Effect of short duration high temperature stress on bambara groundnut (*Vigna subterranea* (L.) Verdc.) plant reproduction

By,

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ABSTRACT

EFFECT OF SHORT DURATION OF HIGH TEMPERATUR STRESS ON BAMBARA GROUNDNUT (*Vigna subterranea* (L.) Verdc.) PLANT REPRODUCTION

Agricultural production is at the mercy of uncertainties driven by climate change, as a result of this most of the major and minor crops are under threat of crop failure due to severity of its effects on 'crop fertility'. Plant reproduction is regarded as highly sensitive to such climatic changes especially under high temperatures stress. Therefore, a key strategy to adapt to ever increasing global temperature is by improvement and promotion of underutilised crops, one such future crop and main focus of our research is bambara groundnut (*Vigna subterranea* [L] Verdc.). The present research was aimed at investigating the effect of temperature stress on plant reproduction, however there is very limited data on how bambara groundnut genotypes differ in their response to temperature stress with respect to plant reproduction. Therefore, the focus of this research was a systematic study to establish firstly a guide to reproductive development and, to quantify the impact of high temperature stress on floral development, pollination to fertilization and pod set in bambara groundnut.

The main methodology applied to establish a first guide to bambara groundnut flower development and pollen formation was through advanced microscopy techniques (fluorescence and scanning electron microscopy [SCM]). Later, the effect of short duration high temperature stress surrounding flowering time (pre- and post-anthesis) in seven bambara groundnut genotypes (IITA-686, S19-3, AHM-753, Uniswa red, Getso, and DipC), was investigated by applying three days of high temperature stress (36/33°C, day/night) at 100% flowering, before the temperature was returned to control conditions (28/22°C) and maintained until pod harvest. Phenotypic characteristics such as flower morphology, pollen viability and pod set, between control and heat stressed groups, were some of the traits observed to assess the effect among the seven genotypes. RNA sequencing experiment was designed and executed

to obtain differential gene expression data under high temperature stress, however breakdown of a freezer prevented the successful completion of the experiment.

Firstly, a scale of bambara groundnut flower development was established based on flower size as unit of measurement, from stage 8 to stage 13, which corresponds to floral organ differentiation and flower opening, respectively. Pollen architecture and development (micro-sporogenesis and micro-gametogenesis) was documented using fluorescence microscopy and SCM. And this study is the first one to link flower phenology to the stages of pollen development through morphological data.

Secondly, the effect of short duration high temperature stress was evaluated, it showed that the effect on *in vitro* pollen germination was significant ($p<0.05$) and was seen within 24 h of the application of the heat stress (HT-1) in all genotypes, and correspondingly pod set was significantly ($p<0.05$) reduced during heat stress. The genotypes IITA-686, AHM-753 and S19-3 recovered, pollen viability relatively faster and produced pods when the plants were returned to control conditions after heat-stress, in contrast to Uniswa red and Getso, where pod set was severely effected. We could conclude that like most of the legumes bambara groundnut pollen is sensitive to high temperature stress, with temperatures 36/33°C producing significant effects on pollen viability, and also display acclimation capability, when returned to control conditions. We advocate that selection for breeding should start at male gamete level, through *in vitro* pollen germination to determine tolerant and sensitive genotypes, and the prospects of bambara groundnut pollen thermo-tolerance should be evaluated.

Many research experts are considering bambara groundnut as a future crop based on its agro-ecological, cultural, genetic and nutritional importance. This research is one step in contributing to the knowledge of this future crop. This study serves as a guide for future researchers interested in flower development and variation in the process observed within and between bambara groundnut genotypes. Assessing pollen thermo-tolerance can give a direct indication of crop success/ seed yield under heat stress and the methodology is not limited to only temperature stress, that is it could also be adapted in photoperiod, drought and water stress. Investigation of other forms of stress tolerance could ultimately contribute to the development of future crop which could cope with future climatic anomalies.

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Table 27: RNA sample log of leaf and flower samples collected at 24h-time point during heat stress. Briefly; The table lists the RIN values from Bioanalyzer reports (RIN < 4 = degraded RNA; RIN < 7 = intermediate; RIN ≈ 7-10 = good quality), heat stress samples show degradation of RNA and control samples show good quality

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CHAPTER 1

1.1. Introduction

Agriculture is driven by numerous factors such as market fluctuations, management practices and biophysical properties like land and water resources, soil fertility, pest and diseases (Kurukulasuriya & Rosenthal 2003). Given its dependence on natural resources, agricultural production is mainly at the mercy of uncertainties driven by climate change. Over the last decade, phenomena such as increase in average temperatures, lower precipitation rates and the occurrence of extreme weather events due to climate change have been recognized as important factors which have influence on the form, scale and production value of Agriculture (Kurukulasuriya, P. & Rosenthal 2003; Pachauri et al. 2014). As a result of this most of the major and minor crops are under threat of crop failure in a number of their current regions of cultivation.

Given the urgency of addressing climate change, a key strategy according to Massawe et al. (2015), to adapt to changing climates is the improvement and promotion of underutilized crops (disadvantageous, lost, orphan, neglected, traditional and understudied crops). Their argument is that, we are neglecting a vast repository of genetic resources and beneficial traits of underutilized crops, as we mainly depend on very limited numbers of major crops (such as rice, wheat, maize etc.,), which have largely been bred for high yield under high inputs. Genes for resilience may have already been lost during the breeding process (Mayes et al. 2012; Massawe et al. 2015). Therefore, exploitation of underutilized crops would provide much needed agricultural diversity and could allow the development of more sustainable agricultural systems in the face of climate change. A strategic approach to suggest/decide on one such underutilized crop is by trait analysis and study of physiological responses of the crop to climate change. Therefore, many research experts are considering bambara groundnut (*Vigna subterranea* [L.] Verdc.) as a future crop based on its agro-ecological, cultural, genetic and nutritional importance (Azam-Ali 2010; Bamshaiye et al. 2011; Mayes et al. 2012; Massawe et al. 2015;

Singh et al. 2015). However despite its importance as future crop, bambara groundnut still lacks complete knowledge on its physiological response to climate change.

It has been well established that environmental signals such as photoperiod and temperature play important roles during reproductive development in plants (Searle and Coupland 2004; Yamashita and Komeda 2010; Pachauri et al. 2014). In particular, plant reproduction is regarded as highly sensitive to temperature stress and is one of the limiting factors in crop productivity (Hedhly et al. 2009a; Rieu et al. 2017). As one predicted result of global warming is an increase in extreme temperature episodes, the reproductive phase could become more vulnerable (flowering, fertilization and seed set), thus reduces crop yields (Porter 2005; Porter and Semenov 2005; Hedhly et al. 2009a; Thuzar et al. 2010; Driedonks et al. 2016; Rieu et al. 2017). Therefore, in our present research our work was aimed at determining the importance of plant reproduction, especially at the gametophytic, pollination-to-fertilization and seed set/fruit formation stages, and their interaction with the current scenario of increasing short episodes of extreme high temperature periods. This will provide a better understanding of the crop itself and how it could perform in future climate conditions.

Bambara groundnut, despite gaining recent popularity for research, still has only few studies on its reproductive development, particularly with respect to temperature stress. Many studies on the effect of temperature stress on bambara groundnut, focuses on the combination effect of drought (Al Shareef et al., 2013, Karunaratne et al., 2011b) and photoperiod (Karunaratne et al., 2011a, Brink, 1997). However, these results were mainly focused on measuring vegetative growth and pod yield, any information on the effect of temperature on its reproductive development is yet to be clearly documented. Therefore, the focus of our present research was primarily on the detailed characterization and fundamental understanding of its reproductive biology with a particular emphasis on inflorescence, flower development and pollen formation, secondly, on the effect of short episodes of high temperature stress on bambara groundnut reproductive biology especially on flower, pollen viability and pod set.

Advances in crop genomics have begun to enable a new cohesive understanding of the biology of many crop species and this has also resulted in the development of a

powerful set of molecular and bioinformatic tools. Genome sequencing is one such method which can be used to generate data, interpret and improve the genetics of crops. Gene expression profiling is widely used to study plant-stress regulatory mechanisms that control cellular processes (Van Verk et al. 2013). RNA sequencing extends the possibility to analyse previously unknown genes through transcriptome analysis and it also offers a dynamic range of quantification at reduced cost, which is particularly helpful in underutilized crop species such as bambara groundnut, without any reference genomes (Chapman 2015). In the case of underutilized crops like bambara groundnut, which serves mainly as a regional food source, there has been limited investment in genetic and genomic tools. Therefore we designed and executed RNA sequencing experiment to generate differential gene expression data at high temperature stress, to identify and annotate differentially expressed transcripts by comparative analysis of expression data of control and temperature stressed samples, obtained at different time points.

The over all hypothesis is that, the plant sexual reproduction phase in flowering plants is an active developmental process subjected to various selective pressures. But the reproductive phase also provides the plant with an opportunity to adjust to environmental variations. Understanding phenotypic malleability/plasticity and gametophytic selection for current temperatures, along with potential genomic/epigenetic variation during this process, could provide new understanding into plant adaptation and the potential for evolution under future global-warming scenarios. The prospect of that gametophytic development and selection might favour the best adapted genotypes to future temperatures is an appealing postulation that deserves advanced research, and it could also explain how plants rapidly adapt to different temperatures while sustaining variability.

1.2. Aims and objectives

Collectively, the main aim of this PhD research was to gain deeper understanding of bambara groundnut reproductive biology and its vulnerability to high temperature stress. The specific objectives of the current study were as follows:

- To study phenology of bambara groundnut flower, mainly flower development, pollen formation and fertilization.

- To assess the effect of short duration of high temperature stress on bambara groundnut plant reproduction with particular focus on flower morphology, pollen viability and podding among seven bambara groundnut genotypes.

- To generate differential gene expression data from flower and leaf tissues under high temperature stress.

1.3. Thesis Outline

- Chapter-2 is a literature review of bambara groundnut, which includes its taxonomy, habitat and growth habit, crop production and agronomic importance. The main topic of temperature stress is discussed in this chapter in relation to effect on global agriculture and crop physiology. An introduction to molecular biology and scope for new discovery in underutilized crop research, are described.

- Chapter 3 contains the details of methodology to study bambara groundnut reproductive biology. This chapter contains comprehensive report on light and scanning electron microscopy experiments to study flower and pollen development. The chapter also provides new insight into bambara groundnut floral formulae, floral diagram and flowering trend.

- Chapter 4 contains methodology adapted and implemented to study effect of short duration of high temperature stress on reproductive biology of seven bambara groundnut genotypes. The results of effect on flower morphology, *in vitro* pollen germination and podding are described in this chapter.

- Chapter 5 documents the detailed experimental design and methodology implemented to obtain total RNA for RNA sequencing. This chapter also details the setbacks during the experimental trials. The results of quality of RNA from Bioanalyzer and future considerations are described in this chapter.

- Chapter-6 discusses the potential applications of the over all results from chapter 3 and 4. In this chapter the recommendations for future research based on the

results and setbacks from chapter 5 are also included. This chapter concludes with discussion of initial aims and objectives, and how the present research study has aimed to achieve the same.

CHAPTER 2

Introduction

2.1. Bambara groundnut (*Vigna subterranea* (L.) Verdc) - taxonomy

Binomial Classification (Cannon et al. 2009)

Family: Leguminosae (Fabaceae)

Subfamily: Papilionoideae

Clade: Millettioid

Tribe: Phaseoleae

Genera: *Vigna*

Species: *subterranea*

Variety: subterraneae (cultivated forms)

Common name: bambara groundnut

Local names: Jugo beans (south Africa), ntoyo cibemba (Zambia), gurjiya (Nigeria), okpa (Ibo, Nigeria), epa-roro (Yoruba, Nigeria), nyimo beans (Zimbabwe) and izindlubu (IsiZulu) (Bamshaiye et al. 2011).

For the first time in literature bambara groundnut was referred under Angolan name as 'mandubi' in the 17th century (Marcgrav de Liebstad, 1648). Later in 1763, it was named as *Glycine subterranea* in accordance with Linnaeus classification of species in the book *Species Plantarum* (Heller et al. 1995). In 1806, Du Petit-Thouars named the crop 'voandzou' in French based on the vernacular name 'voanjo' ('voa' means 'seed' and 'anjo' means 'filling'). Based on this the name *Voandzeia subterranea* (L.) Thouars was proposed and used over a century among researchers (Heller et al. 1995). According to Maréchal et al. (1978), detailed botanical studies showed the closest resemblance of bambara groundnut with other species of the genus *Vigna*. Therefore in 1980, Verdcourt proposed the current name *Vigna subterranea* (L.) Verdc.

(Maréchal et al. 1978). Cytogenetic studies performed on the pro-metaphase and metaphase chromosomes of shoot meristem of bambara groundnut revealed that both wild and cultivated forms are diploid with $2n = 2x = 22$ chromosomes. There was no evident polyploidy observed, but difference in karyotypes (chromosome shape) was observed between accessions (Uguru et al. 2006). The uniformity in number of chromosomes suggests the fact that all the accessions have evolved through homogenous assemblage of chromosomes from a common ancestor. The origin of bambara groundnut is now undisputed as researchers agree that the crop originated from the African continent particularly north-eastern Nigeria and northern Cameroon (Hepper 1963; Begemann 1988). With Diversity Arrays Technology (DArT) molecular markers and phenotypic descriptors, Olukolu et al. (2011) provided new evidence which shows the origin to be Nigeria/Cameroon.

Bambara groundnut belongs to the family Leguminosae, which is a diverse family with 800 genera and about 20,000 documented species, and it is the third largest flowering plant Family next to Orchidaceae and Asteraceae (Doyle and Luckow, 2003). Crop legume species are grouped under three subfamilies Caesalpinioideae, Mimosoideae, and Papilionoideae (Cannon et al., 2009). Bambara groundnut belongs to the largest subfamily, Papilionoideae which diverged from other legumes around 45-50 million years ago (Wojciechowski et al., 2004). Papilionoideae is again sub-divided into four major clades containing economically important food and feed species, such as genistoids, dalbergioids, millettioids/phaseoloids and galegoids (Doyle et al. 2003). *Vigna subterranea* belongs to the Phaseoleae tribe under the Millettioid clade, which also contains the newest model crop *Glycine max* (Soybean) along with other major and minor legumes such as *Phaseolus vulgaris* (Common bean), *Vigna angularis* (Abzuki bean) and *Vigna unguiculata* (Cowpea) (Cannon et al. 2009).

2.1.1. Habitat

Bambara groundnut is a short day plant and can adapt to wide range of climatic zones, ranging from sea level to an altitude of 1600 m. An average temperature of 20-28°C is considered optimal for ideal growth and development. It can also grow and produce considerable yields at high temperatures (up to 40°C) where most of the other crops

fail (Massawe et al. 2005b). It thrives better in deep well drained soil with light and friable sandy loam soil with pH of 5.0 to 6.5 and the crop grows even in low nutrient soil regions (Swanevelder 1998). Many farmers use flat seedbeds and in other countries the use of mounds and ridges is also practiced (Heller et al. 1995).

2.1.2. Habit

Bambara groundnut (*Vigna subterranea* (L.) Verdc) is an herbaceous annual plant essentially grown for human consumption. The general appearance of the plant is similar to peanut (*Arachis hypogaea*), as it appears as a crown of leaves, due to bunched leaves arising from branches above the soil surface (**Figure 1**). The stem is lateral and arise from the main root. Differences in internode and petiole length results in the bunched, semi-bunched (semi spreading) and spreading varieties (IPGRI, 2000). Based on the 4th petiole (P) and internode (I) length ratio ($\frac{P}{I}$) the plants can be classified into bunched ($\frac{P}{I}$ = >9), semi-spreading ($\frac{P}{I}$ = 7-9) and spreading (open) ($\frac{P}{I}$ = <7) types. About a week after germination the stem branching begins, and produces as many as 20 branches. Each branch consists of nodes, where leaves and flowers buds arise alternately. The internodes are differential in length i.e., those near the base are shorter than the more distant ones (Heller et al. 1995). The domesticated bambara groundnut landraces have a well-developed tap root system with abundant geotropic lateral roots which can grow up to 20 cm (**Figure 1**). The root nodules are formed through a symbiotic association with cowpea type *Brayrhizhobium* and/or groundnut type Rhizobia, which helps in nitrogen fixation (Doku 1968; Heller et al. 1995; Molosiwa 2012).

Leaves are trifoliate and pinnate in domesticated varieties and pentafoliate in some wild-types, with smooth epidermal surface. The terminal leaflet is slightly larger than the lateral leaflets with average width of 3 cm and length of 6 cm (**Figure 2**). The petiole is long and erect, thickened and grooved at the base, the petiole colour varies between green, purple or brown among the landraces. Petiole colour is an important morphological character which ranges from green to dark purple, and it can be used in identifying the different landraces at early stages. The leaf is stipulate, where the terminal leaflet has two stipels, while only one is assigned to each of the two lateral

leaflets (**Figure 2**). The shape of the leaflet varies among the landraces/genotypes; these leaflets are attached to the rachis with raised/marked pulvini.

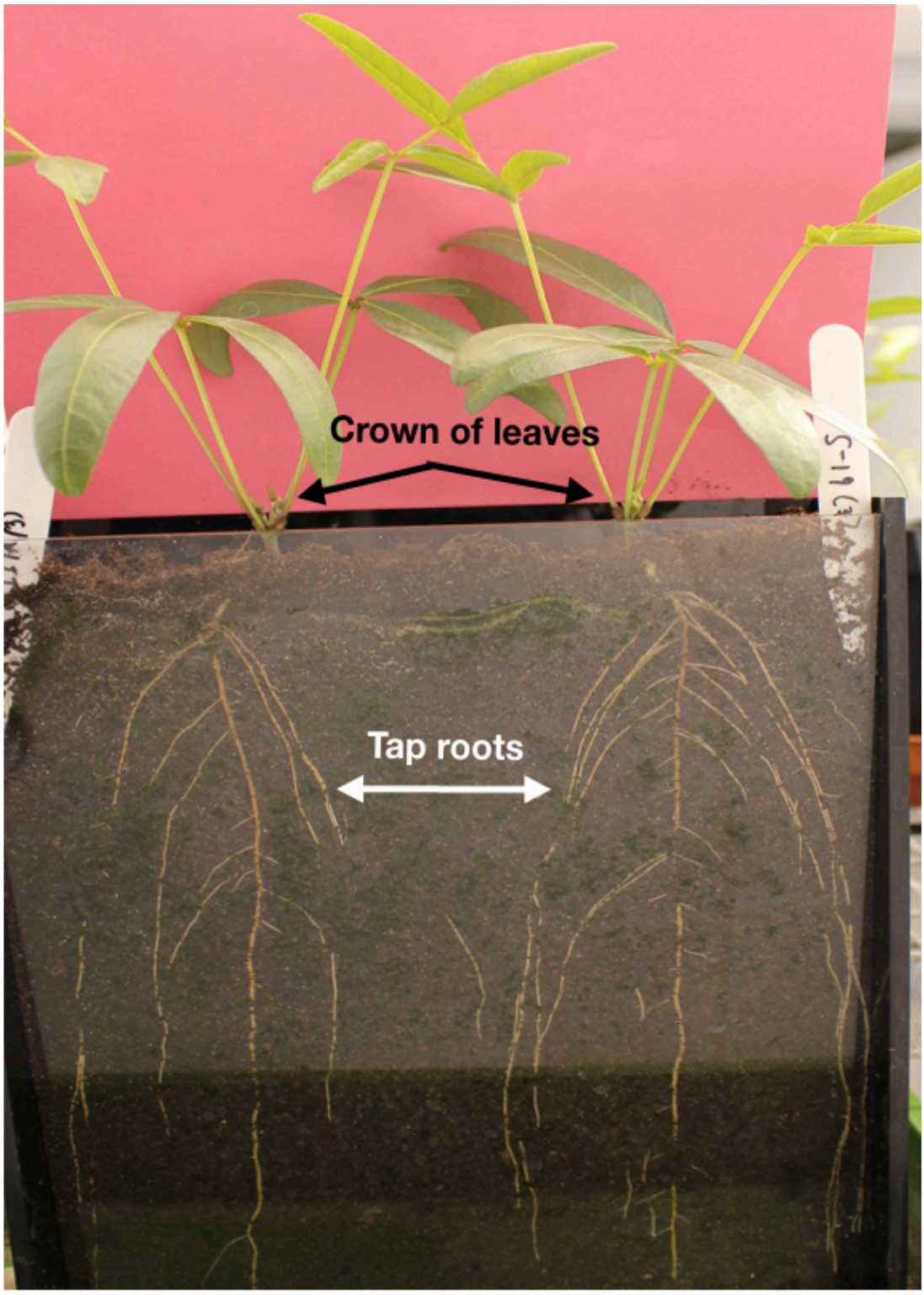

Figure 1:Bambara groundnut plant (genotype S19-3) showing crown at the base of the stem and prominent tap root system (Photo taken from pilot study).

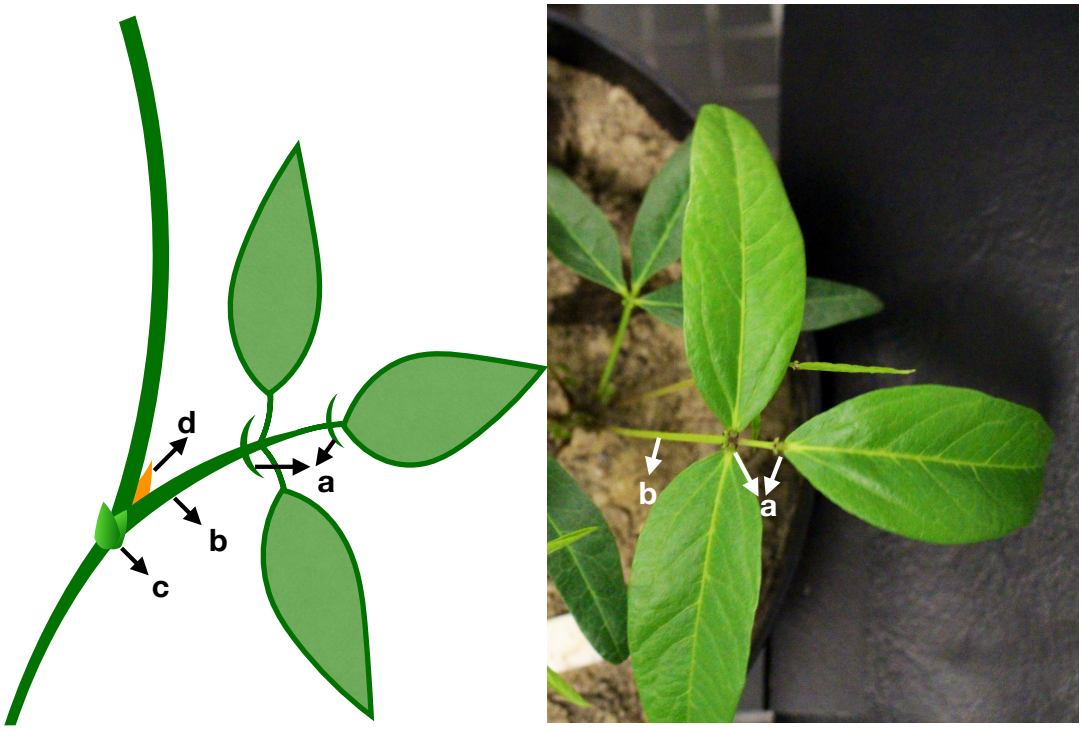

Figure 2: Diagrammatic representation (left side image) of trifoliate leaf of bambara groundnut with stipels (a), long erect petiole (b), stipule (c) and auxiliary bud (d). (scale bar = 1 cm)

2.2. Crop

2.2.1. Origin of domestication

The species *subterranea* is divided into var. *spontanea,* comprising of wild forms, mainly located in northern Nigeria (Yola) and Cameroon (Garuoa), and var. *subterranea* with cultivated forms, mostly around sub-Saharan Africa (Basu et al. 2007b). Many investigators confirmed that the crop originated from the African continent (refer to review by Molosiwa, 2012). More advanced investigations using DArT molecular markers and phenotypic descriptors, showed that the putative area of origin to be Cameroon or Nigeria (Olukolu et al. 2011). They also inferred that bambara groundnut was introduced from west Africa to east Africa and Asia. The crop is believed to be domesticated within the area where the wild forms are found, that is within the area of Cameroon (Garuoa) to northern Nigeria (Hepper, 1963). It is also cultivated in America, few parts of northern Australia, India, Indonesia, Malaysia and Sri Lanka (Heller et al. 1995; Suwanprasert et al. 2006). Although bambara

groundnut germplasm was collected from different regions of Africa and other countries, the major germplasm collection is located at the International Institute of Tropical Agriculture (IITA), Nigeria (Massawe et al. 2005b).

