenging System and Ca 2 + Signal Transduction. Front Plant Sci 12:. https://doi.org/10.3389/FPLS.2021.693690

Zhang Z, Mao B, Li H, *et al* (2005) Effect of salinity on physiological characteristics, yield and quality of microtubers *in vitro* in potato. Acta Physiol Plant 2005 274 27:481–489. https://doi.org/10.1007/S11738-005-0053-Z

Zhao B, Zhang J, Yang C, *et al* (2018) Rapeseed seedling stand counting and seeding performance evaluation at two early growth stages based on unmanned aerial vehicle imagery. Front Plant Sci 9:1362.

Zhao D, Yu Y, Shen Y, *et al* (2019) Melatonin Synthesis and Function: Evolutionary History in Animals and Plants. Front Endocrinol (Lausanne) 10. https://doi.org/10.3389/FENDO.2019.00249

Zhao H, Song Y, Li J, *et al* (2021) Primary restriction of S-RNase cytotoxicity by a stepwise ubiquitination and degradation pathway in *Petunia hybrida*. New Phytol 231:1249–1264. https://doi.org/10.1111/NPH.17438

Zhao H, Zhang Y, Zhang H, *et al* (2022) Origin, loss, and regain of self-incompatibility in angiosperms. Plant Cell 34:579–596. https://doi.org/10.1093/PLCELL/KOAB266

Zheng X, Tan DX, Allan AC, *et al* (2017) Chloroplastic biosynthesis of melatonin and its involvement in protection of plants from salt stress. Sci Reports 2017 71 7:1–12. https://doi.org/10.1038/srep41236

Zhou L, Mu T hua, Ma M mei, *et al* (2019) Nutritional evaluation of different cultivars of potatoes (*Solanum tuberosum L.*) from China by grey relational analysis (GRA) and its application in potato steamed bread making. J Integr Agric 18:231–245.

Zhou X, Zhao H, Cao K, *et al* (2016) Beneficial Roles of Melatonin on Redox Regulation of Photosynthetic Electron Transport and Synthesis of D1 Protein in Tomato Seedlings under Salt Stress. Front Plant Sci 7. https://doi.org/10.3389/FPLS.2016.01823

Zhu H, Li C, Gao C (2020) Applications of CRISPR–Cas in agriculture and plant biotechnology. Nat Rev Mol Cell Biol 2020 2111 21:661–677.

Ziaf K, Amjad M, Pervez MA, *et al* (2009) Evaluation of different growth and physiological

traits as indices of salt tolerance in hot pepper (*Capsicum annuum L.*). Pak J Bot 41:1797–1809

Zsögön A, Čermák T, Naves ER, *et al* (2018) *De novo* domestication of wild tomato using genome editing. Nat Biotechnol 36:1211–1216.

Zsögön A, Cermak T, Voytas D, Peres LEP (2017) Genome editing as a tool to achieve the crop ideotype and *de novo* domestication of wild relatives: Case study in tomato. Plant Sci 256:120–130.

Appendix I: Certificates of analysis of vitamin C, B12 and group 2 nutritional analysis of *Solanum okadae* and *Solanum tuberosum* 'Celandine' provided by Eurofins.

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School of Biosciences
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Certificate Code: AR-22-UD-079304-01
Page Number: Page 1 of 1
Reported On: 14/02/2022
PO reference: None Supplied
Reported By: Michal Lakomy
Analytical Services Manager - Chemistry

Certificate of Analysis

Sample number	400-2022-00067802	Received on	04/02/2022
Your sample code	NZ4DUK210010-02	Analysis started on	05/02/2022
Your sample reference	Solanum okadae		

Test Code	Analyte	Results	Units	Method Ref.
A7291	† Ascorbic acid (vitamin C) @EUAB02	10.69	mg/100 g	

† Indicates that the analysis was subcontracted and accredited to ISO 17025

Opinions and interpretations within this report are outside our accreditation scope.
Pass/Fail criteria or other comments where shown are based on specifications agreed with client or Eurofins general limits and do not take in to account measurement of uncertainty, unless stated
Unless otherwise stated, all results are expressed on a sample as received basis.
This certificate of analysis shall not be reproduced except in full, without the written permission of the laboratory.