2.2.2. Crop cultivation

Bambara groundnut is a short day plant and is mostly intercropped with other major crops such as maize, millet, sorghum, cassava, yam, peanut and cowpea (Azam-Ali et al. 2001; Berchie et al. 2010). The planting density is an important factor which determines the yield due to competition for space. The recommended plant density ranges from 20-30 cm between plants and the recommended spacing is 40-50 cm apart in single rows (Ocran et al. 1998). Since bambara groundnut is normally grown under dry conditions, farmers depend mainly on the seasonal rains to sow the seeds. Bambara groundnut requires moderate rainfall of 500 to 600 mm from sowing till flowering (www.nda.agric.za 2011). The seeds are sown at 5 cm depth and the seedling emergence takes 7 to 21 days in field conditions depending on the landraces. Flowering starts at 35 to 40 days after sowing and the plant continues to flower until the end of lifecycle (Brink et al. 2006), although the level of determinacy depends on landrace.

Upon pollination and successful fertilization, the pedicel of the flowers bends towards soil pushing the young pod into the soil, it takes up to 30 days for the pod to reach its maximum size. The geocarpic pods develop and mature in/on the soil surface within following 10 days; it does not require complete coverage of the pod with soil for the pod development, although different landraces favours different degrees of pod burial. Bambara groundnut completes its lifecycle within 100 to 150 days from the day of sowing to harvesting of mature pods. Some of the landraces in Ghana are reported to have faster growth cycle of 90 days (Berchie et al., 2010). It needs at least 3 to 5 months of frost free climate to complete its lifecycle, and requires no external fertilizers, although is able to make use of them.

Photoperiod (day length) has been reported to influence pod set and seed development. For example, long photoperiods usually delay seed set in some landraces (Linnemann and Craufurd 1994a). The unripe pods vary in colour

depending on the landraces from yellowish green to purple. The fully mature pods are indehiscent, hard and often vary in size depending on the number of seeds it contains, which usually ranges from 1 to 2. The plants are harvested at the end of life cycle when 80% of pods are mature, the plant turn yellow and starts to dry (Brink et al. 2006).

In dry environments, asynchronous maturity is not an issue, as pods dry. However, in wetter environments it can be a problem. The seeds can be harvested by pulling or lifting the plant, most of the pods will remain attached to the peduncle and the detached pods can be collected manually. Harvested pods are left to air-dry for several days or can be subjected to high temperature until the pod shell hardens. The pods can also be harvested when they are still green before they reach maturity as they are more palatable and, to prevent rotting and premature germination in wet conditions. The seeds size on average is 1.5 cm in diameter and it is smooth and very hard when dried. The colour of seeds also varies from, creamy, yellow, red, purple and black with different testa patterns depending the landraces (**Figure 3**). The survey on consumer preferences on seed colour showed that the white and cream-colored seeds were preferred in southern and central districts of Ghana as they command up to 10% premium, whereas red seeds were preferred by those in the northern part close to the Zimbabwean as the seeds were rich in iron and get higher market price (Hillocks et al. 2012). The harvested seeds can be stored well when they are still in pod shells, as they are more prone to weevil damage after shelling. The shelled seeds are treated with insecticide, ash or vegetable oil before storage to prevent insect damage (www.nda.agric.za 2011).

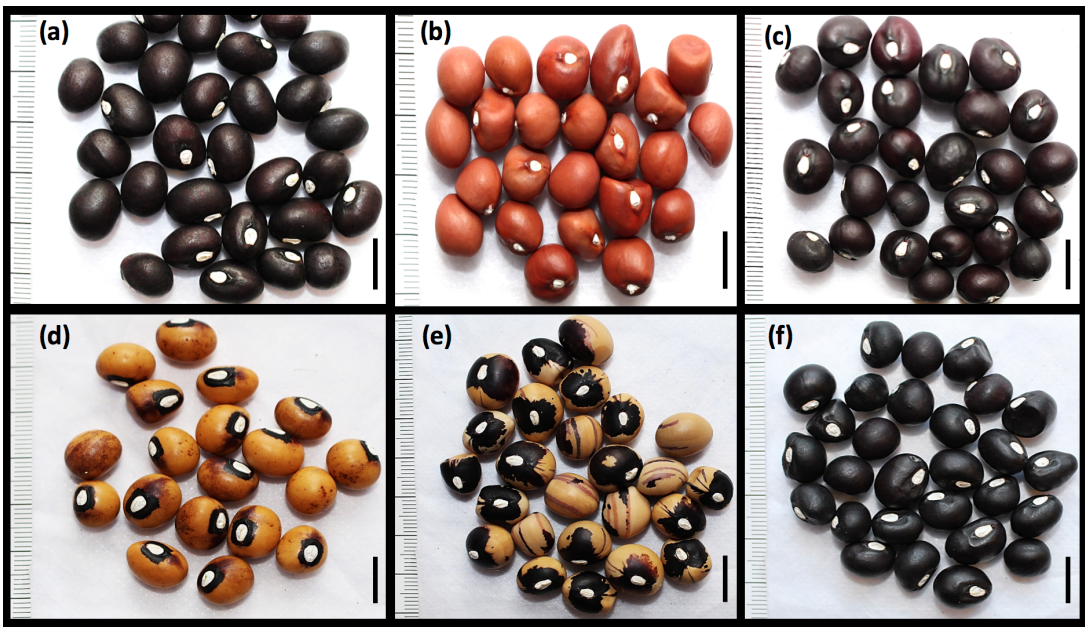

Figure 3: Seed colour and testa pattern of bambara groundnut seeds, (a) IITA-686; (b) Uniswa red; (c) S19-3; (d) DipC; (e) Getso and (f) Gresik. (Scale bar = 1 cm)

2.2.3. Seed composition

The seed of bambara groundnut makes it a complete food because of its biochemical composition, which provides all the essential nutrients for the human diet. On average, the seeds contain 63% carbohydrate, 19% protein and 18% oil. In comparison with other legumes, bambara groundnut seeds are rich in iron and contain high levels of lysine and methionine amino acids in their protein content, which is in par other legumes (Hepper 1963; Heller et al. 1995; Ijarotimi and Esho 2009; Yao et al. 2015). The bambara groundnut could regarded as a major source of dietary proteins in developing countries, where carbohydrates are the principal staple foods and access to meat is restricted. The fatty acid content of bambara groundnut is predominantly linoleic, palmitic and linolenic acids (Minka and Bruneteau, 2000). The black coloured varieties have high tannin content of 14.9 mg CE/g with relatively low levels (4.5 mg CE/g) found in cream coloured varieties (Nti, 2009). The presence of anti-nutrients such as tannin limit the digestibility of proteins by forming indigestible protein complexes, since the seed colour is associated with the presence of tannin, cream verities are preferred (Yao et al. 2015). MudPit (Multidimensional protein identification technology) and Mass-spectrometry analysis revealed between 10 and 12 different types of proteins in malted and dry seeds respectively (Okpuzor et al.,

2010). Seed storage protein B and vicilin are major proteins found both in malted and dry seeds, these were similar to the proteins in *Vigna luteola*. These storage proteins are the main source of nitrogen, sulphur and carbon during the development of the embryo (seed germination). Many proteins showed amino acid sequence homology to the proteins found in *Vigna radiate* and *Vigna unguiculata*. Unlike dry seeds, malted (dried germinated seeds) seeds contain heat shock proteins (HSPs), which shared sequence homology with those found in *Phytophthora melonis*. Okpuzor et al., (2010), reported that the dry seeds bambara groundnut contain dehydrin proteins which may assist in withstanding stress under low water levels (Okpuzor et al. 2010).

The seeds of bambara groundnut are consumed in many ways. The immature seeds can be consumed fresh or can be grilled. The mature hard seeds are boiled to make soup or snacks for consumption. The seeds can be milled to make flour, which can be used to make stiff porridge and also bread. Nutrient rich extruded snacks can also be produced using bambara groundnut flour when mixed with corn starch and cassava, which suggests that there is the potential to make gluten free products ((Ogunmuyiwa et al. 2017). When used with wheat flour, bambara groundnut flour (at 15%) increases the protein content in breads (Erukainure et al. 2016) hence useful in preparation of high protein enrichment foods (Alozie et al. 2009; Ayo and Andrew 2016). The milk produced from bambara groundnut seeds has flavour and composition similar to that produced from soybean, cowpea and pigeon pea. Importantly the milk produced from seeds of bambara groundnut also had low levels of trypsin inhibitor (Poulter and Caygill, 1980) and high protein content in the yogurt (Pahane et al. 2017). The seeds of bambara groundnut can also be used as an animal feed (Mahala and Mohammed 2010; Abake 2015) and, the nitrogen and phosphorus rich leaves can be used for animal grazing (Heller et al. 1995).

2.2.4. Agronomic importance

The crop can be cultivated in a wide range of climatic zones (sea level to 1600 m altitude) and about 20-28°C is an ideal temperature for the crop cultivation. The main countries cultivating bambara groundnut are Benin, Chad, Ghana, Nigeria and Togo. It can also grow in areas which are too dry for other crops to grow such as the Bambara district near Timbuktu on the southern fringe of the Sahara Desert, in well rain-fed

areas and cool moist highland areas of Zimbabwe (www.nda.agric.za 2011). All cultivated lines of bambara groundnut are landraces which have evolved from their wild relatives and are favoured by farmers because of their yield stability even under different environmental conditions (Massawe et al. 2005b).

Like all leguminous crops, bambara groundnut increases the soil fertility through symbiotic nitrogen fixation and reduces soil degradation, therefore cultivation is sustainable and requires low inputs (Massawe et al. 1999). It grows well on well-drained soils, but sandy loams with a pH of 5.0 to 6.5 are most suitable (Swanevelder, 1998). Bambara groundnut symbiotically associate with cowpea type *Brayrhizhobium* and Rhizobia from groundnut (Doku, 1969). The ability to fix nitrogen varies among the landraces of bambara groundnut, the high yielding bunchy type landraces seems to fix more nitrogen than spreading type (Kishinevsky et al. 1996). The nitrogen fixation mechanism is the major source nitrogen during reproductive growth for these plants (Kumaga et al., 1994) and the variation in seed yield seems to be significantly dependant on nitrogen fixation capacity of the plant (Kishinevsky et al. 1996). There seems to be a significant genotypic variation among the bambara groundnut landraces and cultivars in response to symbiotic nitrogen fixation (SNF) and phosphorus use efficiency (PUE) (Andriamananjara et al. 2013). In case of glass house experiments inorganic nitrogen fertilizers (up to 150 kg/ha) should be added due to limitation in the nitrogen fixation in the closed environment (Massawe et al. 1999). In field conditions the recommended use of phosphorus fertilizer (P_2O_2) is 60 kg/ha (Toungos et al. 2009).

The crop can be intercropped along with major crops to reduce soil degradation. In bambara groundnut, like other legumes, there exists a competition for photo-assimilates between vegetative and reproductive phases. Some of the major crops are bred to have smaller vegetative structure, short lifecycle and minimum branching but maximum seed yield due to selection pressure over decades of domestication. The vast number of bambara groundnut landraces produces variable yield, this may be due to variation in successful resource capture and balance between vegetative biomass and seeds. The high yielding variety may defer from low yielding ones, in the perception of environmental signals, such as temperature or photoperiod or they might

be insensitive to these factors. Understanding the influence of such factors on the relationship between vegetative and reproductive development, may uncover important links relating to yield and stress resilient properties (Massawe et al. 1999). The FAO calculated that, in 2010 bambara groundnut production was close to 45,000 Mt and crossed 50,000 Mt in 2008 (FAOSTAT 2017). Global production of bambara groundnut was about 330,000 Mt of which half of the production came from West Africa (William et al. 2016).

Bambara groundnut has a high degree of pest resistance compared to cowpea or groundnut. However in humid conditions, it is more prone to fungal diseases such as Cercospora leafspot, Fusarium wilt and Sclerotium rot (Heller et al., 1997). Apart from the above agronomic traits, bambara groundnut has also been reported to have medicinal importance in some cultures. The water from boiled maize and bambara groundnut is used to treat diarrhea and nausea. The mixture of ground bambara groundnut and Mexican marigold (*Lantana trifolia L.*) leaves are used in washing livestock as an insecticide (prevention against ticks) (www.nda.agric.za 2011).

2.3. Global warming a concern for agricultural production

Agriculture is driven by numerous factors such as market fluctuations, national and international policies, management practices and more importantly biophysical properties like land and water resources, soil fertility, pest and diseases (Kurukulasuriya & Rosenthal 2003). Given its dependence on natural resources, agricultural production is also at the mercy of uncertainties driven by climate change, including extreme temperature fluctuations, drought and flooding. According to NASA records there has been an increase of average global temperature of 0.94°C (1.7°F) from 1880 to 2017 (**Figure 4**). The year 2016 is regarded as the warmest year since the record keeping began in 1880 and from about 2010 every year has been the warmest recorded in history (NASA/GSFC GISS 2017). The study comprising of already available data on temperature and precipitation since 1983 also showed that, there is a significant increase in temperatures especially in semi-arid and dry humid climate (0.044-0.087°C) in the last 5 to 10 years (Singh et al. 2015). The biggest temperature changes will be at higher latitudes (IPCC 2007). Even though, there are

reports of slowdown in the rate of temperature increase since the late 1990s (New et al. 2011), NASA studies predict that the global warming is likely to continue.

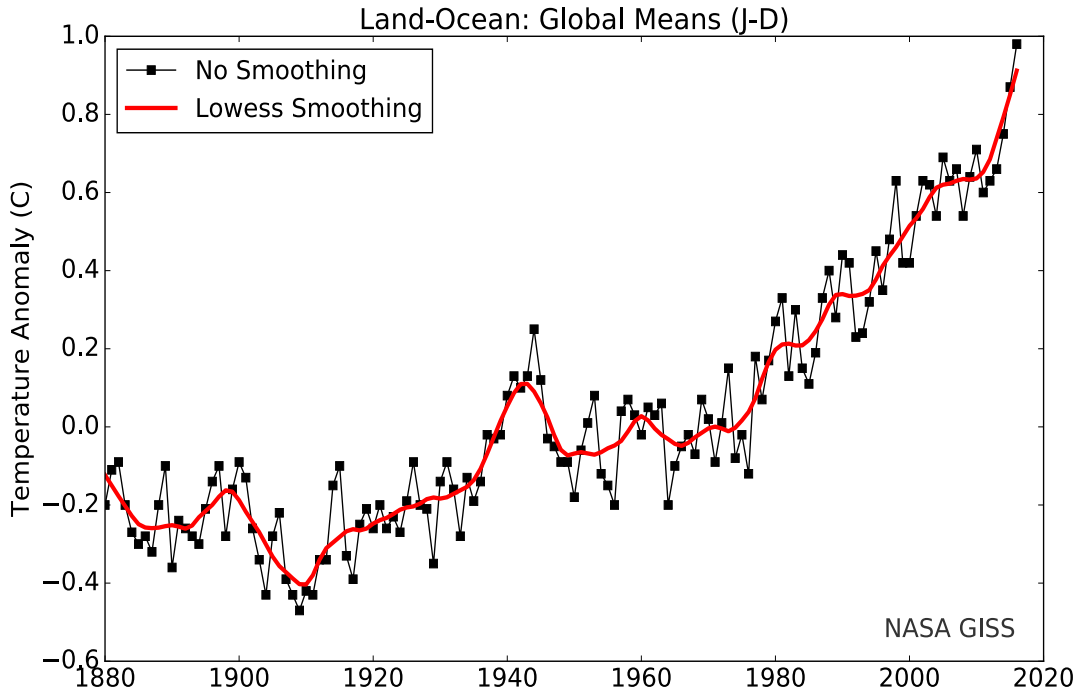

Figure 4: This graph illustrates the change in global surface temperature relative since 1880 (NASA/GISS 2017)

Carbon dioxide is an important greenhouse gas which is also known as a heat-trapping gas (Shindell, 2014). According to NASA reports the carbon dioxide concentration has reached record breaking levels of 406.31 ppm (as of June, 2017), which is far greater than highest historical CO_2 level (300 ppm) ever recorded in centuries (NASA/GSFC GISS 2017). As a result of these greenhouse gas emissions from human activities, the average global surface temperature has continued to increase and is causing warming of the atmosphere and oceans. This in turn has been suggested as the cause for extensive melting of the arctic ice and increases in sea level. Further escalation in greenhouse gas emissions are projected to increase the average earth surface temperature by between 1.5 to 11°C by 2100 across various regions (Stainforth et al. 2005).

2.3.1. Impacts on Agriculture

Over the last decade, phenomena such as increase in average temperatures, lower precipitation rates and the occurrence of extreme weather events due to climate change have been recognized as important factors which have influence on the form, scale and production value of Agriculture (Kurukulasuriya, P. & Rosenthal 2003; Pachauri et al. 2014). The world today faces many great challenges to produce adequate food, feed and fiber, to satisfy 7.5 billion (as of June 2017, www.workdmeters.info, 2017) people on earth. The global population is predicted to increase to between 8 billion and 10 billion by the year 2025 and 2050 respectively (Reddy et al., 2009). This demands an increase of 70 to 100% food production by the middle of the century (Godfray et al. 2010).

Throughout human history agricultural production has been greatly dependent on climate changes and variability across the agricultural regions of the world (Kurukulasuriya, P. & Rosenthal 2003; Zinn et al. 2010; Ramirez-Villegas et al. 2013; Driedonks et al. 2016). Since an obvious result of global-warming would be high temperature periods, extreme flooding and droughts, such changes can alter the agro-ecological zones (AEZ) and timing and length of the growing seasons in different parts of the world (Kurukulasuriya, P. & Rosenthal 2003; Lane and Jarvis 2007; Lin et al. 2013; Pachauri et al. 2014). This can, in turn, affect crop production, as a result of this most of the major and minor crops are under threat of crop failure in a number of their current regions of cultivation (in terms of yield, production and quality).

According to Agro-ecological zone analysis studies, reported that there will be a shift towards north-east in US corn belt for every 1°C temperature rise and a 1.5 to 3.2°C increase would drastically reduce the production of cereal crops in the tropics and sub-tropics (Lobell and Asner 2003; Singh 2017). The same study also proposed expansion of agricultural areas of wheat production to hill regions in order to compensate for the loss of land in plains due to high temperatures. (Lane and Jarvis 2007), in their study using current and predicted future climate data predicted that climate change would reduce the land available for major food crops (rice, wheat, potato) and cash crop (apple, banana, coffee) production. This is supported by a global agricultural suitability study by Zabel et al. (2014). To grow the 16 most

important food and energy crops under future climates (up to 2100), they projected that the crop land availability could increase only in Northern high latitudes (in Canada, China and Russia) whereas the availability decreases in global south regions which comprises of tropical and subtropical climate where most developing world agriculture is practiced (Tubiello et al. 2007; Zabel et al. 2014). A case study by Sarker et al. (2012), shows a reduction in rice yields under high and low temperature stress in Bangladesh. Similarly, according to predictions using FAO-EcoCrop database, global climate change will have negative implications on grain sorghum production in its current regions of cultivation by 2030 and the crop is more vulnerable in the regions where the production is already marginal (Ramirez-Villegas et al. 2013).

The projections of the effect of global warming on crop production are variable depending on the climatic zones, crop type and limitations of the software, therefore a more reliable direct approach used climate data and rice yields between 1992 to 2003 shows that every 1°C increase in temperature there was a 10% decrease in yield (Peng et al. 2004). Similarly, it was predicted that there will be relative reduction in yields of both wheat (*Zea mayes*) and sorghum (*Glycine max* [L.] Merr.) by 17% in US Midwest (Lobell and Asner 2003), and 15% reduction in rice production in Philippines (Peng et al. 2004). Therefore it is a challenge to agricultural scientists to understand the severity of the effects and to improve agricultural production using current/future land and resources available. In our current research we mainly focus on the effect of temperature stress as it is obvious that global warming is primarily associated with increasing temperatures.

2.3.2. Temperature stress and plant reproduction

The effect of temperature stress on yields outlined previously are but some of the examples, with many studies pointing to the reproductive phase as a potential sensitive stage (Rieu et al. 2017). The future high temperatures are likely to shorten the crop growing cycle, during which plants can tolerate only narrow changes in temperatures, which if they exceeded the optimum range can reduce seed set and interfere with development (Porter 2005). Therefore, it is important to understand the mechanism and processes underlying plant reproduction before understanding the effect of temperature stress on the same.

2.3.2.1. Reproduction process in higher plants

The concept of plant sexual reproduction and the relationship between pollen viability and the formation of seeds and/or fruits has been recognized since the dawn of agricultural civilization (Frankel and Galun 2012). All higher plants propagate by asexual or sexual reproduction. The end of 19th century marks the discovery of double fertilization processes in higher plants (Angiosperms) by a Russian observer, Prof. S. Nawaschin (1898), with similar results also published by Guignard a year later (1899) on the same plant *Lilium martagon* (Sargant 1899). A century later, the evolutionary origin of endosperm (embryo-nourishing tissue) which categorizes Angiosperms, was determined (Friedman 1998). Angiosperms are also known as flowering plants and comprise most of the major and minor food, feed and economic crops which reproduce by fertilization of gametes (double fertilization and triple fusion).

The process of fertilization in Angiosperms is illustrated in **figure 5**. The pollen grains carrying male gametes. Pollination, resulting in the transfer of pollen grains from the stamen to the stigma surface, is the beginning of the cascade of events from the delivery of male gametes to the vicinity of egg (ovules). Fusion of male and female gametes takes place when the pollen tube carrying male gametes enter into the embryo sac (/ovary) containing an egg cell (female gamete) (Sargant 2006). The process of fertilization is defined as “the fusion of male gametes (two sperm cells) with a female gamete (an egg cell) and a central cell (polar nuclei) respectively” also known as double fertilization (Pua and Davey 2010). Following successful fertilization, the ovules become seeds and get enclosed within the ovary (which later becomes the fruit).

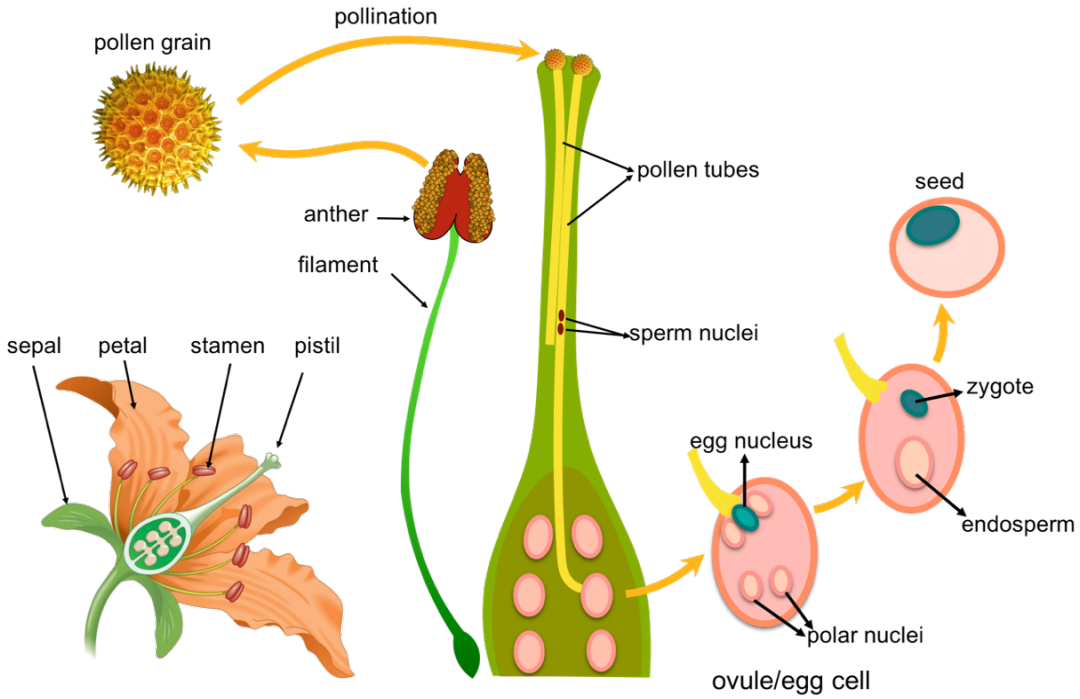

Figure 5: Overview of plant reproduction in Angiosperms (original illustration, Keynote software)

In bambara groundnut, initial studies on floral biology suggested that it is a self-pollinating species, it takes 30 to 48 days after sowing (DAS) for the initiation of flowers and 120-155 DAS to pod maturity, depending on the genotype. Flowers are produced in pairs and the mature flowers open during the early hours of the day (07.00 – 10.00) they appear yellow-white at opening and towards evening they turn from yellow to light brown (Doku 1968; Onwubiko et al. 2011a). The cleistogamous flowers encourage inbreeding, resulting in high a percentage of self-pollination, which has been confirmed through analysis of offspring and single plants with microsatellite markers (Molosiwa et al. 2015) . Upon pollination and fertilization, the pedicel elongates to just below ground level to let the geocarpic pods grow, the pod reaches its mature size about 30 days after fertilization and the seed fully develops in the following 10 days (Doku 1968; Doku and Karikari 1970; Uguru et al. 2006; Basu et al. 2007b; Onwubiko et al. 2011a). A number of publications have focused on the floral biology and flowering time (Doku 1968; Doku and Karikari 1970; Linneman 1993; Linnemann and Craufurd 1994a; Heller et al. 1995; Oyiga et al. 2010b; Onwubiko et al. 2011a) and on fertilization/pollen germination (Oyiga et al. 2010a).

2.3.2.2. Importance of pollination and fertilization in agricultural crops

The statement "pollen is necessary for fertilization and formation of seeds" by 'CAMERARIUS' can be regarded as first published account (1964), stating the universal role of pollen in the plant pollination and fertilization process (Frankel and Galun 2012). From an agricultural perspective, in forage plants (cabbage, onion and potatoes etc.,) the vegetative tissues serve as yield and pollination may be of no importance beyond maintaining material to plant. However, proper pollination and fertilization are of extreme importance for yields where seed set is required (Frankel and Galun 2012). The world's most important crops such as rice, wheat, soybean and other major or minor crop yields depend on the successful pollination and fertilization processes as we depend primarily on their nutritive grains. Global grain production as of 2016/2017 was 1,070 million metric tons (mmt), 483 mmt, 755 mmt and 146.9 mmt of Corn, rice, wheat and barley respectively (www.statista.com 2017).

The final production/yields in crop species cultivated mainly for seeds/fruits are determined by the processes that takes place during the reproductive phase (Olesen and Bindi 2002; Rieu et al. 2017). Considering the socio-economic importance of such crops for our daily consumption, research has to focus on the effects of global warming on plant reproduction. Therefore, here we discuss the effect and implications of temperature stress on the reproductive development of plants from an agricultural and physiological perspective.

2.3.2.3. Implications of temperature stress on plant reproduction

It has been well established that environmental signals such as photoperiod and temperature play important roles during reproductive development in plants (Searle and Coupland 2004; Yamashita and Komeda 2010; Pachauri et al. 2014). In particular, plant reproduction is regarded as highly sensitive to temperature stress and is one of the limiting factors in crop productivity (Hedhly et al. 2009a; Rieu et al. 2017). As one predicted result of global warming is an increase in extreme temperature episodes, the reproductive phase could become more vulnerable (flowering, fertilization and seed set), particularly if heat stress effects crop fertility and thus reduces crop yields (Porter 2005; Porter and Semenov 2005; Thuzar et al. 2010; Rieu et al. 2017). The

direct consequence on the reproductive processes by temperature extremes has been documented previously, and the latest data on physiological processes that are affected by increasing temperatures also emphasize the vulnerability of the reproductive process to a changing climate (Hedhly et al. 2009a; Thuzar et al. 2010; Driedonks et al. 2016; Rieu et al. 2017).

The effect of temperature is extensively studied in many legume species. Heat stress significantly reduced stomatal conductance, leaf water content, chlorophyll, membrane integrity and photochemical efficiency in many Chickpea genotypes (Kaushal et al., 2013). Studies on common bean (*Phaseolus vulgaris* L. cv. Tenderette) showed a large reduction in pod set at 32/27°C (day/night) temperatures due to high levels of abscission of flower buds and young pods. This study suggested that the most temperature sensitive stages of plant development are the onset of Anthesis and pod development (Konsens et al. 1991). Similarly, a study on *Arabidopsis thaliana* (L.) Heynh., showed that a short duration of exposure to temperatures >33°C resulted in abortion of not only flower buds but also complete primary inflorescence (Warner and Erwin 2005).

A study on the relative leaf electrolyte leakage during reproductive stages in cowpea (*Vigna unguiculata* (L.) Walp.), provided information on the effect of heat in cowpea lines. Reduction of yield was observed in cowpea lines when incubated at high night temperatures (Ismail and Hall, 1999). In both cowpea and common bean, the reduction in pod yield is associated with the effect of high temperature on the number of flowers and successful pod set (Hall, 1992). A similar study on the red kidney bean cv. Montcalm, showed that despite the beneficial effects of enriched CO_2 during the reproductive phase of plant development, increases in temperature (>34/24°C) produced a reduction in seed yield (Vara Prasad et al. 2002). Similarly, when groundnut (*Arachis hypogaea* L.) was exposed to short duration (6 days) of hear stress (combinations of 28, 34, 42 and 48°C day and, 22 and 28°C night temperatures), there was a strong negative relationship between day temperature, number of flowers, pod set and pollen viability. However, increases in night temperature had no effect on the number of flowers, but had a negative effect on fruit set, pollen production and viability (Prasad et al., 1999, Prasad et al., 2000). These studies showed that sudden

increase in temperatures even for short duration surrounding reproductive phase severely affect crop fertility thus reducing crop yields.

The plant sexual reproduction process can be separated into three major biological events; gamete development, pollination-to-fertilization and seed development (post zygotic early embryo development). To study the effect of temperature on each of them has been proposed to be critical (Hedhly et al. 2009a). The response to temperature stress differs depending on the plant species, but both female and male reproductive tissues and/or functions are affected, along with the reproductive process itself. However, most of the work has concentrated on the male function, maybe because it is simpler to handle and the impact is most prominent (Rieu et al. 2017).

Many studies have shown that high temperatures can affect male reproductive function, such as quantity, morphology and pollen wall architecture of pollen grains, and anther dehiscence, along with chemical composition and metabolism of pollen (Aloni et al. 2001; Sato et al. 2002; Vara Prasad et al. 2002; Koti et al. 2005; Rieu et al. 2017). Detailed studies to evaluate the effect of temperature stress on pollen are carried out in many legume species to find sensitive stages during floral development. When plants of soybean were exposed high temperatures ($38/33^0$C) in combination with optimum CO_2 and UVB, the stressed plants produced small flower buds and a reduced number of pollen grains; pollen germination was also affected (Koti et al. 2005). Similarly, when plants of groundnut (*Arachis hypogea* [L.]) (6-days before Anthesis (DBA) and 6 days after Anthesis (DAA)) were subjected to high day time temperature treatments (up to 48°C) for a short episode of 1 - 6 days, there was a significant reduction in fruit set depending on the stages of flower buds. 4 DBA coincides with microsporogenesis, and anthesis coincides with pollination or fertilization. These are the most sensitive stages, due to pollen sterility or retarded pollen tube growth (Prasad et al., 2001). In the same study 33°C was determined as the critical bud temperature for groundnut plants, above which fruit set was reduced by 6% for every 1°C increase in temperatures.

Similar studies on heat tolerant and heat sensitive varieties of chickpea (Cicer arietinum L.) showed that the critical temperature for pod set failure was found to be ≥37°C and ≥33°C respectively. Some structural abnormalities such as changes in

anther locule number, anther epidermis wall thickening and pollen sterility were observed as a result of high temperature stress (Devasirvatham et al. 2013). This was supported by Kumar et al. (2013) in their study in which they concluded that heat stress leads to loss of pollen as well as stigma function and induces oxidative stress in the leaves that causes failure of fertilization and damage to the leaves, respectively. The above literature review clearly indicates that the critical temperature during plant reproduction may vary among plant species, however, in legume species ≥37 °C was found to be consistently critical. In our current research we took a temperature range of ≥33 °C to ≥37 °C as a reference point to start our study on the effect of temperature stress in bambara groundnut.

The extent of damage caused by temperature stress depends on the growth stage and type of plant tissue that is stressed (Hedhly 2011). The majority of food produced is a result of successful sexual reproduction of flowering plants. Many studies are based only on economically important plant species, in which the reproductive phase seems to be the most affected by temperature variations. As drastic temperature changes are possible in tropic and sub-tropical regions, understanding how different crops/plants cope with stress during the gametophytic (reproductive) phase is critical in managing the future of agricultural productivity (Hall 2000; Zinn et al. 2010).

2.3.3. Temperature stress studies in bambara groundnut

Bambara groundnut, despite gaining recent popularity for research, still has only few studies on its reproductive development, particularly with respect to temperature stress, the information gathered from the literature is detailed in this section.

The optimum temperature for seed germination of bambara groundnut ranges from 30.2 to 35.3°C (Massawe et al. 2003) and the optimal temperature for the vegetative growth is 20-28°C (Bamshaiye et al. 2011). The optimum temperature for crop development might differ between developmental stages. For example, the equivalent temperatures for soybean are; 15-22°C at emergence, 20-25°C at flowering and 15-22°C at maturity (Thuzar et al. 2010). Bambara groundnut is considered as a drought tolerant crop and known to survive and produce yield at high temperatures (Massawe et al. 2005b). However, there is very limited evidence on how different landraces of

bambara groundnut differ in their response to temperature stress. It is essential to quantify any genotypic and/or phenotypic variation during growth and development under controlled environment studies, allowing the extent to which these traits may be selected for breeding purposes to be investigated.

Many studies on the effect of temperature focuses on the combination effect of drought (Karunaratne et al. 2011b; Al Shareef et al. 2013) and photoperiod (Brink 1997; Karunaratne et al. 2011a). However, these results were mainly focused on measuring vegetative growth and pod yield. Any information on the effect of temperature on pollen viability or fertilization is yet to be clearly documented. A study of pollen germination potential of thirteen bambara groundnut landraces showed the influence of early and late planting in many of the cultivars. In all cases, pollen germinated *in vitro* immediately after harvest had the highest germination percentage, whereas the percentage of germination decreased with the increase in duration of exposure to high ambient temperatures (0, 5, 10 and 15 minutes). Pollen exposed beyond five minutes after harvest did not show any germination. This suggests that bambara groundnut pollen grains have short lifespan due to rapid loss of water under tropical conditions (Oyiga et al. 2010a). However, the direct effect of high temperature stress on bambara groundnut pollen grains is yet to be fully explored.

2.4. Bambara groundnut: prospects as a future crop

Given the urgency of addressing climate change, shifts in food production, and yield loss due to unpredictable and extreme weather events, a key strategy according to Massawe et al. (2015), to adapt to changing climates is the development and promotion of underutilized crops (disadvantageous, lost, orphan, neglected and understudied crops). Their argument is that, we are neglecting a vast repository of genetic resources and beneficial traits of underutilized crops, as we mainly depend on very limited numbers of major crops (such as rice, wheat, maize etc.,), which have largely been bred for high yield under high inputs. Genes for resilience may have already been lost during the breeding process(Mayes et al. 2012; Massawe et al. 2015).

There are more than 200,000 plant species in existence, out of which 80,000 of them are fit for human consumption. Despite the large number only 20-25 species provide for 95% of calories and protein intake (Fuleky 2009). Therefore, exploitation of minor and underutilized crops would provide much needed agricultural diversity and could allow the development of more sustainable agricultural systems in the face of climate change, enhancing nutritional security, generating income for resource poor farmers and promoting the conservation of cultural and dietary diversity. One of the reasons for low investment in research on underutilised crops is that underutilized crops may seem to have low profit/returns compared to the private breeding companies which focus on major crops (Nelson et al. 2004). To over come this barrier, it is important to strategize – there are thousands of underutilised crops in the world. One possible strategy is to look for crops with traits that currently surpasses the corresponding trait in major crops, especially where these traits are apparent in adverse environmental conditions, such as extreme heat and/or drought stress (Mayes et al. 2012). That is, some of the orphan crops can provide good models for traits such as drought tolerance, which are not necessarily found in major crops due to the almost exclusive previous focus on yield in high input environments.

A strategic approach to suggest/decide on one such underutilized crop is by trait analysis and study of physiological responses of the crop to climate change. The study on temperature and precipitation patterns in Arid, semi-Arid and tropical forest environments since 1983 showed bambara groundnut to be one of the resilient crops which can tolerate increased temperatures in semi-Arid regions (Singh et al. 2015). Likewise many research experts are considering bambara groundnut as a future crop based on its agro-ecological, cultural, genetic and nutritional importance (Azam-Ali 2010; Bamshaiye et al. 2011; Mayes et al. 2012; Massawe et al. 2015). There is also a large germplasm collection which is not utilized or characterized in many underutilized crops, which would potentially serve as an allele bank to dig out new trait versions from.

Through this literature review, we have shown that the sexual reproduction phase in flowering plants is an active developmental process subjected to various selective pressures. But the reproductive phase also provides the plant with an opportunity to

adjust to environmental variations. Understanding phenotypic malleability/plasticity and gametophytic selection for current temperatures, along with potential epigenetic variation during this process, could provide new understanding into plant adaptation and the potential for evolution under future global-warming scenarios. The prospect of that gametophytic development and selection might favour the best adapted genotypes to future temperatures is an appealing postulation that deserves advanced research, and it could also explain how plants rapidly adapt to different temperatures while sustaining variability. Therefore, in our present research our work is aimed at determining the importance of plant reproduction, especially at the gametophytic, pollination-to-fertilization and seed set/fruit formation stages, and their interaction with the current scenario of increasing extreme high temperature periods. This will provide a better understanding of the crop itself and how it could perform in future climate conditions.

2.5. Molecular biology

Once a suitable crop has been identified with end-user potential, the most productive improvements that can be made are in the genetics of the crops (Mayes et al. 2012). Advances in crop genomics have allowed a new cohesive understanding of the biology of many crop species. This also resulted in the development of a powerful set of molecular and bioinformatic tools. Genome sequencing is one such method which can be used to generate data, leading to an improved interpretation of the biology of the crop. A common approach could be, to sequence the underutilized crops genome using next generation sequencing technique and aligning or organizing the sequence scaffold with the use of reference genomes (soybean, *Lotus*, or *Medicago*) (Cannon et al. 2009). However, this process may depend on high investments for a single species, therefore NGS and other high throughput technologies to produce links between species without a full genome sequence potentially alleviates the absolute need for such efforts and allow us to import some of that knowledge from major and model species into underutilized species and test the potential of the approach, rather than having to generate data *de novo* for each species (Mayes et al. 2012).

2.5.1. Next generation sequencing (NGS)

The automated Sanger method is considered as first generation sequencing technology and later technologies are known as next-generation sequencing (NGS) (Metzker 2010). Over the last five to ten years there has been a significant change in the progress of crop genomics through the technological advancement of Next Generation Sequencing (NGS) and a reduction in their cost (Varshney et al. 2009b; Prabhu Dhanapal 2012; Dijk et al. 2014). This is bringing more plants into the range of genomic and transcriptomic analysis reach, which could help to generate substantial sequence data (RNA or DNA) where there has been little to no sequence information available especially in underutilized species (Michael and Vanburen 2015). With the completion of a complete genome sequence of many major and minor crop species, there are opportunities to accelerate legume crop improvement (Prabhu Dhanapal 2012; Kudapa et al. 2013). In this way, there is a shift from working on a single species to multiple species as a related complex in a cost effective manner. This will possibly allow us to identify what the genetic issues and potentials of such crop species are. The main NGS platforms are, Illumina HiSeq (Illumina Inc, San Diego, CA, USA), MiSeq; Roche 454/FLX (454 Life Sciences, Branford, CT, USA); ABI SOLiD (Applied Biosystems, Carlsbad, CA, USA) and Invitrogen Ion Proton (Invitrogen, Carlsbad, CA, USA) (Varshney et al. 2009b; Metzker 2010; Thudi et al. 2012; Edwards et al. 2013). Some of the third-generation sequencing technologies such as Pacific Biosciences (PacBio, Menlo Park, CA, USA) and Nanopore Technologies (Oxford, UK) can also assist in large scale genomic resource development (Metzker 2010; Munroe and Harris 2010; Thudi et al. 2012). These NGS technologies rely on a ‘combination of template preparation, sequencing and imaging, and genome alignment and assembly methods’ (Metzker 2010).

Application of advanced genomic tools has led to the construction of whole genome sequences of many legume species which are listed in the **table 1**. Genomic approaches such as transcriptomics, gene expression microarrays, map-based cloning and RNA-seq based gene expression profiling could be used in the discovery of stress (abiotic or biotic) related genes in several crop legumes (Kudapa et al. 2013).

Table 1: Draft genome sequences of legume species with potential as reference genome for bambara groundnut

Legume crop	Total estimated genome size (Mb)	Genome assembly size (Mb)	Total genes covered	Coverage (%)	Type of sequencing	References
Lotus (*Lotus japonicas*)	472	315	30799	67	Clone-by-clone and shot-gun sequencing	(Sato et al. 2008)
Soybean (*Glycine max*)	1115	973	46430	87	Sanger sequencing	(Schmutz et al. 2010)
Alfalfa (*Medicago Truncatula*)	454	262	62388	58	Illumina shotgun sequencing And BAC	(Young et al. 2011)
Pigeon pea (*Cajanus cajan*)	833.07	605.78	48680	72.70	Illumina (NGS) and sanger based-BAC	(Varshney et al. 2012a)
Chickpea (*Cicer arietinum*)	738	532	28269	72	Illumina sequencing	(Varshney et al. 2013)
Common bean (*Phaseolus vulgaris*)	637	521.1	31638	81.8	Pacific Bioscience Platform	(Phytozome 12)

Table 2: Expression Sequence Tags (ESTs) generated in legume crop species with NGS technologies under different stress conditions

Legume crop name	Stress	No. of ESTs generated	Type of sequencing	References
Soybean (*Glycine max*)	None	51,529	Illumina	(Libault et al. 2010)
Pigeon pea (*Cajanus cajan*)	Fusarium wilt	54,426	FLX/454	(Dubey et al. 2011)
Chickpea (*Cicer arietinum*)	Drought	80,238	Roche's 454 sequencing – SuperSAGE	(Molina et al. 2011)
	Drought and Salinity	103,215	FLX/454	(Hiremath et al. 2011)
	Drought and Salinity	20,162	Sanger sequencing	(Varshney et al. 2009a)
Common bean (*Phaseolus vulgaris*)	Drought	4219	Whole genome shotgun approach	(Blair et al. 2011)
	phosphorous stress	3344	Whole genome shotgun approach	(Blair et al. 2011)
	Leaf rust infection	6202	ABI 3730xl DNA sequencer	(Thibivilliers et al. 2009)

Huge number of transcript reads (ESTs [Expression Sequence Tags]) have been developed from different crop legumes using a range of developing and stress-responsive tissues, with the help of different NGS platforms (**Table 2**). The vast amount of data available in major crops on the genetic control of traits and their interactions with environment also allows in-depth comparisons to be made between underutilized crops with their major staple crop relatives (Dawson et al. 2009; Mayes et al. 2012; Michael and Vanburen 2015). A combination of Genome Wide Association Studies (GWASs), particularly using Genotype-by-Sequencing (GBS) approaches such as DarT Seq, which require no prior sequence information from the species and next generation mapping populations will improve our ability to link phenotypes and genotypes. Through genomic selection and improved genomic-technologies future crop improvement can be achieved (Prabhu Dhanapal 2012).

In the case of underutilized crops like bambara groundnut, which serves mainly as regional food source for the developing world, the investment investment towards the generation of genetic and genomic tools has been more limited. With whole genome sequence available from the reference genomes, Soy bean (*Glycine max*), *Medicago truncatula* and *Lotus japonicas*, the information can be deployed into minor species research. For instance, gene based markers can be developed for an orphan crop, using the reference genome of model species as a proxy by aligning its ESTs (Deokar et al. 2011) and/or GeneChip (Chai et al. 2017). Such approaches have been used in common bean, groundnut, barrel medic and some wild relatives of major crops (Deokar et al. 2011; Ford-Lloyd et al. 2011; Prabhu Dhanapal 2012; Varshney et al. 2012b), as well as bambara groundnut (Chai et al. 2017)

2.5.1.1. Model crop for bambara groundnut

The taxonomic relationship among the legume genera suggests that *Glycine max* (Soybean) and common bean (*Phaseolus vulgaris*) as the closest model crops for the genus *Vigna*, as it belongs to the same millettioid clade (**Table 3**). The extensive study based on *Matk* gene in legumes revealed the evolutionary sub-divisions in the millettioid clade,

which shows the divergence between the model crop (i.e., *Glycine max*) and bambara groundnut along with other legume species (Wojciechowki et al. 2004).

Table 3: Taxonomic position of major and minor crop species of the Millettioid Clade. Showing the relative closeness between the model crop, Glycine max (bold text) and bambara groundnut (underlined) in the tribe Phaseoleae (Cannon et al. 2009)

Clade	Tribe	Binomial name	Common name
Millettioid	**Phaseoleae**	***Glycine max***	**Soybean**
Millettioid	Phaseoleae	*Pachyrhizus erosus*	Jicama/yam bean
Millettioid	Phaseoleae	*Phaseolus lunatus*	Lima bean
Millettioid	Phaseoleae	*Phaseolus vulgaris*	Common bean
Millettioid	Phaseoleae	*Psophocarpus* spp.	Winged bean
Millettioid	Phaseoleae	*Vigna angularis*	Adzuki bean
Millettioid	Phaseoleae	*Vigna mungo* and *radiata*	Black gram; mung bean
Millettioid	Phaseoleae	*Vigna subterranea*	Bambara groundnut
Millettioid	Phaseoleae	*Vigna unguiculata*	Cowpea/black-eyed pea

The year 2009 marks the completion of the genome sequencing of three legume model crops, soybean (*Glycine max*), barrel medic (*Medicago truncatula*) and birds-foot trefoil

(*Lotus japonicas*). Both *Medicago truncatula* and *Lotus japonicas* being forage crops, serves as model crop for forage legumes. Soybean (*Glycine max*) is an economically important protein and oil crop, and is one of the best suited model crop to study seed and other developmental processes (Cannon et al. 2009). Gepts et al. (2005) recommended, soybean (*Glycine max*) as an effective model for many crop species in the Tribe Phaseoleae to which bambara groundnut belongs (**Table 3**). No single model crop is an only biological or genomic model for all the plants. For example, soybean have been used as a model to understand seed development, mineral uptake and protein and oil biosynthesis (Vodkin et al., 2008) . *Medicago* and *Lotus* have been used in the studies related to nodulation and signalling mechanisms during Rhizobial nodulation (Young et al. 2011). Common bean (*Phaseolus vulgaris*) is mostly used to determine molecular mechanism during photoperiod response and Scarlet runner bean (*Phaseolus coccineus*) has been used in the study of embryo development (see for review, Cannon et al. 2009).

There is a major qualitative difference between model organisms and major economic organisms (Jonkers 2009), therefore it may be helpful to think of more than one model crop for bambara groundnut depending on the area of interest. Since the main aims of the present study was to study the reproductive biology of bambara groundnut which includes development of flower and pollen grains, and RNA-seq gene expression profiling studies under temperature stress, multiple model crops can be considered for biological and genomic studies. The advancement in high throughput and low cost NGS technologies made a way to sequence orphan/underutilized crops, which itself can be established as new model crop (Michael and VanBuren 2015). With the advent of RNA-seq (parallel sequencing of cDNA), researchers can adopt this approach for non-model organism research, to quantify expression profiles in high throughput and cost effective manners (Ellegren 2014; Finseth et al. 2014).

2.5.1.2. A new approach for underutilized crops improvement without reference genome

Gene expression profiling is widely used to study plant-stress regulatory mechanisms that control cellular processes (Van Verk et al. 2013). RNA-seq extends a possibility to analyse previously unknown genes through transcriptome analysis and it also offers a dynamic range of quantification at reduced cost, which is more favourable in the species (orphan/underutilised crop species such as bambara groundnut) with no reference genome. Until recently researchers mainly used the Roche 454/FLX (Roche Diagnostics Corporation) platform for *de novo* transcriptome assembly (Ellegren 2008; Wheat 2010). However, a comparative study using Roche 454/FLX and Illumina platforms for de-novo transcriptome analysis and downstream RNA-seq application in the absence of reference genome (in non-model species) showed that Illumina platform alone produce high quality reads appropriate for RNA-seq gene expression analysis (Finseth et al. 2014).

An outline of RNA-seq experiment includes steps illustrated in the **figure 6**. A typical RNA seq experiment starts with a research question and a good experimental design to address the later. Followed by isolation of RNA and subsequently conversion of this RNA into cDNA to form the RNA-seq library. By sequencing the millions of DNA fragments (also known as reads) in the library with NGS platforms, an accurate measure of the relative abundance of each transcript and splice variant can be obtained. The final step and perhaps the most challenging one of all would be to process the sequence data and translate into information on the gene expression levels using bioinformatic tools (Metzker 2010; Cresko Lab 2012).