Key: cfu colony forming units
< denotes less than
> denotes greater than
~ estimated value

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Certificate Code: **AR-22-UD-076209-01**
Page Number: **Page 1 of 1**
Reported On: **14/02/2022**
PO reference: **None Supplied**
Reported By: **Manpreet Sagoo**
Analytical Service Manager

Certificate of Analysis

Sample number	400-2022-00067819	Received on	04/02/2022
Your sample code	NZ4DUK210010-02	Analysis started on	05/02/2022
Your sample reference	Solanum tuberosum		

Test Code	Analyte	Results	Units	Method Ref.
A7291	† Ascorbic acid (vitamin C) @EUAB02	7.34	mg/100 g	

† Indicates that the analysis was subcontracted and accredited to ISO 17025

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Certificate Code: **AR-22-UD-076671-01**
Page Number: **Page 1 of 1**
Reported On: **14/02/2022**
PO reference: **None Supplied**
Reported By: **Michal Lakomy**
Analytical Services Manager - Chemistry

Certificate of Analysis

Sample number	400-2022-00067803	Received on	04/02/2022
Your sample code	NZ4DUK210010-02	Analysis started on	05/02/2022
Your sample reference	Solanum okadae		

Test Code	Analyte	Results	Units	Method Ref.
DJCDE	† Vitamin B12 @EUAB02	0.28	mg/100 g	

† Indicates that the analysis was subcontracted and accredited to ISO 17025

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Certificate Code: AR-22-UD-076672-01
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Reported On: 14/02/2022
PO reference: None Supplied
Reported By: Michal Lakomy
Analytical Services Manager - Chemistry

Certificate of Analysis

Sample number	400-2022-00067820	Received on	04/02/2022
Your sample code	NZ4DUK210010-02	Analysis started on	05/02/2022
Your sample reference	Solanum tuberosum		

Test Code	Analyte	Results	Units	Method Ref.
DJCDE	† Vitamin B12 (cyanocobalamin) @EUAB02	<0.25 (LOQ)	μg/100 g	

† Indicates that the analysis was subcontracted and accredited to ISO 17025

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Certificate Code: AR-22-UD-083901-01
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Reported On: 16/02/2022
PO reference: None Supplied
Reported By: Manpreet Sagoo
Analytical Service Manager

Certificate of Analysis

Sample number	400-2022-00067801	Received on	04/02/2022
Your sample code	NZ4DUK210010-02	Analysis started on	06/02/2022
Your sample reference	Solanum okadae		

Test Code	Analyte	Results	Units	Method Ref.
Nutrition				
UD08D	Moisture	75.9	g/100 g	H/091
UD001	Crude Protein (Nx6.25) (Dumas)	2.1	g/100 g	Z/001
UD007	Ash	1.2	g/100 g	Q/001
UD017	Carbohydrates (available)	18.47	g/100 g	Q/035
UD08W	Fructose	0.9	g/100 g	CHROM/344
UD08W	Galactose	0.5	g/100 g	CHROM/344
UD08W	Glucose	1.3	g/100 g	CHROM/344
UD08W	Lactose	<0.1	g/100 g	CHROM/344
UD08W	Maltose	<0.1	g/100 g	CHROM/344
UD08W	Sucrose	0.2	g/100 g	CHROM/344
UD08W	Total sugars	2.6	g/100 g	CHROM/344
UD08C	Total Fat	<0.1	g/100 g	H/090
B7039	Total Dietary Fibre (AOAC 991.43)	2.3	g/100 g	H/085
UD771	Energy value (kcal)	87	kcal/100 g	Q/035
UD771	Energy value (kJ)	368	kJ/100 g	Q/035
UD815	Salt (via sodium x 2.5)	< 0.025	g/100 g	ICP/003
Fatty Acids				
UDFA1	Trans fatty acids	<0.02	g/100 g fat	CHROM/215
UDFB1	Monounsaturated fatty acids	< 0.1	g/100 g	CHROM/215
UDFB1	Polyunsaturated fatty acids	< 0.1	g/100 g	CHROM/215
UDFB1	Saturated fatty acids	< 0.1	g/100 g	CHROM/215
UDFB1	Trans Fatty Acids	< 0.1	g/100 g	CHROM/215
Elements				
UD015	Sodium	<0.01	g/100 g	ICP/003

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Analytical Service Manager

Certificate of Analysis

Sample number	400-2022-00067818	Received on	04/02/2022
Your sample code	NZ4DUK210010-02	Analysis started on	06/02/2022
Your sample reference	Solanum tuberosum		