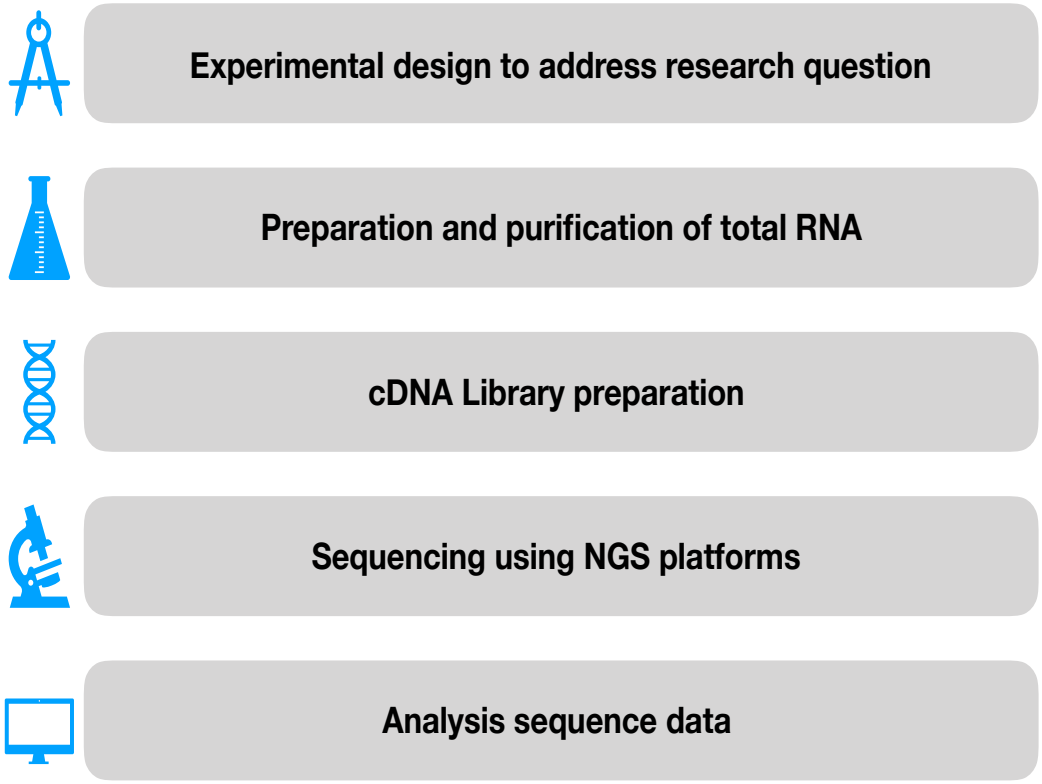

Figure 6: Experimental workflow of typical RNA sequencing experiment.

a) Experimental design

This step of the experiment is the most important but often receives least attention (Conesa et al. 2016). The main objective of the experimental design is to identify the primary research question / goal or to test the null hypothesis of no difference between or among the different treatment groups. There are typically two types of information/data that can be gained from an RNA-seq experiment such as qualitative (annotation) and quantitative (differential gene expression [DEG]) (Metzker 2010; Cresko Lab 2012). Qualitative data includes identifying expressed transcripts, exon/intron boundaries, poly-A sites and transcriptional start sites (TSS). Quantitative data includes measuring differences in the expression, alternative splicing and TTS, and alternative polyadenylation between treatment groups. Some of the differences and criteria to choose between the two types are described in **table 4**. Even though the two types, annotation and quantification are to

some extent related and inseparable, it is important to realize that experiments that are designed to measure DEG and annotation have different requirements. Therefore, it is important to design the experiment to maximize the objective, with respect to our research goal DEG was preferred in our present research, detailed in **chapter-5**.

A good experimental design considers aspects such as library type, number of replicates appropriate for the treatment and sequencing depth (Conesa et al. 2016). The number and type of replicates are driven by extrinsic factors and intrinsic factors and it is important to build the experimental design around them. Some of the extrinsic factors are, availability of samples, cost and feasibility of the experiments. However, intrinsic factors are more ambiguous and difficult to grasp without prior knowledge, such as degree of transcriptional variability among tissues and ambiguity in the presence of genes in tissue of interest. Therefore, careful consideration was made in our present study based on the afore mentioned factors, to allocate our resources to both biological and technical replicates. 'Differential expression studies using RNA-seq data need to be replicated in order to estimate within- and among-group variation' (Cresko Lab 2012; Conesa et al. 2016).

Table 4: Criteria for Qualitative and Quantitative data, and recommendations for RNA-seq (Cresko Lab 2012).

Criteria	**Qualitative (Annotation)**	**Quantitative (Differential Gene Expression)**
Objective	Identifying genes and genetic architecture	Quantitative measure of differentially expressed reads between treatment groups
Biological replicates	Not necessary but can be useful	Essential*
Coverage across the transcript	Important for de Novo transcript assembly and identifying transcriptional isoforms	Not as important; only uniquely mappable reads are useful
Depth of sequencing	High enough to maximize coverage of rare transcripts and transcriptional isoforms	High enough to infer accurate statistics
Long reads (>80 bp)	Important for de Novo transcript assembly and identifying transcriptional isoforms	Short-reads are preferred, especially without reference genome*
Paired-end reads	Important for de Novo transcript assembly and identifying transcriptional isoforms	Important if reference genome is not available *
Role of sequencing depth	Obtain reads that overlap along the length of the transcript	Get enough counts of each transcript such that statistical inferences can be made
Limitations	Unable to interpret short-read sequences without reference genome	Simple read counts alone are not enough for accurate measurement

Similarly, one of the simplest type of differential expression study design can be to compare two groups, that is, experimental and control. If the design is more complex it is important to introduce sufficient replication at each level of comparison. By considering all the above factors, once the experiment set up is completed it is recommended to sequence a subset of the replicates from a larger experiment, to get a handle on coverage level and sequencing depth, this would leave plenty of remaining samples for additional sequencing (Cresko Lab 2012).

b) RNA preparation and purification

The success of RNA-seq experiment is greatly dependant upon recovering pure and intact RNA (Conesa et al. 2016). Since the goal of the experiment is to measure the transcriptome levels, the first step involved is the isolation and purification of RNAs from the selected tissue type. The first step in the isolation of RNA is homogenization by disruption of tissue either by mechanical or chemical disruption. The homogenized cell-lysate can then be used to extract total RNA, which later can be enriched into mRNA (messenger RNA). The Quality and quantity/integrity of RNA needs to be assessed before proceeding to the next step, some of the commonly used methods are UV absorbance (to check the quantity) and Agilent Bioanalyzer (to check quality and integrity). Some of the recommendations and precautions needs to be taken while working on RNA are listed in the **table 5**.

c) Library preparation:

After obtaining quality RNA it must be converted into double-stranded complementary DNA (hereafter 'cDNA'). Currently available NGS technologies require DNA with platform-specific adaptors. This step is performed by professionals before sequencing, by adding the adaptors and amplifying the cDNA. This marks the completion of library preparation. Some of the new NGS platforms are capable of sequencing multiple samples and generate millions of reads in one reaction, therefore it is more desirable to pool libraries from multiple experiments into a single reaction, this process is also known as 'multiplexing'(Cresko Lab 2012)

Table 5: List of considerations while working with RNA (ThermoFisher 2017)

Topics	Consideration and Precautions
RNA reagents	• Keep separate reagents and consumables and avoid storage with kits (including Rnases) • Keep the RNA on ice and process quickly • Use Rnase free water or water treated with diethyl pyrocarbonate (DEPC) • Use only autoclaved glassware or NaOH/EDTA treated plastic wear
Starting material	• Tissue samples must be stored frozen in liquid Nitrogen immediately after harvest. • Alternative method is to store tissue samples in reagents to preserve RNA in fresh tissue. Eg., RNAstable® (Biomatrica) and RNAlater® (Qiagen) • Tissue concentration is critical therefore do not start with too much starting material
Homogenization and organic extraction	• Ensure complete disruption of tissue and solubilization in buffers • pH needs to be maintained using TRIS and sodium acetate buffers during RNA solubilization • homogenates from high-fat tissues needs to be extracted with chloroform to remove phenol contamination
Extraction methods	• Organic extraction is cheaper and easier to scale up for larger amounts of starting material • Silica columns (RNA extraction kits) are expensive but easier to use. They are more amenable in processing multiple samples in parallel. • Protocols for extraction of mRNA are not optimized for small RNA such as siRNA, piwiRNA and miRNA.
DNA contamination	• Traces of DNA can be removed by digestion with Dnase I

d) Sequencing

It is critical to choose sequencing platforms when designing an RNA-seq experiment. The current leading sequencing platform for RNA-seq is undoubtedly Illumina (Metzker 2010). The Illumina Hiseq preferably produces longer read-strands (currently 120-150bp) and the sequences can be directly assembled into a de-novo transcriptome. In order to increase the sequence coverage of a gene, NGS platforms allow sequencing from both the ends, this is known as 'paired end sequencing'. However, Corley *et al.* (2017) reported that Single-end reads used in accordance with gene set enrichment can generate accurate biological results.

e) Analysis of sequence data

The analysis and interpretation of the sequence data obtained from sequencing are among the most complex problems for research scientists. The nature of questions people can address using the RNA-seq data is practically infinite, therefore there are more aspects to analyse the data than there is to generate one (Cresko Lab 2012). There are practically two types of computational methods available for transcriptome reconstruction (Grabherr et al. 2011; Conesa et al. 2016) (**Figure 7**). One is mapping-first approaches, in which the reads are aligned to reference genome first and merged with over-lapping alignments. The second one is assembly-first method (*de-novo*), in which the transcripts are assembled first using the reads generated. The key differences and points of these two methods are listed in the **table 6**. The RNA-seq analysis pipeline for species with and without reference genome is illustrated in the **figure 7**. Both the approaches commonly share a number of steps, including; demutliplex, filtering and trimming sequence reads, during which each read can be traced back to its original sample using index sequence and filtered, and later binned accordingly using the respectively platform software available (eg., Illumina TruSeq). The adaptor cDNA sometimes are sequenced if the read length is small, therefore cDNA inserts are trimmed to improve mapping of sequencing reads (Cock et al. 2009). Normalizing of reads is a necessary step for assembling millions of reads de-novo into reference assembly. The common method to normalise and screen the data is k-mer

distribution statistics, this helps to address issues such as sequence variants and errors, and redundancy (Catchen et al. 2011; Brown et al. 2012).

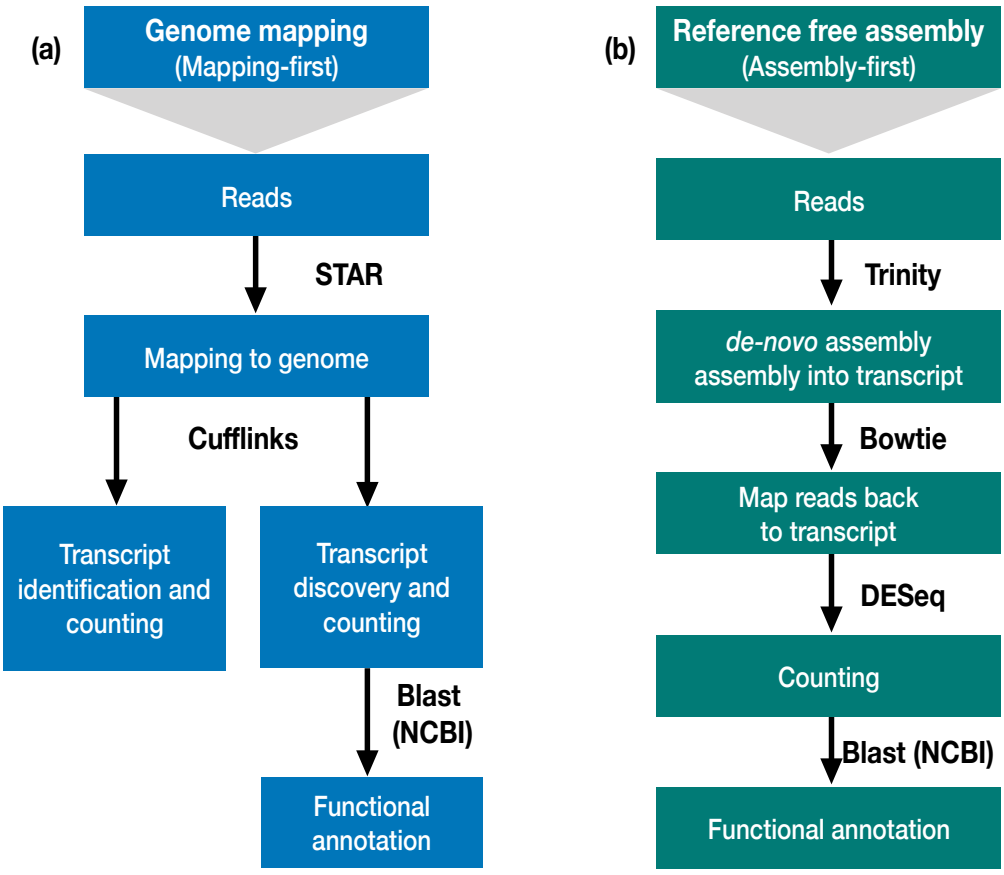

Figure 7: Basic strategies of RNA-seq experiment; (a) Genome mapping (mapping-first approach) and (b) Reference free assembly (Assembly-first approach).

De-novo transcriptome assembly is the general approach for the species without a well-assembled reference genome (Conesa et al. 2016), which holds true in many orphan/underutilized crop species. This approach allows a reference transcriptome to be build from of RNA-seq reads, by assembling and reconstruction of contiguous sequences (hereafter 'contigs'). Construction of the reference transcriptome with contigs is a powerful way to discover and annotate transcribed regions of the genome. If the objective of the study is to quantify and compare transcript abundance between samples or

treatments this approach can be useful (Li and Dewey 2011). Some of the softwares used during this step are, Velvet/Oases (Zerbino and Birney 2008; Schulz et al. 2012), Trans-AbySS (Simpson et al. 2009; Robertson et al. 2010) and Trinity (Grabherr et al. 2011). Out of all these, Trinity produces a transcriptome assembly through a three step process, with each step named as "Inchworm", "Chysalis", and "Butterfly" (Grabherr et al. 2011). The first step Inchworm, builds initial contigs through k-mer graphs. Chrysalis groups these contigs with sufficient k-mer overlap and builds groups. Butterfly simplifies these graphs and reconciles these graphs with the original read data, to output unique splice variants and paralogous transcripts. Oases and trinity assemblers are the most commonly used. The Trinity approach was used to analyse the transcriptome data of bambara groundnut and three other under-utilised legume species by Chapman (2015).

Table 6: Key differences and important points between the mapping-first approach and assembly-first approach (Grabherr et al. 2011)

Criteria	**Mapping-first approach**	**Assembly-first approach (de-novo assembly)**
Overview	Map the reads to reference genome first	Assemble the reads into transcripts first
Approaches	Scripture (Guttman et al. 2010) and Cufflinks (Trapnell et al. 2010)	AbySS (Birol et al. 2009) and SOAPdenovo (Li et al. 2009)
Characteristics	•Highly sensitive •Depend on the read-to-read reference alignment	•Do not need any reference alignment •Best for the species without reference genome
Reference genome	Required and highly dependant	Not required and if available mapping can be done subsequently

The next step during analysis is mapping or aligning the sequence reads to the reference genome. In the case of species without reference genomes the sequence reads are aligned with the transcriptome constructed via *de-novo* assembly. The objective of aligning RNA-seq reads to the reference genome is to discover the origin or location of each read with respect to the reference. Some of the aligner software are Bowtie, BWA and Gsnap (Shang et al. 2014). The next step is to annotate the transcripts assembled and this is followed by counting the abundance of transcripts. In the event that a reference genome is available annotation is relatively straightforward, as many databases will have genomic and biological information on the reference genome. Where as *de-novo* transcriptome assembly needs to be annotated from scratch using NCBI – supported Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) and "BLAST+" (Camacho et al. 2009). The NCBI and Gene Ontology databases give useful information on transcripts based on their "biological process, cellular component and molecular function", via available sequence entries in the database (NCBI 2017; Stover and Cavalcanti 2017). However, they do not usually give a confirmed orthologous identity.

The ultimate objective of RNA-seq study is to identify differential expression patterns between treatment groups or tissues. The most commonly used robust differential expression packages are DESeq (Love et al. 2017) and edgeR (Robinson et al. 2010). Clearly there are many choices when it comes to the packages available to estimate transcript abundance, hence careful consideration based on some parameters such as sample size and over-dispersion need to be made. There are several reviews which compared these models, yet these have not determined a "clear leader" , instead it has been proposed to combine and compare multiple methods to diminish bias (Kvam et al. 2012; Rapaport et al. 2013; Conesa et al. 2016; Zhang et al. 2017).

2.5.2. Scope for new discovery in bambara groundnut

Bambara groundnut (*Vigna subterranea* [L] Verdc.) has 22 (2n=2x) chromosomes (Forni-Martins 1986). The crop still lacks a fully annotated and complete genome, even though efforts are underway by the African Orphan Crops Consortium (AOCC) to sequence the

bambara groundnut genome in future, it will be some time before the complete genome is available (AOCC). There are several of studies dedicated to developing high density linkage maps, to facilitate the identification of loci (regions) containing important genes for downstream applications such as marker-assisted selection (MAS) during crop-breeding and positional cloning (Chai et al. 2017). The first interspecific linkage map of bambara groundnut was 520cM (centimorgan) in length and consists of 20 linkage groups. It was constructed using an F_2-segregation population derived from the crosses between VSSP11 (wild-type) and DipC (Domesticated line) (Basu et al. 2007b). The map was constructed using 65 amplified fragment length polymorphism (AFLP) markers and one single sequence repeats (SSR) marker. This interspecific map was applied to localise four Quantitative Trait Loci (QTL) markers with respect to seed weight (100 seeds), specific leaf area, number of stems per plant and carbon isotope (Basu et al. 2007a). The first intraspecific linkage map using an F_3 population obtained from the cross between domesticated lines 'Tiga Nicuru' and 'DipC' of bambara groundnut, was reported by Ahmad et al. (2016). This intraspecific map covered 608.6 cM length in 21 linkage groups and comprises of 29 SSR and 209 micro-array based DarT marks. The same map was used to map 36 QTL markers for 19 agronomic traits which includes internode length, peduncle length and biomass (Ahmad et al. 2016).

Most recently a Gene Expression Marker (GEM) map for bambara groundnut was constructed using the Affymetrix Soybean Genome GeneChip (Chai et al. 2017). This GEM map is 982.7 cM in length, mainly consisting of 13 linkage groups with 208 GEMs. Qualitative Trait Loci (QTL) analysis using the GEM map under drought conditions suggested the presence of 31% unique QTL for treatment group alone. The current GEM map in bambara groundnut provides one of the routes for translational genomic studies from major and model plant species to underutilised and resource-deficient crops (Chai et al. 2017; Ho et al. 2017). That is using the extensive resources available in major and model crops such as soybean (*Glycine max*) and common bean (*Phaseolus vulgaris*), potential candidate genes that control important agronomic traits in bambara groundnut could be detected, by projecting the QTLs onto genetic map or physical map of a model crop of choice (Ho et al., 2017).

Chapman (2015) reported leaf transcriptome development, analysis, annotation and mined SSR and Conserved Orthologous Set (COS) markers. The transcriptome was developed from RNA extracted from the true leaves of bambara groundnut seedlings, grown at control environment (16h day length). The NGS platform, Illumina-HiSeq 2500 platform was used for sequencing a partial lane with 100bp paired-end (PE) reads with shallow sequence depth. The sequence data was normalized using k-mer coverage of 30 and then analyzed by assembly-first approach (*de-novo* method), using 'Trinity'. The reads were then mapped back to previously generated transcriptome to assess heterozygosity. The functional annotation of transcripts was assessed by BLASTn aligning the transcripts with the common bean genome and SSR markers were developed. COS markers were also developed through 'reciprocal BLAST'. The in-depth details of genetic information available is highlighted in **table 7**.

Still a great deal of work remains; to develop mapping populations for underutilized crops to study traits of interest such as drought tolerance, photoperiod and temperature stress, and to characterize more traits and genes in model crops, to extend new genomic tools to underutilised crops. Our current research is one step closer to achieve the goal, by implementing advanced technologies and resources available into bambara groundnut research.

Table 7: Summary of bambara groundnut genetic information. [[1]Chapman (2015) and [2]Bennett MD and IJ (2012)]

Descriptors	**Bambara groundnut (Vigna subterranea [L] Verdc.)**
No. of reads	7,887,745 [1]
No. of normalized reads	2,821,597 [1]
No. of genes	34,401 [1]
No. of transcripts	47,759 [1]
Total no. of assembled bases	37,938,088 [1]
Total no. of SSR markers	1305 [1]
DNA C-value	880.20 Mbp [2]
% of GC	42.82 [1]

Over all, this chapter provides a detailed research review of bambara groundnut crop, an underutilized crop with superior agronomic values, such as drought tolerance and yield resilience under low input system, and we have also emphasized the potential of bambara groundnut as a future crop. There exists a knowledge gap in understanding reproductive biology and its interaction with high temperature stress, in bambara groundnut crop, these were the focus of the future chapters.

CHAPTER 3

Reproductive biology of bambara groundnut

3. Introduction

Plant reproduction, particularly pollen formation, is regarded as highly sensitive to environmental conditions such as temperature, photoperiod and drought, especially high temperature is one of the limiting factors in crop productivity. Since the major result of global warming is an increase in average temperature, this may have an effect on the reproductive phase of many crop plants (Thuzar et al. 2010). However, knowledge on plant reproductive biology in bambara groundnut is limited to only few studies (Doku 1968; Doku and Karikari 1970; Linneman 1993; Linnemann and Craufurd 1994a; Heller et al. 1995; Oyiga et al. 2010b; Onwubiko et al. 2011a), which lack detailed characterization and fundamental understanding of its reproductive biology. Therefore, this chapter describes the study undertaken to deepen understanding of bambara groundnut reproductive biology, with a particular emphasis on inflorescence, flower development and pollen formation.

3.1. Experiment 1: Flower

Flowers are the most complex structures of the plants, the flower encloses specialized structures such as stamens (male organs) and carpels (female organs) (Alvarez-Buylla et al. 2010). Eudicot flowers are arranged into four concentric whorls of organs, such as sepal (calyx), petal (corolla), stamen (androecium) and carpel (gynoecium) (Scott et al. 2004). The ABC model of floral development and morphology provides a basis for understanding the genetics behind the formation of these four whorls in the flower (Bowman et al. 2012). The flowers also distinguish the most important and recently diverged plant lineages, Angiosperms (Alvarez-Buylla et al. 2010). The important function of the flower is to ensure fertilization of the ovule and development of fruits containing seeds.

Arabidopsis consists of typical eudicot flowers which are studied extensively and the main processes and stages of flower development provides a fundamental framework to study the same in other flowering plants (Smyth et al. 1990; Bowman et al. 2012). Similarly *Medicago truncatula* being a model legume crop also provides a fundamental framework to study the flower development process in legumes (Benlloch et al. 2003), in this case bambara groundnut. Both *Arabidopsis* and *Medicago* flower development scales were reviewed and used to understand the bambara groundnut flower development in our study.

3.1.1. Materials and methods

3.1.1.1. Plant materials and growing conditions

Seeds of bambara groundnut (*Vigna subterranea* (L.) Verdc) genotype 'IITA-686' were germinated in modules with Levington seed and modular compost (F2) in a Controlled Experiment (CE) room, University of Nottingham (Sutton Bonington Campus, UK) with 28/22ºC as day/night temperatures and 12 hours of photoperiod. The detailed growth conditions are listed in the **table 8**. Fourteen days after seed germination these seedlings were transplanted into 5 L pots with a mixture of John Innes No. 3 compost: sand (50:50%). The plants were watered regularly to keep the top soil surface moist throughout the lifecycle.

Table 8: Experimental conditions maintained in CE room

Criteria	Experimental conditions
Soil composition	JI03 compost: sand (50:50)
Photoperiod (day/night)	12/12 h
Temperature (day/night)	28/22 ºC
Light intensity	20,000 Lux (400 to 700 nm photo-synthetically active radiation)
Relative humidity	58±2 %

3.1.1.2. Tissue preparation

Bambara groundnut flower buds ranging in size from ≤1 mm (buds) to 9 mm (opened flower), were used to study flower development using light microscopy and scanning electron microscope (SEM). Flower buds were freshly harvested and dissected using forceps and scalpel, to obtain photographs using the light microscope (LEICA model). The flower size was measured using Image J software (National Institute of Health, US, Version 1.4). For SEM, the specimens were prepared by following the protocol described by Benlloch et al. (2003); freshly harvested inflorescences were vacuum infiltrated for 15 min with FAE solution (formaldehyde: acetic acid: ethanol: water, 3.5:10:50:26.5; v/v/v/v) and fixed overnight at 4^0C in fresh solution. The samples were dehydrated in a 30, 60, 90 and 100 (v/v)% ethanol series. The samples were then critical point dried with liquid CO_2 in a SAMDRI-780 (Tousimis). The brittle samples were mounted onto a self-adhesive carbon disk (12mm-diameter, TAAB) attached to the cylindrical studs (Agar Scientific), and the outer whorls of calyx and corolla were carefully removed under a dissecting microscope using a fine needle. The mounted samples were then coated with a gold-palladium sputter coater (SCD-030, Balzers). SEM was performed using a JSM-840 scanning microscope (Advanced microscopy division, QMC [Queens Medical college], Nottingham, UK). The parameters used were 22.0 kV and 6×10^{-10} Amp of probe current. The digital images were captured and stored in JPEG format, before formatting and processing for publication. The process of sample preparation and microscopy is illustrated in **figure 8**. During SEM imaging phenological characteristics such as size of flower buds and, underlying anther and stigma development patterns were noted.