Test Code	Analyte	Results	Units	Method Ref.
Nutrition				
UD08D	Moisture	85.1	g/100 g	H/091
UD001	Crude Protein (Nx6.25) (Dumas)	1.1	g/100 g	Z/001
UD007	Ash	1.0	g/100 g	Q/001
UD017	Carbohydrates (available)	10.61	g/100 g	Q/035
UD08W	Fructose	0.8	g/100 g	CHROM/344
UD08W	Galactose	<0.1	g/100 g	CHROM/344
UD08W	Glucose	0.6	g/100 g	CHROM/344
UD08W	Lactose	<0.1	g/100 g	CHROM/344
UD08W	Maltose	<0.1	g/100 g	CHROM/344
UD08W	Sucrose	<0.1	g/100 g	CHROM/344
UD08W	Total sugars	1.5	g/100 g	CHROM/344
UD08C	Total Fat	<0.5	g/100 g	H/090
B7039	Total Dietary Fibre (AOAC 991.43)	2.2	g/100 g	H/085
UD771	Energy value (kcal)	51	kcal/100 g	Q/035
UD771	Energy value (kJ)	216	kJ/100 g	Q/035
UD815	Salt (via sodium x 2.5)	< 0.025	g/100 g	ICP/003
Fatty Acids				
UDFA1	Trans fatty acids	0.23	g/100 g fat	CHROM/215
UDFB1	Monounsaturated fatty acids	-0.2	g/100 g	CHROM/215
UDFB1	Polyunsaturated fatty acids	-0.2	g/100 g	CHROM/215
UDFB1	Saturated fatty acids	<0.1	g/100 g	CHROM/215
UDFB1	Trans Fatty Acids	<0.1	g/100 g	CHROM/215
Elements				
UD015	Sodium	<0.01	g/100 g	ICP/003

Opinions and interpretations within this report are outside our accreditation scope.
Pass/Fail criteria or other comments where shown are based on specifications agreed with client or Eurofins general limits and do not take in to account measurement of uncertainty, unless stated
Unless otherwise stated, all results are expressed on a sample as received basis.
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Key: cfu colony forming units
< denotes less than
> denotes greater than
~ estimated value

0342

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Appendix II: Partial S-locus F-Box protein (SLF) sequences identified from S_2 haplotype of *Solanum okadae*

*Solanum*_okadae S-locus F-box protein type-7 (SLF-7), partial

IQSTTFIHLHLNYQTTIEHEFILFKHSIKEAPNEFINALSFLSGDDDAFNPLFPDINITYM
SSNFNCTFYPLVGPCHGLIVLTDLTTIILFNPATRNFRLIPPSPFGCPQGFHRSVEGIGFG
FDSIAKYYKIVRISEVFWNPWDDYPGPKESKIDVYDFSMDCWREVEHVNLPLIYWLP
CSEMLYKEVVHWFATTDMSMVILCFDMCTEIFRIIKMPDVCNILTNEQYYGLVILRES
LTLISYPNPMSPIDP

*Solanum*_okadae S-locus F-box protein type-12 (SLF-12), partial

RFKCVTKTWCNLMQSFSFINLHHNYTSSKKDEFILFKRSLKEQNVFTNPLSFLRTPNG
DDDLDYITRDLEVPYLSTGYGSIFHQFNGPCHGLIVLTDYVNFVILNPATRNYRLLPK
SPFVCPRGFYRAIGGVGFVYDAIQRTYKVVRISEISGEEPFNDPSVVDWIGEVYDFSV
DSWRNLPFGEEEFPWPYNCPFAEMYYKGVFHWYAHRNLVAILCFDSSTEVFRIMQV
PEMCSLYDEKVHCLTILDECLTFICYPDPRRVSSPVQEITDIWIMKEYNVNDSWIKKF
TIRCPPIESPLAI

*Solanum*_okadae S-locus F-box protein type-23 (SLF-23), partial

MKKLPQDVVIFMLLKLPLKSLLRFKSISKIWYTLILSDSFFELHLKRTTTTKDEFILFIR
TLREDDPYKLTSIASFFSGDDKNNLTTLFPDVDVTHLTSSCCTIFNELIGPCHGLIALTD
SYIIIVLNPGTRKYFVIPPSPFECPKGYYRYIEAVAFGFDSIVNDYKIIKLSDVYWDPPT
DDRGPRGSRVEIYNLSIDSWREHNIEFPSIYFSHCSEIYYKEAVHWFTIKDDLVILCFDI
SS