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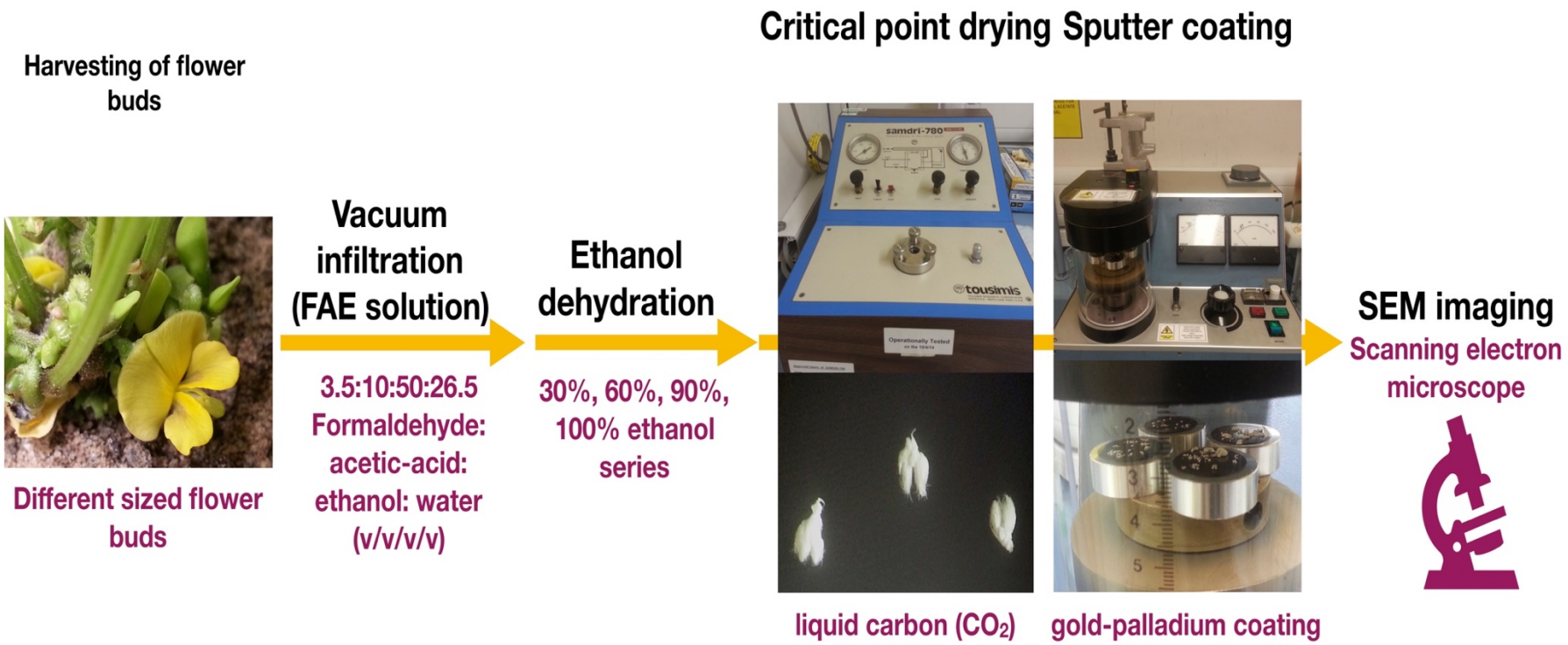


Figure 8: Flow-chart of sample preparation steps for SEM (Scanning electron microscope) imaging; briefly, the harvest flower buds (different size) were vacuum infiltrated (FAE solution) and dehydrated with an ethanol series, followed by critical point drying and sputter coating with liquid CO_2 and gold-palladium respectively, and SEM imaging follows.

3.1.1.3. Model flower development scale

A flower development scale of Arabidopsis and the model legume Medicago truncatula was adapted following the observations by Alvarez-Buylla et al. (2010) and Benlloch et al. (2003) respectively (**Table 9**), to determine the landmark events in bambara groundnut flower ontogeny. The stages 1 to 7 of flower development show similar development events in both model species (Arabidopsis and Medicago). From stage 8 to 14, the Arabidopsis development scale was more descriptive, whereas the Medicago development scale summarizes all those events into stage 8. Careful considerations were made to adapt and compare both the scales to the development pattern in bambara groundnut flower. The floral formulae and floral diagram were developed in accordance with unicode system based on standardized descriptors outlined by Ronse de Craene et al. (2014), Prenner et al. (2010) and Beentje (2010)

Table 9: Landmark events during early flower development in Arabidopsis and Medicago truncatula.

Stage	*Arabidopsis thaliana* (Alvarez-Buylla et al. 2010)	*Medicago truncatula* (Benlloch et al. 2003)
1	First sign of flower primordia formation	Unidirectional initiation of primary (I_1) and secondary (I_2) inflorescence meristem and leaf primordia (L)
2	Hemispherical growth of flower primordia	I_2 differentiates into floral meristem
3	Sepal primordia becomes visible and overtops the flower primordia	Sepal primordia initiated at abaxial (ab) and lateral sides
4	Elongation of pedicel and completely covered flower primordia	Petal and stamen primordia becomes visible. Adaxial (ad) sepals become visible
5	Petal and stamen primordia becomes visible	Differentiation of abaxial (ab), adaxial (ad) and lateral petal primordia. And abaxial, lateral and vexillary stamen primordia
6	Sepal primordia completely covers floral bud and stamens become distinct by bulging out	Sepal elongates to completely cover floral bud. Antesepalous and antepetalous stamen differentiation.
7	Stamens become stalked and forms anther lobes	Stamens are stalked and anther locules are visible. Corolla differentiates into standard and wings. Keel petals fuses enclosing carpel and stamens
8	Anther locules become distinct and appear convex	Stigmatic papillae and staminal tube form. Formation of Staminal tube. Corolla elongates and overlaps flower-bud Pollination and fertilization Carpel forms into pod and fertilized ovule develops into seeds.
9	Petal primordia elongates. rapid lengthening of all organs	
10	Stigma cap forms and petal reaches lateral stamen length	
11	Formation of stigmatic papillae. Petal reaches half the stamen length	
12	Rapid lengthening of petals and sepals. Gynoecium and stamens lengthen coordinately.	
13	Visible calyx between corolla. Elongation of stamen filaments and receptive stigma. Pollination and pollen tube growth	
14	Fertilization and seed development	

3.1.2. Results-1

3.1.2.1. Phenotypic description of bambara groundnut inflorescence and flower

Bambara groundnut (*Vigna subterranea* (L.) Verdc.) inflorescence is depicted in **figure 9a** by an original illustration. The inflorescence is racemose (pseudoreceme), which terminates with two flowers at each nodal region. The bambara groundnut shoot apical meristem (I_1) produces lateral compound leaves (L) with three leaflets (tri-pinnate) and an axillary inflorescence meristem (I_2) (**Figure 9a**). Each axillary meristem (I_2) differentiates into a secondary inflorescence terminating with two flowers and the peduncle of I_2 terminates in a collar of tissue with an abaxial bract and a pair of adaxial bracteole. The floral buds in bambara groundnut develops in the axil of each bract.

The floral formulae of bambara groundnut is **B Bt_2 % ↓ ♂ $K_{(5)}$ $C_{1+2+(2)}$ $A_{1+(9)}$ $\underline{G}_{(1)}$** and the floral diagram is depicted in the Figure 1b, designed in accordance with Unicode system, shows the flower is bracteate (B), bracteolate (Bt) with 2 bracteoles and zygomorphic (%) with a single plane of symmetry in median direction (↓). The flower was pentamerous with alternating whorls of sepals (K), petals (C), two sets of stamen (A) whorls and a single carpel/gynoecium (G) with superior ovary. The characteristic aestivation of this flower is vexillary imbricate in a pattern called descending cochleate (imbricate) (**Figure 9b**). The flower consists of a prominent corolla (arrangement of petals) with three petal types, arranged along the dorsoventral (DV) axis (**Figure 9b and c**). The corolla of the flower consists of a posterior Standard or Vexillum petal (St), which overlaps the two lateral wings or Alae (bambara), and two boat shaped petals towards the anterior side called keel or carina (k) which in turn are over lapped by the Alae (**Figure 9c**). The wing (bambara) and keel (ke) petals remain enclosed inside the large standard petal, until the flower opens. The standard petal is bilaterally symmetric and usually coloured bright golden-yellow in the cultivated varieties, whereas a few wild varieties show dark pigmentation. The pale yellowish keel encloses the stamens and carpel. The Bambara groundnut androecium is asymmetric with diadalphous stamens arranged in two whorls with single vexillary stamen and 9 stamens with partly fused filaments (**Figure 9d**). The corolla is

surrounded by calyx (sepals), the five sepals are fused together in valvate aestivation. Both the calyx and epicalyx (arrangement of bracts) enclose the corolla completely during the early developmental stages.

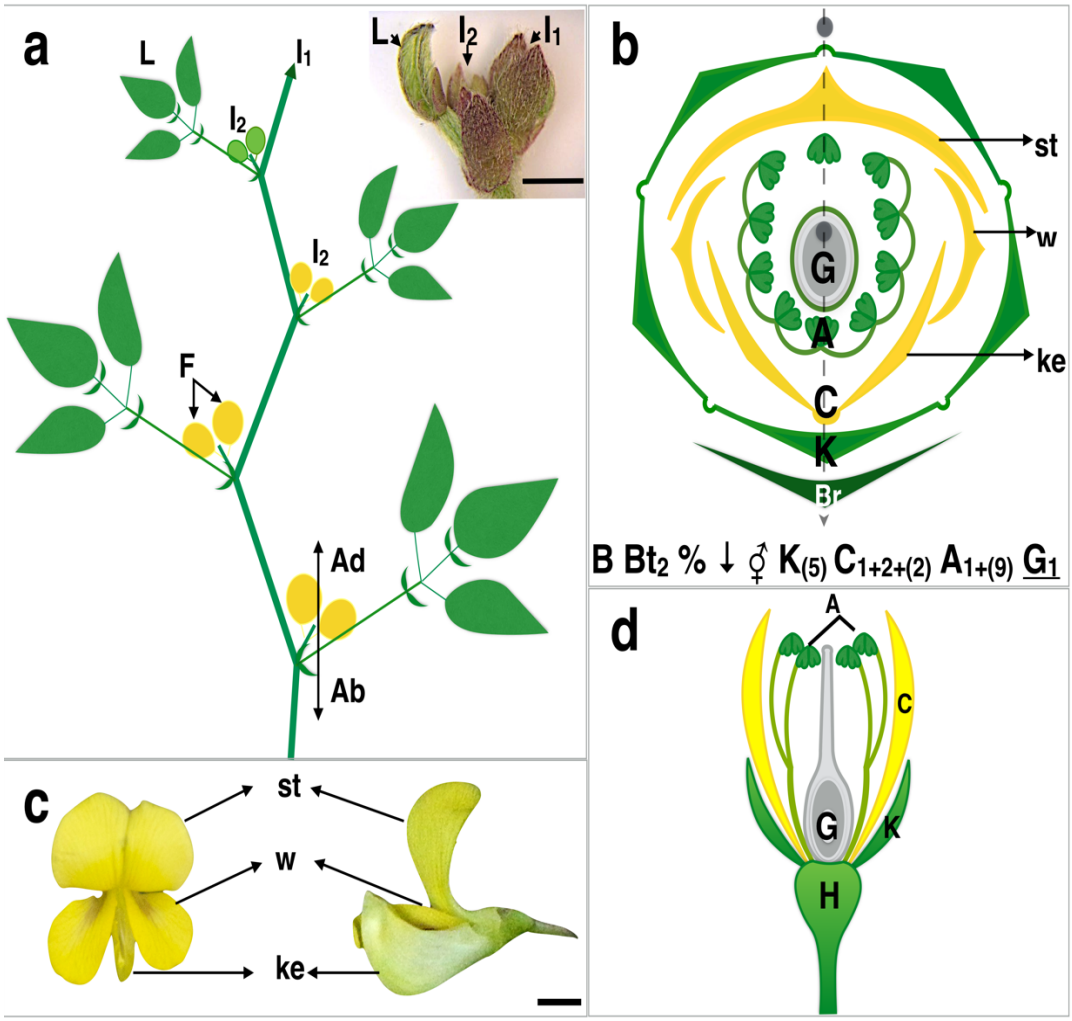


Figure 9: (a) Diagrammatic representation of bambara groundnut lateral shoot depicting axillary meristem (I_1), lateral inflorescence meristem (I_2), inflorescence with two flowers (F) [adaxial (Ad) & abaxial (Ab) side], at right hand top corner shoot apical meristem of bambara groundnut [scale bar = 2 mm]; (b) Floral diagram and floral formulae of bambara groundnut flower showing pentamerous arrangement of sepals (K) and petals (C) (St- standard, w- wing and keel petals), diadalphous ([9]+1) arrangement of stamen (A), gynoecium (G) and outer bract (Br) and Bracteole (Bt); (c) The flower showing arrangement floral parts of bambara groundnut flower, standard petal (st), two wing petals (w) and boat shaped keel (ke) [scale bar = 2 mm]; and (d) illustration showing ovary position as superior with hypanthium (H).

3.1.2.2. Flower development scale in bambara groundnut

In the current study, based on light microscopy and SEM observations we have identified the different stages of flower development in bambara groundnut from the time the flower buds were visible at I_2. A progression of floral development through characteristic stages is as shown in closed and dissected flower buds along with SEM images (**Figure 10**)

The flower development stages were determined from the smallest flower bud of 1-2 mm size and still surrounded by hairy protective bracts (**Figure 10a**). The first whorl of stamens becomes distinct and stalked at the base, the filaments for the first whorl of stamens are longer than the second whorl (**Figure 10a [& j]**). It is also defined with fully developed anther locules, which are characterized by visible convex protrusions on the anther lobes in both stamen whorls. At the centre of flower primordium, the carpel/gynoecium grows into a hollow tube. Sepals and bracts remain completely covering the flower buds. The gynoecium growth keeps pace with long stamens and morphologically changes from symmetric to bilateral symmetry forming a small protrusion at the tip. This stage corresponds to **Stage 8** of both *Arabidopsis thaliana* and *Medicago truncatula* flower development. **Figure 10b [& k]**, marks the completion of **stage-9** and **stage-10** of flower development i.e., the growth of sepals keep pace so that the bud remains completely closed and enclosed by bracts. The rapid lengthening of all organs such as petals and stamens filaments also occurs. The tip of the gynoecium differentiates into cap-like papillae that will constitute the stigma cap. **Stage-11** (**Figure 10c [& l]**), begins when the upper surface of gynoecium starts to form a small rounded protuberance, also known as stigmatic papillae, at the ventral side of the stigma and the c-shaped cap begins to form. At the end of the stage 11 the bud starts to appear visibly asymmetric (with single plane of symmetry) and the calyx tip start to open. **Stage 12** (**Figure 10d [& m]**), all five petals continue to lengthen rapidly and cover stamen and carpel. The gynoecium elongates while the stamen filaments grow slowly. The upper part of the gynoecium becomes differentiated into the C-shaped cap and short style with the constricted base forming a small cylinder to give space to the developing ovary. At the end of this stage the calyx starts to open, giving way to the extending petals and both stamens and gynoecium are enclosed

inside a keel petal surrounded by wing petals and standard petals.

Stage 13 is where the petals become visible between the sepals and continue to elongate rapidly (**Figure 10e [& n] to10h [& q])**. At first the standard petals start to extend, but when the bud reaches 4 mm (calyx + corolla) in length, both the keel petals and wing petals start to extend. The anthers become opaque yellow in colour. Simultaneous to the keel elongation, the style of the gynoecium starts elongating and reaches twice the length of the stamen filaments. The stigmatic hairs are completely developed on the ventral side 0.5 mm below the c-shaped cap (**Figure 10s**). All the stamens have separate filaments at this stage (**Figure 10 [n & o]**). At the point when the flower bud reaches 6 mm in length the anther filaments start extending (**Figure 10p**). The filaments of the nine stamens facing the stigmatic hairs on the ventral side of the gynoecium are partially fused to form a tube-like structure enclosing the gynoecium, leaving one separate prominent stamen facing the c-shaped cap. At this stage the diadelphous anthers along with the gynoecium bend into the form of C/L-shape to accommodate the inside of the keel petal (**Figure 10q**). By the time the flower bud reaches its maximum (8-9 mm) length all the filaments have reached the height of stigma and remain in contact with stigmatic hair and cap. **Figure 10** (s) shows the carpel of the bambara groundnut flower at **stage-14** which marks anthesis and fertilization.

The mature gynoecium is long and curved at the tip, with stigmatic hairs (**Figure 10s**). In the young flower buds, the stigma is slightly above the anther, while in the mature flowers the filaments elongate to place the anthers at the level of stigma (**Figure 10**). The ovary consists of usually two ovules or, rarely, three ovules, with a hypogynous superior ovary. It is attached directly to the receptacle, as are the other floral parts (**Figure 9d and 10s**). The last stage of early flower development is fertilization and pod formation.

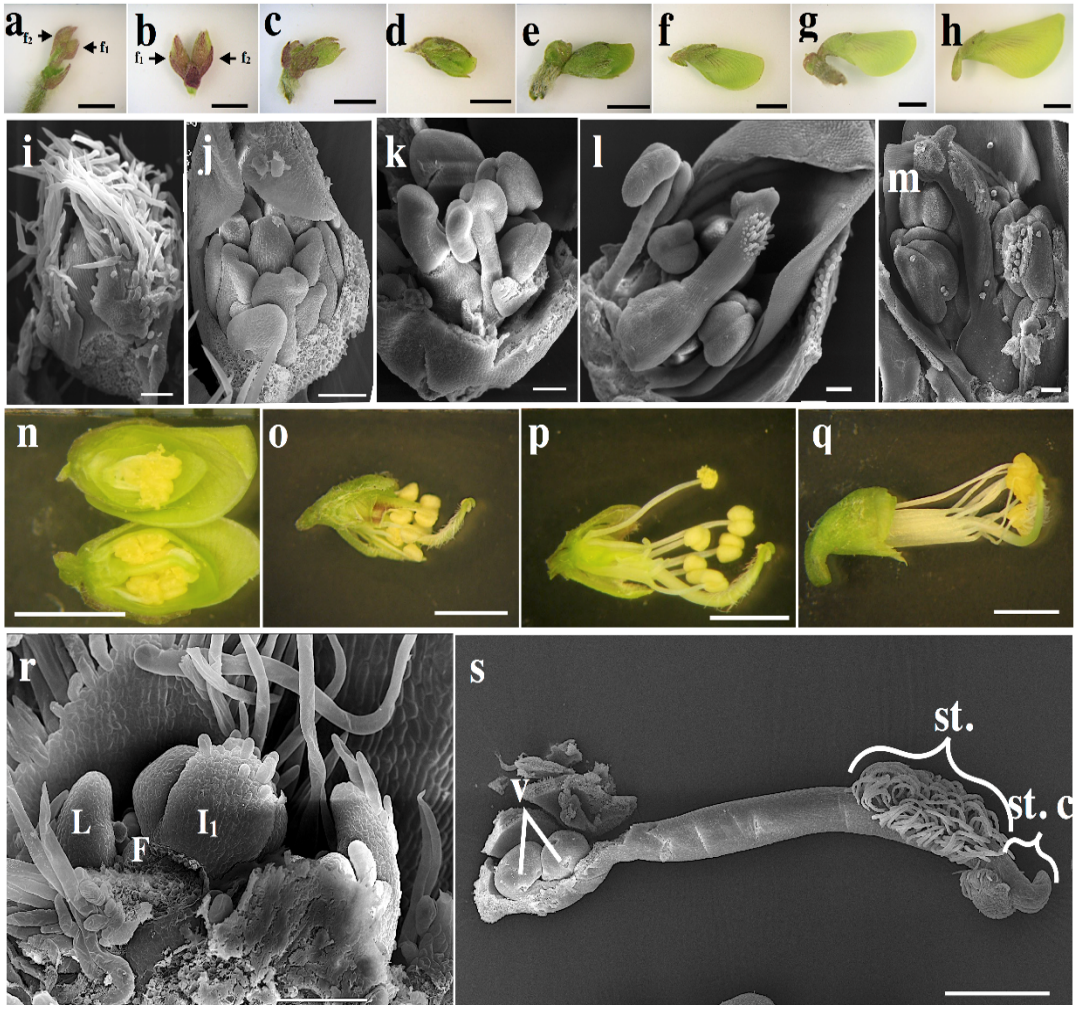


Figure 10: Progression of flower development (a) to (h) and underlying flower morphology (i) to (q); (i) SEM image of flower bud enclosed in hairy bracts; (j) SEM image of flower morphology corresponding to (a) ; (k) SEM flower morphology corresponding to (b) shows fully formed anther lobes; (c) & (I) SEM image of flower with slighly bent stigma and stigmatic hair forming at the dorsal side; (d) & (m) SEM image of corresponding flower morphology showing anthers with haploid microspores and stimatic hairs with C-shaped stigma cap; (e) & (n) SEM image of flower morphology; (f) & (o) flower sectioning showing elongated carpel with anther filaments roughly half the length; (g) & (p) flower section showing elongated anther filaments; (h) & (q) fully developed flower with anther lobes touching stigma cap; (r) SEM image of shoot apical meristem with leaf primordia (L), flower primordia (F) and axillary meristem inflorescence (I_1); (s) carpel from fully developed flower with two ovules (V), stigma hair (st. h) and stigmatic cap (st. c). [(a) to (h) & (n) to (q) scale bar = 2 mm; (i) to (m) and (r) scale bar = 100 μm; (s) scale bar = 500 μm].

3.1.3. Discussion-1

3.1.3.1. Inflorescence and flower

The sequence of events in the process of early flower development has been extensively studied in the Papilinoideae subfamily to which bambara groundnut belongs (see a review by Tucker 2003). As observed, the axillary meristem (I_1) differentiates to produce lateral meristems (I_2), it is continuous in spreading and semi spreading bambara groundnut genotypes before terminating with tripinnate leaves, whereas in bunchy genotypes I_1 it differentiates into only one or maximum two axillary meristem (I_2) to form a crown at the base. The number of flowers at each inflorescence is fixed in bambara groundnut unlike other legumes in the same subfamily (Millettioid clade) (Tucker 2003).

The ABC model hypothesis of floral organ identity applies to flowers such as Arabidopsis, in which all organs of a whorl initiate simultaneously, the order of initiation is sepals, petals, stamens, and carpels, and where whorls do not overlap in time of initiation (Tucker 2003a). The floral ontogeny of legumes is different from *Arabidopsis thaliana* mainly because the initiation of floral organ primordia on the axillary meristem is centripetal and sequential in Arabidopsis, whereas it is unidirectional starting from the abaxial side of the flower in legume species such as *Medicago truncatula* (Benlloch et al. 2003) and *Pisum sativa* (Tucker 2003), which both belong to the same subfamily, papilinoideae. Therefore, ABC model does not satisfactorily explain a system in which more than one type of organ is being initiated at the same time which is prominently seen in papilionoideae, where the carpel initiate concurrently along with petals and stamens.

Medicago truncatula is a model species for legumes such as bambara groundnut, however the whole of flower development was assigned into only 8 stages (Benlloch et al. 2003) i.e., stage 8 includes the formation of stamen filaments, carpel and pollination, whereas in bambara groundnut a distinct pattern was observed at stage 8. Therefore, in the present study there was a need for a more comprehensive and complete guide for early flower development, hence the basic framework of eudicot flower documented in *Arabidopsis thaliana* (Alvarez-Buylla et al. 2010) was adapted.

Section (3.1.2.2.) describes the flower development stages following stage-8 with flower size ≤2 mm, the longest being stage 13, with flower size ranging from 4 mm to 9 mm, the main landmark events in this stage are elongation of style (Gynoecium) followed by elongation of stamen filaments and petals.

The current bambara groundnut flower scale was developed based on the flower size as unit of measurement to identify different stages. This was because the system of categorising the flower based on size was developed along the way when we noticed, the time of initiation of flower buds was difficult to identify. This was because the in semi spreading varieties (IITA-686 and S19-3) inflorescence was small and develops at nodal region of 9 or 10^{th} leaf, where as in bunchy type (DipC) the initiation of inflorescence was near to crown of leaves. In order to develop a universal scale of flower development for spreading, semi-spreading and bunchy type bambara groundnut genotypes, it was more comprehensible through size of flower buds as it was easy to identify the underlying stages. The current bambara groundnut flower development scale can be applied in plant breeding trials to identify best stages for emasculation of flowers. The scale can also be extended to bunchy, semi-spreading and spreading type genotypes of bambara groundnut. The established scale depicts bambara groundnut flower development from stage 8, the early development stages from 1 to 7 are yet to be defined, hence our scale can serve as a guide in terms of flower size below which the observations should be made to learn earlier stages.

In this study, it was also noted that only the fertilized flower remains stalked and the flowers with unsuccessful fertilization wither and fall off within 24 hours. This phenomenon can be used in field trials to get initial indication of success of fertilization, there is no need to wait until pod formation. This can be applied to study relative number of flowers formed which can produce successful pods. Bambara groundnut produces geocarpic fruits, further classified as active geocarpy by Barker (2005), where the plant physically responds and grows peduncle to bury the seeds without any interference from the environment. The pod grows first, and reaches its mature size and the seed develops later. The pods are indehiscent and are mostly geocarpic (developed on or inside the soil), although in some landraces it is not a requirement for development of mature pods. After fertilization, the ovary eventually

forms geocarpic fruit (pods) and the ovules form the seed. Mean temperature during the seasons influences the time taken to achieve physiological maturity; bunch types tend to mature earlier than spreading types. Fruit development has been reported to be influenced by photoperiod (Linneman 1993; Linnemann et al. 1995). The current scale contributes to the comprehensive knowledge on the early flower development in bambara groundnut, the last stages such as fertilization and pod formation requires further investigation. The future researchers can use *in vitro* aniline blue staining to study fertilization and, SEM or transmission electron microscopy (TEM) to study pod formation in bambara groundnut.

3.1.3.2. Floral formulae

Floral formulae can summarise wide range of flower features such as acropetal sequence of organ initiation, number and symmetry of each whorl of floral organs (bracts, sepals, petals, androecium, gynoecium, ovules), position of the organs relative to each other, partial and/or complete fusion of organs (Prenner et al. 2010). The bambara groundnut floral formulae was designed according to the updated formal taxonomic descriptors (unicode), it follows a typical quadripartite 'KCAG' system, they are listed in the order from periphery (base) to center (apex) of floral axis: with sepals (K), petals (C), androecium (A) and Gynoecium (G) (Prenner et al. 2010; Ronse de Craene et al. 2014). There are 5 sepals which are fused depicted by $\mathbf{K_{(5)}}$ and three types of petals shown as $\mathbf{C_{1+2+(2)}}$, one standard petal, two free wing petals and (2) two fused boat shaped keel petals. The next inner whorl consists of androecium ($A_{1+(9)}$) with diadalphous stamens, one free and nine partially fused, followed by a gynoecium with superior ovary and single locule ($\underline{\mathbf{G_1}}$).

Bambara groundnut is a self-pollinating species, with typical papilionaceous zygomorphic flowers with uniform ontogeny. Like most of the zygomorphic flowers, bambara groundnut flowers also have a bilateral symmetry, which typically manifests as two kinds of asymmetries, the dorsoventral (DV) for the floral planes and organ internal (IN) asymmetry in reproductive organ planes (**Figure 9b**). As documented by Wang et al. (2008) and Tucker, (2003), the asymmetric development of the floral organ primordia of the floral meristem results in this DV asymmetry. However, IN is variable among petals, the dorsal standard petal is IN symmetric, the two wing petals

and the two ventral petals which are united on the lower edge to form keel are IN asymmetric. The phenomenon of late asymmetry is a characteristic feature of genus such as *Vigna* and *Phaseolus,* the same pattern was observed in the current study as well, the young flower buds of bambara groundnut shows symmetry during early development, but differentiates into asymmetry late in the ontogeny (**Figure 10**). Changes such as the upward turning of style and stamens, differential elongation of sepals and the positioning of the entire flower in the fascicle are the characteristic features of late zygomorphy (Tucker 2003a). Like most of the Papilionoideae species, the bambara groundnut androecium is asymmetric with 10 stamens in two whorls (**Figure 9b**). The distinct asymmetry in the taxa, *Vigna* is a result of late floral ontogeny (Prenner 2004).

International code of botanical nomenclature requires minimum levels of character-based information (including floral formulae) in any formal taxonomic description of the species, our research presents the first recorded floral formulae for bambara groundnut flower in recent literature. They can usually be applied within as well as between species, to compare wild type versus cultivated verities and different ontogenetic stages (Prenner et al. 2010). Prenner et al. (2010), also recommended that floral formulae can become a routine component of diagnoses in protologues and other formal taxonomic (re)descriptions, functioning as a logical phenotypic counterpart to the DNA barcode.

3.2. Experiment 2: Flowering trend

The characteristic feature of angiosperms is the flower (Alvarez-Buylla et al. 2010). Bambara groundnut is continuously flowering type, i.e., the plants produce flowers throughout the lifecycle even after podding begins. The flowering trends in a number of genotypes were measured by counting the number of completely opened flowers each day from the date of first flower opening. In each landrace/genotype there exists a diversity in terms of number of days to flower opening and the number of flowers completely opened each day. These data have been documented in this chapter to collect and compare among the bambara groundnut genotypes.

3.2.1. Plant materials and growth conditions

As bambara groundnut only exists in the form of more or less heterogenic landraces, in order to reduce the risk of genotypic variation F_2 generation seeds from self-pollination of landraces were used in the experiments. These seeds were kindly provided by Dr. Presidor Kendabie, University of Nottingham (Sutton Bonington campus, UK). Since landraces are generally regarded as being adapted to their areas of cultivation and thus able to cope with the predominant stresses of a particular environment, a total of seven genotypes potentially differing in their capability to heat stress were chosen based on their region of origin (**Table 10**). The seed characteristics colour and pattern are presented in the **figure 11**, with each genotype representing a selection from the more heterogenous landrace.

The plants were grown in a controlled environment (CE) room, University of Nottingham (Sutton Bonington campus, 52° 50' N, 1° 15'W). The CE was equipped with temperature control unit and two benches (left and right side of the entrance) to accommodate pots (**Figure 12**). The temperature was maintained at 28/22°C (day/night) with 12 hour photoperiod (light conditions). Relative humidity of CE was at 58 to 60% and the light intensity was 20,000 Lux and above (400-700nm photo-synthetically active radiation). The seeds were soaked for 3-4 hours (as this increases the seed germiability) then sown for germination in the Levington seed and modular compost (F2) (**Figure 12)**. 14 days after germination (DAG) the seedlings were transplanted into 5 L wide pots with equal volumes of sand and JI-03 (John Inns no.

3) compost. The pots were arranged in randomized design distributing seven pots per genotype between two benches on the either side of the room (**Figure 12**). The plants were watered into the saucers of each pot regularly throughout the lifecycle, to avoid any water stress.

Table 10: List of bambara groundnut landraces and their respective environmental conditions at the point of origin. (*- www.whetherspark.com & **- www.whether-and-climate.com.)

Landrace	**Seed Colour**	**Region of Origin**	**Temperature range (Minimum-Maximum°C)**	**Average day length (hr.)**	**Average humidity**
IITA 686	Dark	Tanzania	19-33*	12	60%
S19-3	Dark	Namibia (Windhoek)	7-30* (up to 36)	12	50%
DipC	Cream (butterfly testa)	Botswana (Maun)	10-33*	12	55%
Gresik	Dark	Indonesia	22-33**	12	55%
Uniswa red	reddish	Swaziland	11-28*	12	55%
AHM-573	reddish	Namibia	7-30* (up to 36)	12	50%
Getso	Cream (purple testa pattern)	Nigeria (Yelwa)	15-33** (upto 38)	12	60%

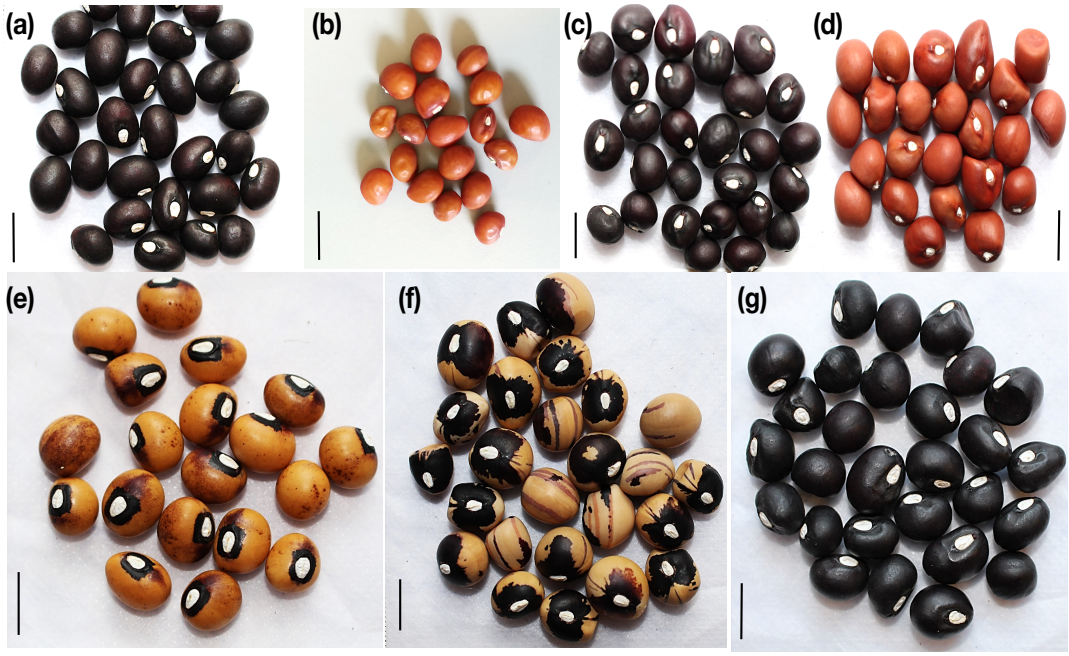


Figure 11: Seed colour and testa patterns of different genotypes of bambara groundnut; (a) IITA-686; (b) AHM-573; (c) S19-3; (d) Uniswa red; (e) DipC; (f) Getso & (g) Gresik. (Scale-bar = 1 cm)

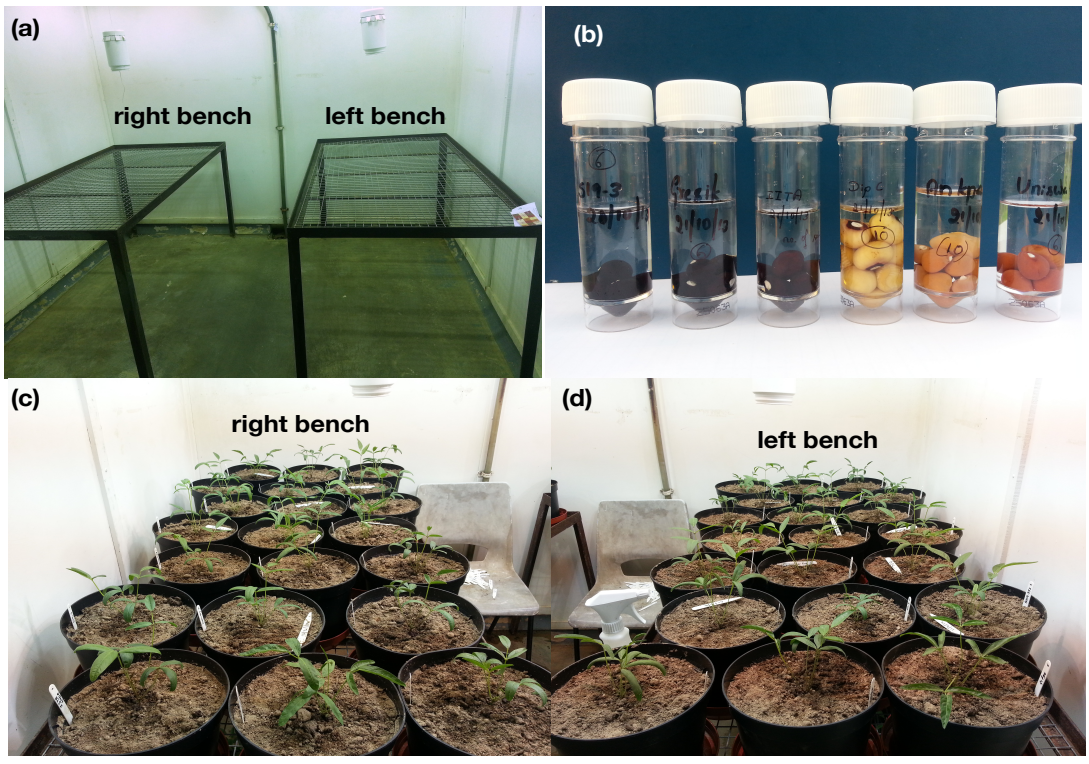


Figure 12: (a) The CE room work space with right and left bench; (b) Bambara groundnut seeds soaked in water before germination; Right bench (c) and left bench (d) with bambara groundnut seedlings.

3.2.2. Methodology

To measure the flowering trend, first the flowering time of all seven genotypes were determined followed by defining flowering pattern that is measured by the number of flowers opened each day from the day of first flower opening measured in DAG (days after germination/emergence). The total number of flowers opened on the day were counted in all seven genotype and seven biological replicates per genotype.

3.2.2.1. To determine flowering time

Flowering time was calculated by counting the number of days to first flower opening in all the landraces/genotypes. A one-way ANOVA test was performed on flowering time data, to test the null hypothesis (H_0) i.e., there is no significant difference between the genotypes in terms of flowering time. Otherwise to provide evidence for the alternative hypothesis (H_1) is correct i.e., there is a significant difference in flowering time between the genotypes. The multiple comparison 'Bonferroni post-hoc t-test' was carried out to determine the specific significant differences between genotypes. During the Bonferroni correction an adjustment was made to p-value by diving the critical p (0.05) value by the number of comparisons made to obtain P-value (t). Then the significance was analysed by comparing the P-value (t) to the p-value (T) from a two-tailed t-test, the parameter followed was T<=t. The flowering time of each genotype was plotted in a simple box and whisker chart to show the distribution of time range (refer to **section 3.2.3.1.**). The one-way ANOVA with post hoc t-test was carried out using data analysis tools from Microsoft excel-2015 (Version 15.16) and the box plot chart was created using the function stacked bar chart in Microsoft excel (version 15.16).

3.2.2.2. To determine flowering trend or pattern

The pattern of flowering was observed by simply plotting the number of flowers produced each day in all the genotypes (refer to **section 3.2.3.2.**). The line graph was plotted using number of days after germination (DAG) on the x-axis against the number of flowers opened on the corresponding days, on the y-axis. Standard deviation was added as error bars to each point in the graph.

3.2.3. Results-2

The bambara groundnut flowers were considered completely open when Standard petal and wing petals which covers the keel petals were completely open (**Figure 13**).

Figure 13: Bambara groundnut flower showing completely opened flower with standard and wing petals. (Scale bar = 1 cm)

3.2.3.1. Flowering time

There was a statistically significant difference in terms of flowering time between genotypes (p-value < 0.05). The results of the one-way ANOVA tests are presented in the **table 11**. Therefore, the null hypothesis "there is no significant difference between the genotypes in terms of flowering time", was rejected.

The one-way ANOVA only shows that there was a significant difference ($p<0.05$) in flowering time among the genotypes but does not identify the genotypes which are significantly different from each other. Therefore, a stepwise multiple comparison 'post-hoc t-test' was carried out with a Bonferroni correction. All possible comparisons between the six genotypes were made and a two tailed t-test was performed assuming equal variances (**Table 12 & Figure 14**). The box plot (box and whisker chart) shows the simple representation of flowering time between genotypes with standard deviation plotted as error bars (**Figure 14**). The flowering time of IITA-

686, S19-3, DipC, AHM-753, Getso, Gresik and Uniswa red genotypes were 43±2.73, 42±2.21, 48±3.69, 48±3.29, 53±3.53, 46±3.59 and 42±3.64 DAG, respectively. The one-way ANOVA shows that the flowering time of genotype Gresik was significantly different from IITA-686, S19-3 and Uniswa red, similarly S19-3 differed from AHM-753. This concludes that Gresik flowers later when compared to IITA-686, S19-3 and Uniswa red.

Table 11: One-way ANOVA output of days to flowering in different bambara groundnut genotypes (* statistically significance at the 0.05 level). SS-sum of squares, df- degrees of freedom, m.s.-mean squares, p- probability.

Source of Variation	**SS**	**df**	**m.s.**	**F-value**	**p-value**
Between Genotypes	702.694	6	117.1157	10.862	**<0.05***
Within Genotypes	452.8571	42	10.7823		
Total	1155.551	48	1155.551		

Table 12: The list of genotype comparisons showing significant differences at 0.05 level during Bonferroni post-hoc t-test. (The indicators are plotted on figure 14)

Landrace comparison	**Significance value**	**Indicators (graph 1)**
IITA-686 & Gresik	<0.05	a
S19-3 & AHM-753	<0.05	b
S19-3 & Gresik	<0.05	c
Gresik & Uniswa red	<0.05	d

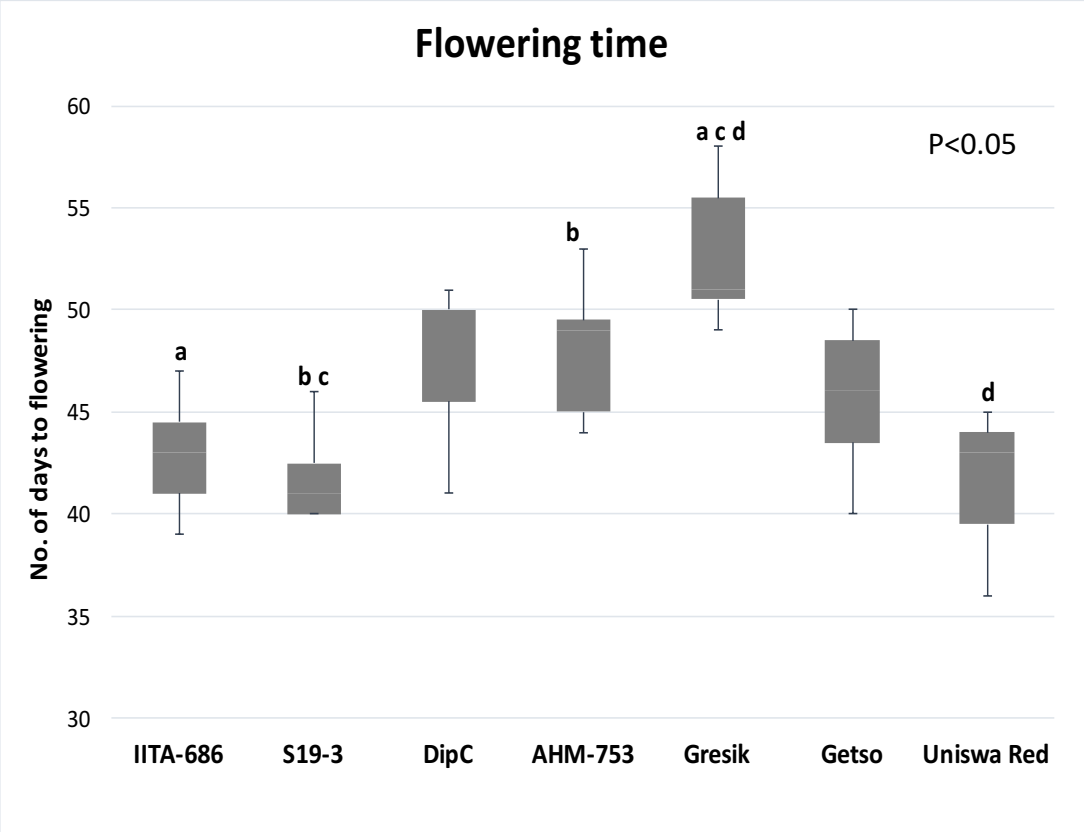


Figure 14: The box plot represents Flowering times of six landraces of bambara groundnut. The Bonferroni post-hoc t-test multiple comparison show significant differences at 0.05 level ($p<0.05$), between (a) IITA-686 & Gresik; (b) s19-3 & AHM-753; (c) s19-3 & Gresik; (d) Gresik & Uniswa red, genotype.

3.2.3.2. Flowering pattern or trend

Bambara groundnut is a continuous flowering type; it flowers throughout the lifecycle under field conditions. Under CE room conditions the flowering pattern/trend in bambara groundnut genotypes are as depicted in **figure 15**. The flowering usually starts around 35 to 40 DAG depending on the genotypes. Flowering pattern shows a steep increase in the number of flowers followed by gradual decline. The pattern of flowering seems to be similar in the genotypes under investigation growing under CE conditions (IITA-686, S19-3, AHM-753, Uniswa red, Gresik, Getso and DipC). This pattern was important to assess the window of flowering during which all the plants are at 100% flowering.

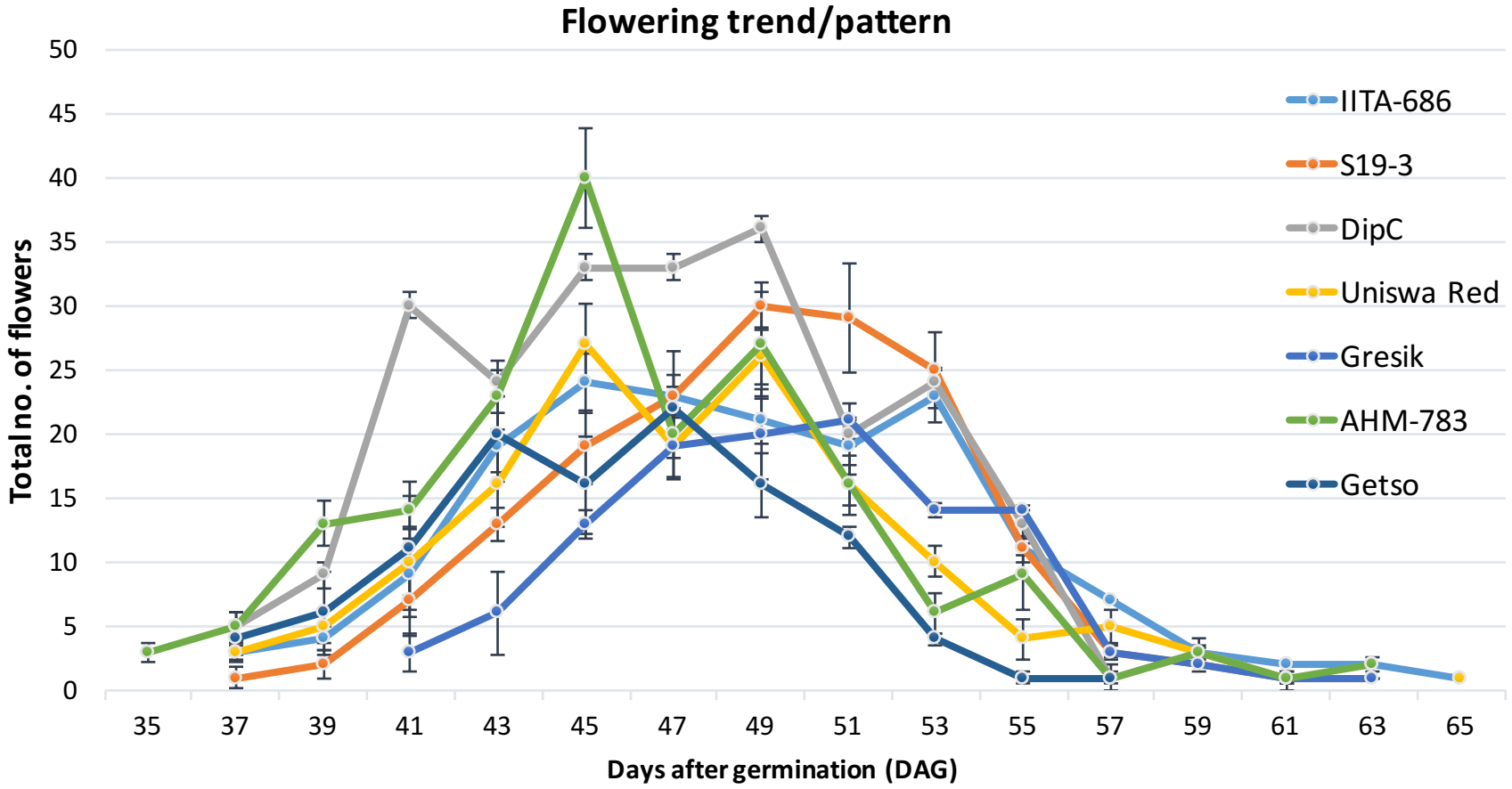


Figure 15: Flowering pattern of bambara groundnut genotype. Briefly; the number of flowers increase steeply after first flower opening and decrease gradually. (error bars - standard deviation)

3.2.4. Discussion-2

Our results show that bambara groundnut is indefinite flowering type that is, it continues to produce flowers throughout its lifecycle even after the plant enters maturation stage during which pods are formed. These findings provide updated evidence on type of bambara groundnut flowering, this also is in agreement with relatively old literature (Doku 1968; Linneman 1993; Brink 1997). Our main deduction was that, two weeks after the first flower opened was the time when the plants produced the maximum number of flowers. This time window was used to determine an ideal time frame for a short duration high temperature stress, which will be explained in detail in **chapter 4**.

Bambara groundnut genotypes shows both definite and indefinite flowering trends (Personal communication, Dr Feloye Ben [IITA, Nigeria] December 2017), this study can be extended to determine flowering pattern in different bambara groundnut genotypes. The application being, flowering time and pattern data gives a time frame before sowing to avoid dry/heat season, which might reduce the risk of crop failure.

3.3. Experiment 3: Pollen development

In Angiosperms or flowering plants, sexual reproduction involves the formation of male and female gametes. Stamens are the male reproductive organs of flowering plants which mainly consists of anthers, the site of pollen development, and filaments, which are positioned to aid in nutrient supply and pollen dispersal (Scott et al. 2004). Pollen development involves an array of events, including cell division and differentiation, which is independent of that of meristem tissues.

The process of pollen development mainly involves two events, microsporogenesis (pollen formation) and microgametogenesis (pollen maturation) (Scott et al. 2004). Microsporogenesis, is the formation of haploid microspores (n) as a result of meiosis of diploid pollen mother cells (PMC)/microsporocytes. Microgametogenesis is the maturation phase where haploid microspores undergo mitosis to form bicellular pollen grains, upon maturation which forms tricellular mature pollen grains. A mature pollen grain is an unusual vegetative cell in itself as it contains sperm cells complete with cell wall and plasma membrane (Edlund et al. 2004). By capitalizing on available microscopy techniques it is possible to dissect and magnify cellular structure and observe the formation of pollen grains (Edlund et al. 2004). Scott *et al.* (2004), indicates that stamen and pollen development processes are closely linked. The available literature in model species (such as *Arabidopsis*) also allows comparative studies across taxa (Scott et al. 2004), here we observed and compare the landmark events during pollen formation (microsporogenesis and microgametogenesis) in bambara groundnut.

3.3.1. Materials and methodology

3.3.1.1. Plant growth conditions

Seeds of bambara groundnut (*Vigna subterranea* (L.) Verdc) genotype 'IITA-686' were germinated in modules with Levington seed and modular compost (F2) in a Controlled Experiment (CE) room, University of Nottingham (Sutton Bonington Campus, UK) with 28C/22°C as day/night temperatures and 12 hours of photoperiod. 14 days after seed germination these seedlings were transplanted into 5 L pots with the mixture of John Innes No3 compost: sand (50:50%). The plants were watered

regularly to keep the top soil surface moist throughout the lifecycle.

3.3.1.2. Fluorescence microscopy and SEM

To study microsporogenesis and microgametogenesis, different sized flower buds were freshly harvested and anthers were separated under a dissection microscope, after taking photographs for image analysis to measure flower size. The anthers were then stained with DAPi (4',6-diamidino-2-phenylindole) (1µg/ml) solution for 2-3 hr and observed under a UV filter on a fluorescent microscope (LEICA model). Three samples each of similar sized flowers were prepared for DAPi staining to determine the stage accurately. The pollen morphology was studied by SEM and Alexander staining (Alexander 1969). The pollen grains from freshly opened flowers were dabbed on to the self-adhesive carbon disk attached to the cylindrical studs (Agar Scientific) and sputter coated with gold-palladium (4:1) using a sputter coater (SCD-030, Balzers). SEM was performed on a JSM-840 scanning microscope and digital images were captured and saved in JPEG format. Images were processed using keynote (mac OS El Capitan) for publication.

3.3.2. Results-3

3.3.2.1. Pollen formation

Simultaneous microsporogenesis, as seen in a true eudicot, was observed in bambara groundnut (**Figure 16**). The detailed processes of microsporogenesis, the formation of haploid microspores from the diploid pollen mother cell and microgametogenesis, formation of mature tricellular pollen grains, were recorded in bambara groundnut anthers using fluorescent microscopy with DAPi staining of samples (**Figure 16**). The underlying processes of simultaneous microsporogenesis are, the diploid (2n) pollen mother cell (PMC), undergo meiosis I & II to produce tetrads with haploid (n) microspores. These released microspores undergo the process of microgametogenesis also known as pollen maturation. The haploid microspores undergo mitosis I to produce bicellular pollen with vegetative (V) and generative cells (G). The PMC stage is as shown **figure 16b** and tetrad and microspores are shown in **figure 16 (c & d** respectively). The early bicellular stage is shown in **figure 16e** with the generative

cell towards the pollen wall and **figure 16f** shows the late bicellular stage with V and G in the middle. The generative cell of bicellular pollen undergo mitosis II to produce two sperm cells (S) in addition to the V cell, on the day of flower opening during anther dehiscence (**Figure 16g**).

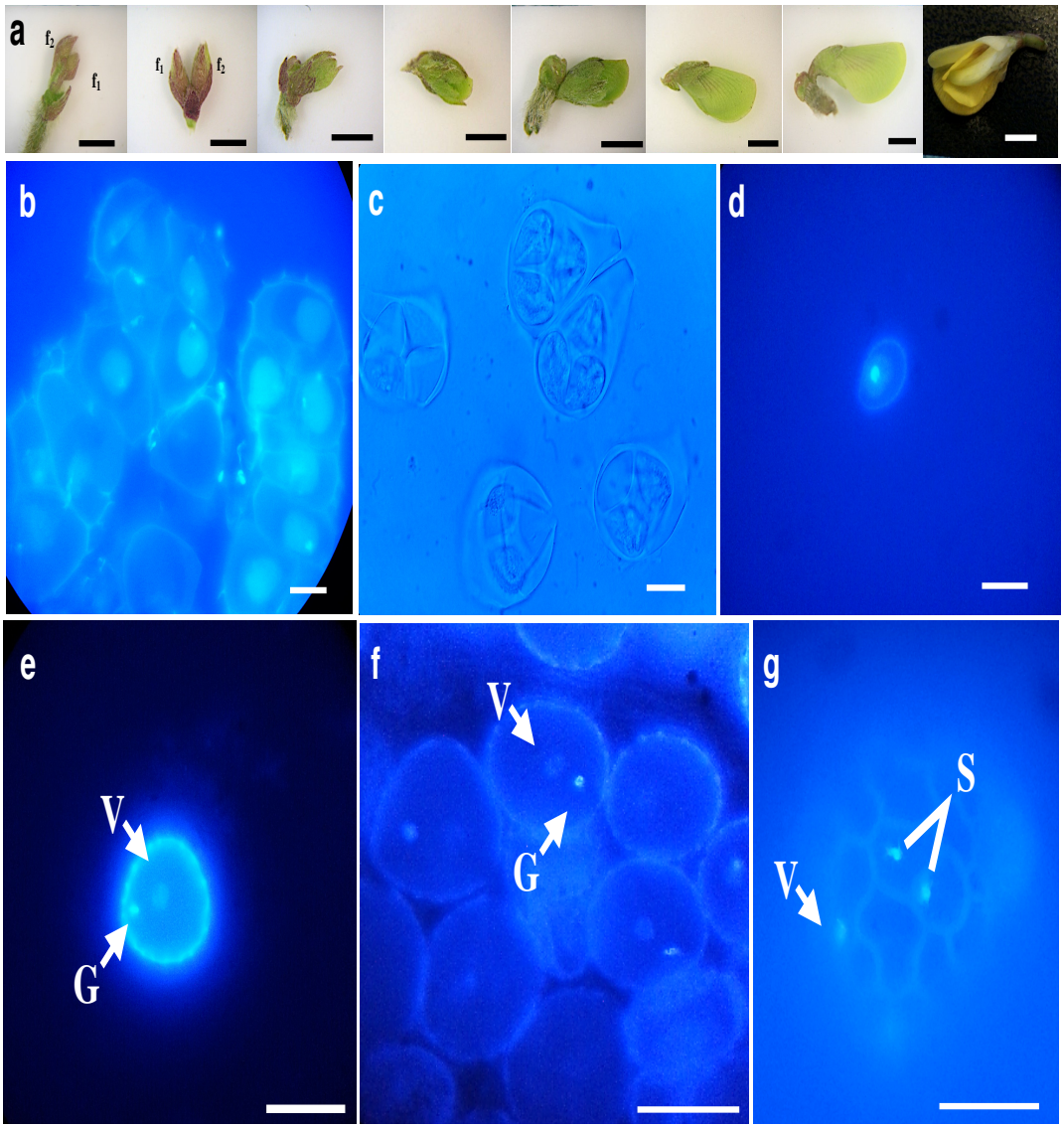


Figure 16: (a) Progression of inflorescence development from bud to mature open flower in bambara groundnut (example from IITA-686 genotype); simultaneous microsporogenesis and microgametogenesis in bambara groundnut (b)-(i); DAPi staining of (b) Pollen mother cell (PMC); (c) tetrad; (d) haploid microspore; (e) early bicellular pollen with generative (G) and vegetative cell (V); (f) late bicellular stage; (g) tricellular pollen grain at the time of anthesis with two sperm cell (S) and vegetative cell (V). Scale bars in a= 2 mm; b = 100 µm; c-f= 50 µm; g = 30 µm.

3.3.2.2. Anther dehiscence and pollen dispersal

Bambara groundnut anthers dehisce longitudinally and release the viable pollen grains on to stigma surface. Upon observation, it was noticed that bambara groundnut stamens have dorsifixed anthers (**Figure 17a**) and dehisce longitudinally (**Figure 17b**).

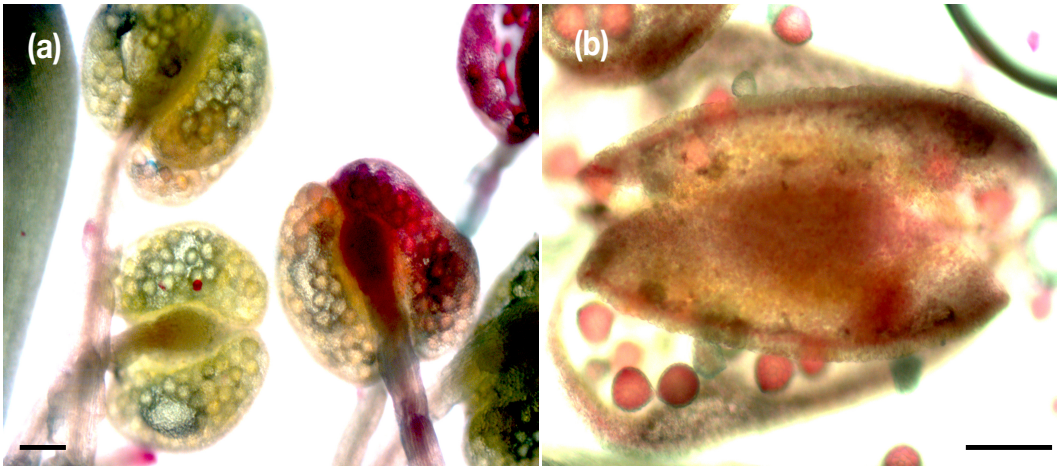


Figure 17: Bambara stamen (a) Dithecous dorsifixed anther; (b) longitudinal dehiscence with viable pollens. Scale bar = 100 μm.

3.3.2.3. Pollen grain architecture

SEM imagining of mature pollen grains reveals that the bambara groundnut pollen grains are circular with triangular ends/edges and tricolpate with three circular pores (**Figure 18**). Schematic diagrams of tricolpate pollen grains as seen in bambara groundnut is illustrated in **figure 18b**. The most striking structural feature of the pollen grain is its tough, resistant outer coat, known as the exine, this surrounds an internal wall layer, known as intine. The exine layer of bambara groundnut is reticulate or tectate with ridges and furrows on the surface. In addition to the purely mechanical function of the exine in protecting the reproductive cells from environmental injury, exine sculpturing plays a role in adhesion to stigmatic surfaces. The elaborately sculptured exine of bambara groundnut pollen grain is seen in SEM image **figure 18**. The exine does not develop over the regions that define the positions of the germination apertures known as colpi, bambara groundnut pollen grains have three colpi as revealed by the SEM imaging (**Figure 18c**).

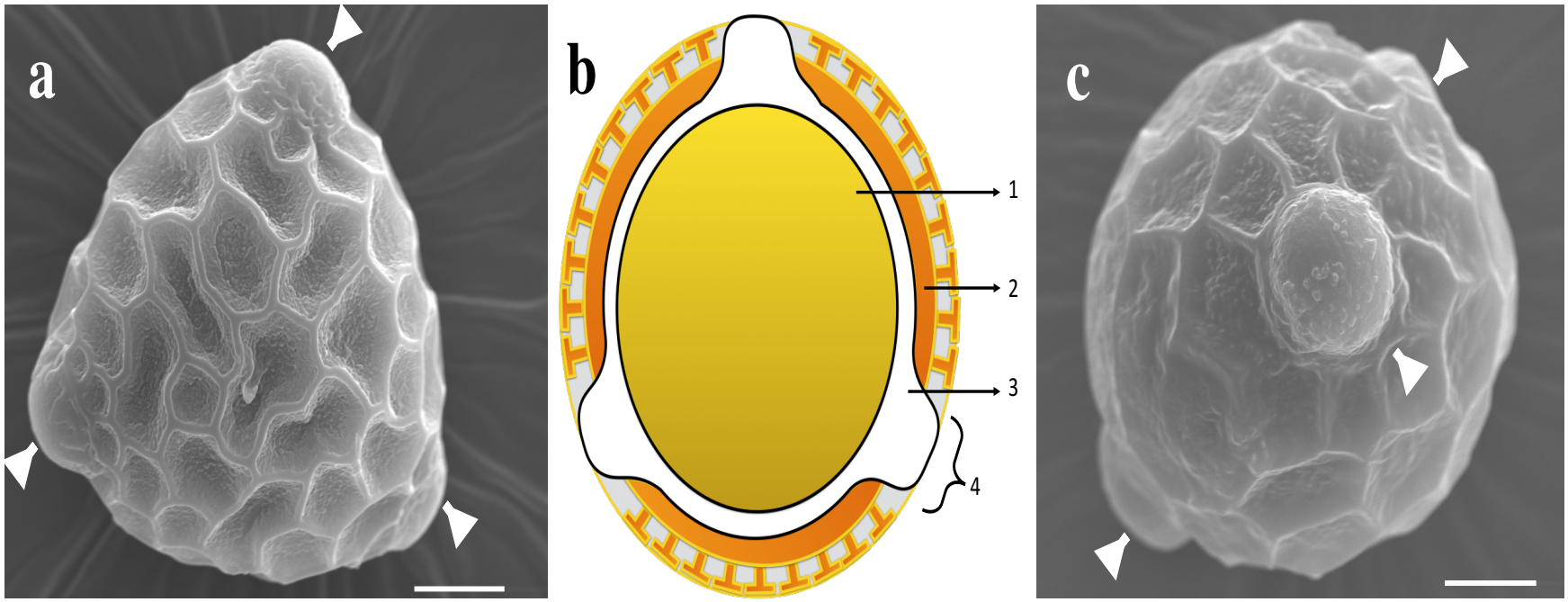


Figure 18: (a) SEM image of tricolpate pollen grain with arrows pointing to 3 colpi/apertures and close-up view of colpus / aperture of pollen grain (c); (b) Diagrammatic representation of mature pollen grain, cytoplasm [1], exine [2], intine [3], aperture/colpi [4], sperm cells (S) and vegetative cell (V). scale bars (a) and (b) = 10 µm.

3.3.3. Discussion-3

3.3.3.1. Linking bambara groundnut pollen and stamen development

Pollen and stamen development in plants are intimately linked (Scott et al. 2004). At anther stage 4, the anther locules usually contain sporogenous cells which gives rise to pollen grains. During the same anther stage, other cell types which aid in pollen development and release, which constitute the microsporangium, also develops. Stage 5 of anther development marks the differentiation of sporogenous cells which develop into pollen mother cells (PMC). In bambara groundnut, anther stage 5 was identified with PMC (**Figure 16b**). These PMC are released into locules during anther stage 7, to undergo meiosis to produce tetrad and haploid microspores. Anther stage 7 in bambara groundnut was identified with tetrads (**Figure 16c**) and microspores (**Figure 16d**). By anther stage 12 the pollen grains are at tricellular state and anther stage 13 marks the anther dehiscence with mature pollen grains. The observation of bambara groundnut pollen grains showed that the bicellular pollen grains (**Figure 16 e & f**) become tricellular (**Figure 16g**) during anther dehiscence. The tricellular pollen grains are short lived, therefore successful pollen tube germination and fertilization depends on the efficiency of pollination (Williams et al. 2014). This supports the conclusion by Oyiga *et al.* (2010), that the bambara groundnut pollen grains are short lived if left under ambient conditions. These findings can be applied during cross pollination (plant breeding trials) during which the time frame between pollen harvest from fresh flowers and transfer to stigma surface should be less than 5 minutes.

Whether the flowers are protandrous (the anthers release their pollen before the stigma matures) or protogynous (stigma matures before anther dehiscence) needs to be deduced. The phenomenon of protandry is a characteristic feature of cross-pollinating species, since bambara groundnut is highly self-pollinating it can be considered as protogynous. However Doku and Karikari, (1971), sighted that pollen maturity and stigma receptivity occurs just before or immediately after the flower opens (Doku and Karikari, 1971). Similar observation suggests that the anthers dehisce as the stigma becomes receptive even before the flowers open and sometimes fertilization takes place on the same day as anthesis (Linnemann and Craufurd, 1994). During our observation the stigma became receptive well before the anther dehisce, this gives

flexibility of manual cross pollination, that is, freshly harvested pollen grains can be transferred on to the stigma surface before anthesis and the emasculation of anthers can still be carried out just before the anther dehiscence.

3.3.3.2. Bambara groundnut pollen architecture and anther dehiscence

There exists a tremendous diversity in the pollen grain structure, which is mainly due to species specificity and female selectivity (Edlund et al. 2004). Mature pollen grains are unusual vegetative cells that contain within themselves sperm cells, complete with cell walls and plasma membranes. Bambara groundnut pollen grains are circular with triangular ends/edges and are known to be trinucleate when they are shed (**Figure 18**). The pollen grains are contained within a unique pollen wall, whose formation starts when the haploid microspores/meiocytes form (Edlund et al. 2004). The function of pollen wall is to protect the cell from desiccation after release and protection against UV radiation, this also involved in adhesion, signalling and compatibility with stigma. The pollen wall at maturity can be divided into three strata, an outer exine wall which mainly composes sporopollenin and interrupted with openings called apertures/colpi. Bambara groundnut pollen grain has three such apertures, which are circular in shape. the exine wall itself is multi-layered and the composition and architecture are unique for each species. The inner wall of the pollen grain is called intine, which mainly composed of cellulose. The third strata of the pollen wall is mainly composed of proteins, pigments and aromatic compounds found to be accumulated in the outer exine cavities (Edlund et al. 2004). The outer layer of bambara groundnut pollen grain show reticulate or tectate pattern with ridges and furrows on the surface, apart from pure mechanical function it also contain microchannels, which are the sites of water egress and ingress during desiccation and hydration. This intern determine progressive desiccation limits, pollen viability and life expectancy.

As presented in the results (**section 3.3.2.2.**) the anthers of bambara groundnut dehisce longitudinally and release pollen grains onto the stigma surface (**Figure 17**). The stamens of bambara groundnut are diadalphous i.e., the filaments of nine stamens are partly fused (connate) leaving one isolated vexillary stamen also known as the monosymmetric androecium. These connate stamens have an adaptive advantage associated with pollination mechanism in the Papilionoid flowers. This permanent

fusing into the papilionoid flowers is due to intercalary growth (Tucker, 2003). The stamens are didynamous, with alternately arranged long and short filaments with dorsifixed anther lobe (**Figure 17**). The growth pattern of bambara groundnut pollen tube was nonlinear and mainly represents a sigmoid growth curve with initial exponential growth, followed by a lag and a stationary growth phase. Bambara groundnut pollen showed vigorous pollen tube growth immediately after harvest, which increases the chances of fertilization (Oyiga et al. 2010a). This suggests that pollen grains from freshly harvested flower is more vigorous and viable, which intern increases the rate of successful pollen tube formation and fertilization.

3.4. Comparative scale of bambara groundnut flower and pollen development

Our study establishes the relationship between flower size and the stages of pollen development in bambara groundnut (**Table 13 and figure 19**). These documented stages of flower development and corresponding stamen/pollen development are similar to that observed in *Arabidopsis thaliana* (Scott et al. 2004), i.e., the flower stage-8 corresponds to anther stage 4 in which Pollen Mother Cells (PMC) are present **(Figure 19 [a] & [f])**. The smallest flower buds that were identified and tagged was at this stage, it took 14 to 16 days for this flower bud to reach anthesis and complete flower opening (**Table 13**).

Similarly, stage 9 of flower development corresponds to anther stage 5 during which PMC undergo meiosis to a tetrad of cells, which are enclosed in a thick callose wall. /microspores). **Figure 19b** shows the tetrad extruded from the anther of bambara groundnut. The callose wall disintegrates to release haploid microspores by the action of callase enzyme produced from tapetal layer of anther (**Figure 19c**). The microspores enlarge and each undergoes asymmetric mitosis to for a large vegetative cell and a small generative cell (**Figure 19d**). In *Arabidopsis thaliana,* at flower/anther stage 12, the anthers contain tricellular pollen by the division of generative cell through mitosis II, however in bambara groundnut, tricellular pollen grains were formed only at the end of stage-13 (on the day of anthesis). It takes total of 9 days to for flower bud at stage 12 to reach the end of stage-13, during which anthesis occur and tricellular pollen grains are formed.

Table 13: Comparative scale and characteristic features during different stages of bambara groundnut flower and pollen development.

Flower size (≈)	No. of days to anthesis	Flower stage	landmark developmental events	Anther stage	Pollen development stage
<2 mm	**14 to 16**	**Stage-8** (Figure 19f)	Stamens become distinct and stalked at the base Carpel/gynoecium grows into a hollow tube Sepals and bracts remain completely covering the flower buds	**Stage-4**	PMC (2n) (Fig 19a)
2-3 mm	**12 to 14**	**Stage 9-10** (Fig 19g & h)	Rapid lengthening of stamen filaments and petals become stalked	**Stage-5 to stage-7**	Tetrads/microspore (n) (Fig 19[b & c])
2-3 mm	**10 to 11**	**Stage 11** (Fig 19i)	Gynoecium forms a stigmatic papillae (c-shaped protuberance) and slightly open calyx tip	**Stage 12**	Bicellular pollen (n) (Fig 19d)
<4 cm	**1-9**	**Stage 12** (Fig 19i)	The gynoecium extends and is longer than stamen plane The calyx starts to open, giving way to the extending petals	**Stage 12**	Bicellular pollen (n) (Fig 19d)
4-9 mm		**Stage 13** (Figure 19j)	Corolla becomes visible between the sepals and begin elongation Anther filaments starts extending and reach the height of stigma Anther dehiscence	**Stage 13**	Tri-cellular pollen (Fig 19e)

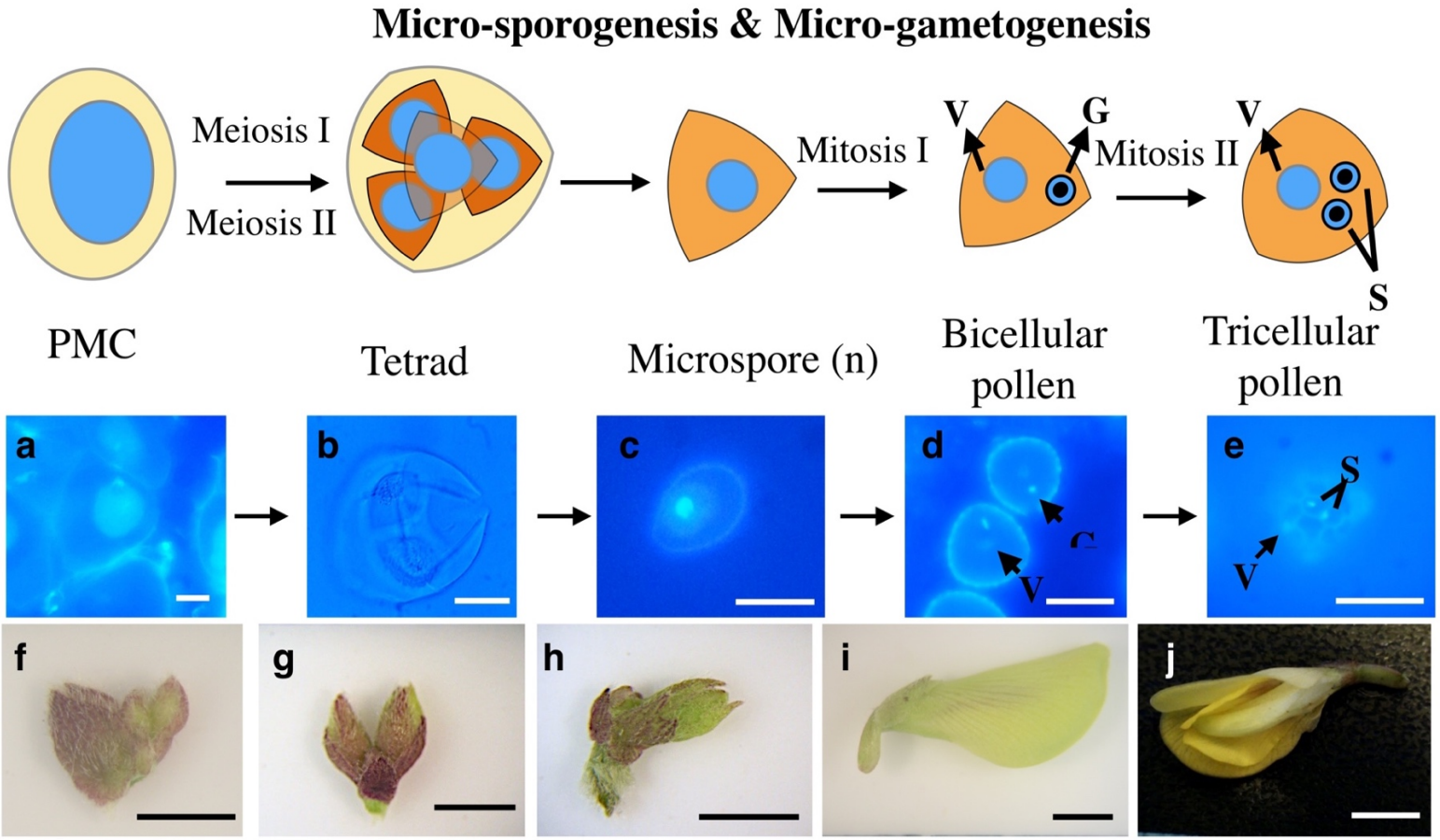


Figure 19: Schematic diagram showing the simultaneous microsporogenesis and microgametogenesis observed in bambara groundnut; (a) Pollen mother cell (PMC) stage and corresponding flower at the same stage (f). (b) Tetrad stage and corresponding flower at the same stage (g). (c) Haploid microspore and showing the corresponding flower at stage (h). (d) Bicellular pollen with generative (G) and vegetative (V) cells; corresponding flower morphology (i). (e) & (j) tricellular pollen grain with two sperm cell (S) and vegetative cell (V). [scale bar: a to e -50 µm; f to j – 2 mm]

The timing of this second mitosis varies among the plant families, in most (higher angiosperms) it occurs during anthesis (pollen release) and pollen tube growth (McCormick 2004). This was confirmed by independent experiments in which pollen grains from the flowers of one day prior to anthesis and on the day of anthesis, were observed under fluorescent microscopy with DAPi staining. This shows that bambara groundnut pollen grains are indeed "bicellular" and reach "tricellular" at the time of anthesis.

For plant breeders, the interval between flower initiation and anthesis can be of the interest. According to Onwubiko et al. (2011a), the average interval between flower initiation and anthesis was 4 days, as they have considered the emergence of flower bud from the bracts covering inflorescence as the day of flower initiation. However, the term flower initiation refers to the initiation of flower primordia from the primary inflorescence (I_1). Therefore, our findings contradict the results presented by Onwubiko et al. (2011a), as we have shown that the flower buds at stage 8 with PMC took 14 to 16 days to reach anthesis (tricellular) at stage 13.

Figure 19e shows the tricellular state in bambara groundnut at anthesis just before the flower was completely opened (**Figure 19j**). *Arabidopsis* flower stage 14, corresponds to 0 hour after fertilization (HAF), while in bambara groundnut it has already been established that the fertilization occurs on the day of flower opening (Linnemann and Craufurd, 1994) and unfertilized flowers usually fall off within 24 hours. The observation on the timing of tricellular pollen grain formation shows that they are indeed formed at the time of anthesis/flower opening, this gives plant breeders a solid proof to consider a window of time (1 or 2 days before flower opening) for emasculation of anthers. This increases the chance of successful fertilization and seed formation through manual cross pollination. However, we could not able to determine whether the pollen grains become tricellular on the stigma surface or within pollen tubes, this would be interesting to uncover through future investigations.

3.5. Conclusion

The flower is one of the complex structures of plants, which is critical for seed and subsequently crop yield, the ontogeny and morphological study of the same can be adapted into a physiological and molecular understanding of the species itself. Although the research aim was to study flower initiation and flower development, we were only able to establish flower development from stage-8; one of the limitations being that the smallest flowers collected from the inflorescence which were visible at I_2 were 1-2 mm in size and it was practically difficult to collect and dissect flowers smaller than 1mm during this study. However, our study can serve as a guide point for future researchers interested in establishing a complete flower development guide and variation in the process within and between genotypes. Nonetheless, this study is the first one to link flower phenology to the stages of pollen development through morphological data obtained from advanced microscopy studies; as such we also provide documented formal taxonomic descriptors of bambara groundnut floral morphology.

The present study provides a series of reference observations and a first guide to study bambara groundnut flower and pollen development, these findings can be extended in the future to further our understanding of reproductive development in bambara groundnut. Bambara groundnut is a potential future crop, currently receiving much needed attention to study plant-environment interaction especially involving temperature, photoperiod and drought stresses. Since it is evident from studies across plants that the reproductive stage is the most sensitive to stress, the knowledge from the present study is vital to be able to effectively study the effect/interaction of abiotic stress on the reproductive development of bambara groundnut.

CHAPTER 4

Effect of high temperature stress on reproductive development

4.1. Introduction

It has been well established that environmental signals such as photoperiod and temperature play an important role during reproductive development in plants (Searle and Coupland 2004; Yamashita and Komeda 2010; Rieu et al. 2017). Particularly plant reproduction is regarded as highly sensitive to environmental conditions such as temperature and it can be one of the limiting factors in crop productivity (Hedhly et al. 2009a; Rieu et al. 2017). Bambara groundnut (*Vigna subterranea* (L.) Verdc.) is often considered to be a drought tolerant crop and known to survive and produce yields at high temperatures (Massawe et al. 2005b). The prospect that selection of gametophytic development might be used to favour the best adapted genotypes for higher future temperatures deserves further research. Therefore, there is a need to experimentally evaluate the weakest link during the reproductive phase (gametophytic development) under high temperature stress in bambara groundnut.

The focus of many studies in bambara groundnut were of the combination effect of drought and photoperiod on plant growth and development (Linnemann and Craufurd 1994b; Brink 1997; Brink et al. 2000; Karunaratne et al. 2011a). Uncoupling the effect of high temperature on fertility from photoperiod and drought stress could be key to understanding plant reproduction under heat stress in this species. Linnemann and Craufurd (1994), in their study on photo-thermal effects on the phenology of bambara groundnut landraces established that onset of flowering was controlled by temperature and flowering occurred irrespective of different photoperiods. Subsequently, the effect of photoperiod on flowering was confirmed to be landrace dependent, although the major effect on phenology and development of photoperiod was on pod set and pod filling, rather than flowering time (Berchie et al. 2013). This clearly indicates that onset of flowering is temperature dependent and seed set is photoperiod dependent. However, there is very limited evidence on how bambara groundnut genotypes differ in their response to temperature stress with respect to reproductive success.

The example from other crops suggests that pollen development is often the most vulnerable developmental stage to heat stress when gametes are placed separately under high temperature stress (Zinn et al. 2010; Rieu et al. 2017). From the literature review (Refer to chapter 2, **section: 2.3.2.3.**) it was clearly indicated that the critical temperature during plant reproduction may vary among plant species, however, in legume species, temperatures ranging from ≥33°C to ≥37°C were found to be critical. In our current research, we took the temperature range of ≥33°C to ≥37°C as a reference point for investigating the effect of temperature stress on the reproductive biology of bambara groundnut. Short episodes of high temperature stress (≥35°C) proved to be detrimental during flowering and pod formation and caused a significant reduction in chickpea seed yields (Wang et al. 2006). Similar studies on short episodes (5 days) of temperature stress (32/27°C) also caused a lack of anther dehiscence and pollen sterility in common bean (Gross and Kigel 1994a) and high night temperatures reduced pollen production in groundnut (Prasad et al. 1999).

Similarly when soybean flowers were subjected to temperature stress, two stages of flower development were found to be affected which resulted in abnormal tetrad and pollen development deficiency which intern lead to poor pod set (Ohnishi et al. 2010). The first stage which was affect was an early flower development stage at 12 days before anthesis which corresponds to tetrad/microspore stage, and the second stage was 3 to 4 days before anthesis. Therefore, we decided to apply high temperature stress during flowering (reproductive stage) for a short period of 3 days. The objective of this research was to determine the effect of short durations of temperature stress (day/night) surrounding flowering time (pre- and post-anthesis) in different genotypic lines derived from landraces of bambara groundnut.

4.2. Materials and methodology

As bambara groundnut primarily exists in the form of more or less heterogeneous landraces, to remove genotypic variation seeds from a selfing-pollination of single plants derived from landraces were used in upcoming experiments. Previous studies using microsatellites have shown these to be largely inbred (average residual heterozygosity = 0.02 (Molosiwa et al. 2015). A total of six genotypes potentially differing in their capability to tolerate high temperature stress were chosen based on

the climate at their region of cultivation. These seeds were kindly provided by Dr Presidor Kendabie, University of Nottingham (SB campus). The seeds of bambara groundnut genotypes were soaked for 3-4 hours before germination in seed modules containing Levington seed and modular compost (F2). At fourteen days after germination (DAG) the seedlings were transplanted into 5L wide pots with equal volumes of sand and JI-03 (John Innes no. 3) compost. The plants were watered on regularly to keep the top soil surface moist throughout the lifecycle to avoid water stress.

4.2.1. Experimental outline

The trials mainly consist of one control (C) and two high temperature (HT) stress experiments, which were established and conducted independently. These experiments were conducted in a Controlled Experiment (CE) room, University of Nottingham, UK (location: 52° 50' N, 1° 15'W). The CE room was equipped with artificial light source (with light intensity = 20,000 Lux), 12 hr. of Photoperiod (day/night) with 1-hour transition period between day and night, temperature control unit (Conviron). In both the control and high temperature stress treatments, the pots were arranged in completely randomized design with 7 biological replicates/genotype.

a. Control

The outline of the control experimental flow is illustrated in **figure 20**. The control experiment was conducted under ideal temperatures of 28/22°C (day/night) and 12h photoperiod through the life cycle of plants without imposing stress. Seeds of total of seven bambara groundnut genotypes ((IITA-686, S19-3, DipC, AHM-753, Uniswa red, Gresik and Getso) were germinated and transplanted as described in the previous **section (4.2)**. Phenological measurements such as seed germination, 4-leaf stage, and flowering time of all genotypes were noted. The freshly opened flowers were used to determine *in-vitro* pollen germination percentages for each landrace/genotype. For the control experiment, no heat stress was imposed on the plants.

b. Short-duration high-temperature stress treatment-1 and treatment-2

The six genotypes, IITA-686, S19-3, DipC, Uniswa red, AHM-753 and Getso, were grown under control conditions (28C/22°C; day/night) until flowering. The observations from the flowering pattern (chapter 3; section 3.2.) concluded that 2 weeks after the first flower opens was the time when the plants produced the highest number of flowers. This time window was used to determine an ideal time frame for the application of the heat stress. A short duration (3 days) of high temperature stress of 36°C (day/night) was applied two weeks after the first flower had opened. At the time of imposition of the heat-stress, the temperature was increased with a 30 min transition period to reach the new set temperatures. The temperature was brought back to control conditions i.e. 28/22°C (day/night) after three days of stress and was maintained under this regime throughout the plant life-cycle. The photoperiod was maintained at 12:12 hr. (light/dark) throughout and careful watering was done to ensure that moisture was not a limiting factor in either of the temperature regimes. The frequency of irrigation was every alternative day and the amount of water irrigated into each pot was 200 mL until flowering and increased to 300 to 400 mL during podding.

The three days of high temperature (HT) stress, hereafter will be referred as HT-1 (24 h into heat stress), HT-2 (24h - 48 h into heat stress) and HT-3 (48h - 72 h into heat stress). The days after HT stress were referred as recovery day-0 (R-0; 0 h to 24 h), day-1 (R-1; 25h to 48 h), R-2 and so on. Both the high temperature stress treatments were conducted the same way with the only difference being that during treatment-2, three plants/genotype were placed in growth chamber under control temperatures of 28C/22°C (day/night) and 12hr photoperiod. These plants were considered as a control within treatment group to obtain data on pollen germination and pod production under permissive conditions.

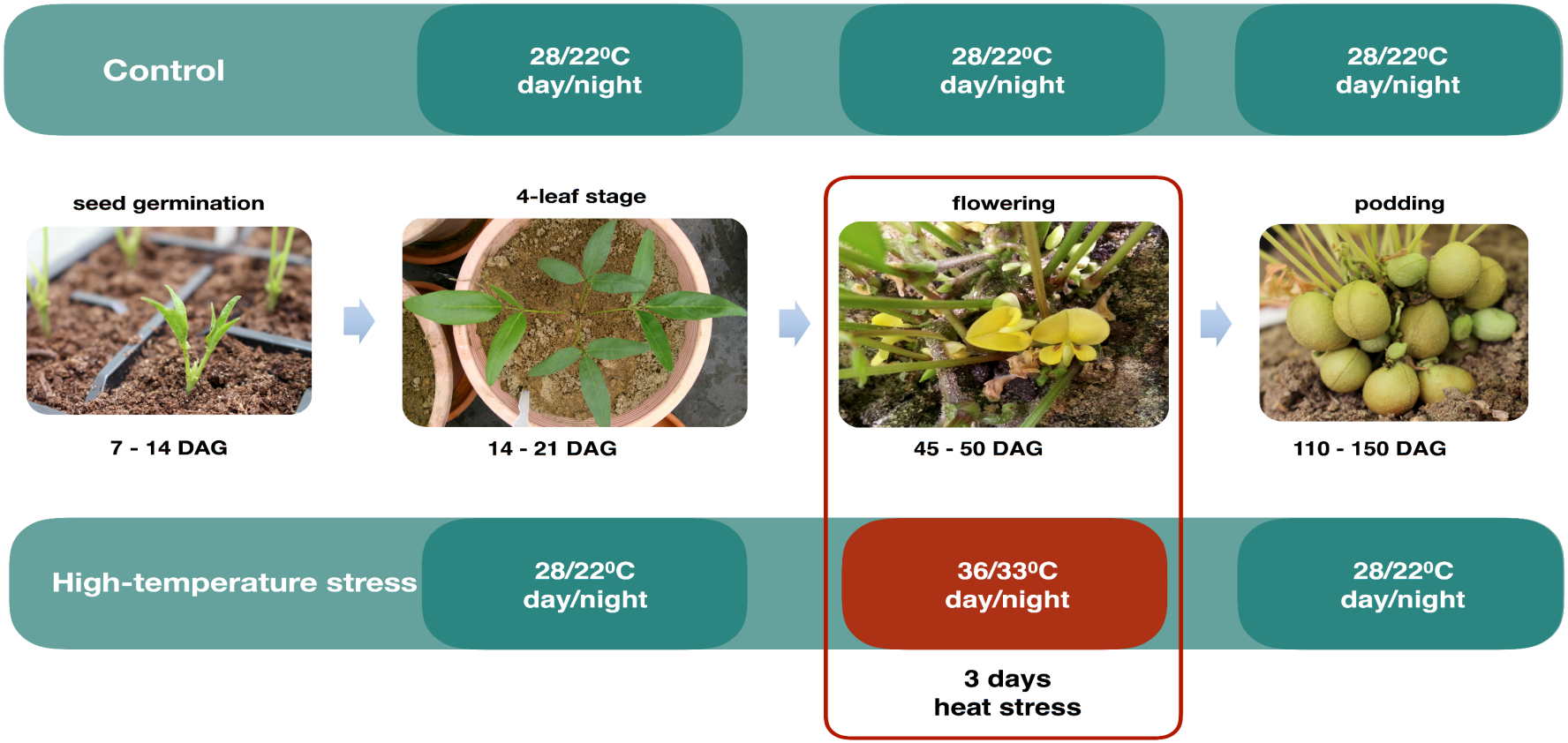


Figure 20: Outline of the experiment work flow. Briefly; the temperature regime throughout control experiment was 28/22°C, whereas the high-temperature stress treatment regime was 28C/22°C from seed germination until maturity, with the exception of 36/33°C applied for three days at 14 days after first flower opening.

4.2.2. To determine effect of high temperature stress on flower morphology and flowering pattern

The flower morphology was evaluated based on whether the flowers were open on the three days of heat stress (HT-1, HT-2, HT-3) and during the recovery period (R-0, R-1 so on). The bambara groundnut flowers are considered completely open when the Standard petal and Wing petals are completely open (Onwubiko et al. 2011b). The results are presented in the results **section 4.3.3.1**.

To measure the flowering trend under high temperature stress, the flowering data was collected from the day of first flower opening (measured in DAF; days after first flower opening) in all seven genotypes. For each genotype the flowering trend was determined using seven biological replicates. The pattern of flowering was observed by simply plotting the average number of flowers opened each day in all the genotypes. The control and heat stress flowering trend was plotted using stacked bar-charts. With the number of days after first flower opened (DAF) in x-axis against the number of flowers opened or unopened on the corresponding days in the bambara-axis. Standard deviation was added as error bars to each data point on the graph.

Anthesis (anther opening to shed the pollen grains) was also observed, by recording completely opened and un-opened anthers in the flowers collected from control and heat stressed plants. In bambara groundnut anthesis usually occurs during the morning 8-10 am (Oyiga *et al.* 2010). Five flowers from each genotype were observed and this observation was made under 10X of a light microscope (Leica microsystems) in the heat stress (HT-day 1) period of treatment-2. Pollen viability was tested using Alexander stain on the day of heat stress (HT-1) (Alexander 1969). The results are presented in the **section 4.4.3.1**.

4.2.3. To determine effect on *In vitro* pollen germination

4.2.3.1. Improved methodology to study *in vitro* pollen germination

To study the difference in effect between control and high temperature stress treatments it was imperative to develop standardized pollen germination media (PGM). *In vitro* pollen germination medium (PGM) stock (10X) was prepared using

the recipe from Boavida and McCormick (2007). Working solution (1X) of liquid PGM (hereafter l-PGM) was prepared by adding 2.5 mL of 10X PGM stock and 22.5 mL deionised water. For solid PGM (hereafter s-PGM) medium, 0.5% of low-melting Agarose (A6560, Sigma Aldrich), was added to l-PGM medium and microwaved until the agarose melted. Both types of media were prepared with a 5, 10, 15 and 20% (bambara/v) series of sucrose, to determine the ideal concentration and the pH was adjusted to 7.5 using 0.1 M NaOH. A rectangle of 20 x 30 mm was drawn on the slides (size 40 x 20 mm) and filled with about 300-400 μL s-PGM after melting the agarose, this will built a flat agarose pad after solidification.

The flowers from bambara groundnut genotype, IITA-686, were used in this study subjected to availability. Fresh flowers were harvested on the day of anthesis (flower opening) between 8-10 am, the pollen were scattered on the liquid PGM medium and spread on the rectangular agarose pad of solid PGM medium. To achieve good pollen density and minimise biological variation pollen of three flowers from different plants was used per slide. The slides were placed immediately in a moisture chamber for incubation at room temperature (25°C) for 3-4 hours under dark conditions. For every 5, 10, 15 and 20% sucrose concentrations in l-PGM and s-PGM media, there were three replicates each (one replicate = one slide).

The observations of pollen tube growth were made with light microscope unit at 10X (Leica microsystems). The pollen germination percentage was calculated by counting the number of germinated pollen grains and total number of pollen grains per slide. The pollen grain was scored as germinated when the pollen tube length was longer than the pollen grain diameter. Around 500 pollen grains per replicate were counted to determine the pollen germination percentage for all three replicates (one replicate/one slide). The best suited media with ideal sucrose concentration was used to establish pollen germination percentage of all other genotypes during the control treatment.

Statistical analysis-1: Using the statistical analysis tool kit (Microsoft excel, version 15.16), a Two-factor ANOVA with replication was performed to test whether there was any difference in pollen germination between liquid and solid PGM medium with varying concentrations of sucrose (5, 10, 15 and 20%). The results are presented in

the **section 4.3.1**. The ANOVA analysis only shows whether or not there was a significant difference ($p<0.05$) in pollen germination with respect to the PGM media and sucrose concentrations, but does not identify where the difference comes from. Therefore, a stepwise multiple comparison 'post-hoc t-test' was carried out with Bonferroni correction. During the Bonferroni correction, an adjustment was made to the p-value by diving the critical p-value (0.05) by the number of comparisons made to obtain p-value (t). Then the significance was analysed by comparing the p-value (T) from a two-tailed t-test, to the p-value (t), the parameter followed was T<=t. The two-tailed t-test was performed assuming the equal variances and all possible comparisons between the sucrose concentrations and PGM media were made.

4.2.3.2. To determine *in vitro* pollen germination of seven bambara groundnut genotypes during control treatment

In the control experiment, three replicates per genotype for all six genotypes (IITA-686, S19-3, DipC, Uniswa red, Gresik and Getso), were used to observe and determine pollen germination. *In vitro* pollen germination was assessed using the best suited pollen germination media, the 1-PGM with 20% sucrose was prepared and the pH was adjusted to 7.5. The solid PGM (s-PGM; 0.5%) was prepared by microwaving the medium containing the appropriate percentage of low melting agarose (A6560, Sigma Aldrich). Fresh flowers were collected between 8-10 am (at the completion of anthesis) and outer floral whorls (calyx and corolla) were removed. These dissected flowers with pollen grains were immediately dusted onto the agarose pad on the slides and incubated in an inverted position in moisture chambers for 4-5 hours at ≈ 26±1°C under dark conditions.

Statistical analysis-2: The pollen germination percentages of each genotype was plotted in simple box and whisker chart using the stacked bar chart feature in Microsoft excel (version 15.16). Using the statistical analysis tool kit (Microsoft excel, version 15.16), a one-factor ANOVA was performed to test whether there was any difference in pollen germination percentages between the seven bambara groundnut genotypes under the control conditions (refer to **section 4.3.2.**). The post hoc t-test was carried out using data analysis tools from Microsoft excel-2015 (Version 15.16) and

Bonferroni corrections were applied as described previously in statistical analysis method-1.

4.2.3.3. To determine *in vitro* pollen germination during high temperature stress treatment-1 and treatment-2

Pollen tubes were observed from the flowers of the heat stressed and control plants following the same methodology in the **section 4.2.3.2**. The outer whorls of flowers were dissected and removed, the pollen grains were dusted on the slide and anthers were mounted using 50% glycerol. The observation was made using a light microscope under 10X (Leica microsystems). The results are presented in the **section 4.3.3.2**.

During high temperature stress treatment-1, the pollen germination percentage was determined following the same protocol described in the section **4.2.3.2.**, on flowers from heat stress day (HT-1), and recovery days (R-3, R-8 and R-12). In addition, pollen germination was also determined one day prior to heat stress, this was treated as within treatment control (C). Similarly, during high temperature stress treatment-2, the pollen germination percentage was determined on heat stress day (HT-1) and recovery days (R-0, R-3 and R-8). During treatment-2, flowers from control plants (growing at ideal conditions, 28/22°C) were used to determine the pollen germination percentages of bambara groundnut genotypes and compare it with the treatment-2.

In both the experiments the pollen germination was evaluated for IITA-686, S19-3, AHM-753, Uniswa red and Getso genotypes. From the observations of the control experiments, DipC and Gresik were left out, DipC because the pollen grains did not germinate in the media reliably, which may be due a genotypic effect (even though a number of parameters such as sucrose, agarose and pH were evaluated for DipC), and Gresik because the availability of seeds were limited, and pollen germination was low (<45%) even under control conditions.

Statistical analysis-3: Using the statistical analysis tool kit (Microsoft excel, version 15.16), a Two-factor ANOVA with replication was performed to test whether there was any difference in pollen germination between genotypes and during heat stress day (HT-1) and recovery days (corresponding to treatment-1 and -2). The post hoc t-test was carried out using data analysis tools from Microsoft excel-2015 (Version

15.16) and Bonferroni corrections were applied as described previously in statistical analysis method-1.

4.2.4. To determine successful pod set under high temperature stress

The flowers, before heat-stress, during heat-stress and during the recovery periods were tagged to observe successful pod set (pod formation and filling) during both heat treatments. Podding was considered successful when the pod was formed with the seed formed inside. Pod formation was determined at 15 days after tagging. The time period during which podding was observed during heat stress experiment-1 was, prior to heat stress (10 to 15 DAF days after first flower opening), during days of heat stress (HT-1, HT-2 & HT-3) and recovery days (R-0 to R-23). During heat stress experiment-2, the time period during which podding was observed was, prior to heat stress (9 to 15 DAF days after first flower opening), during days of heat stress (HT-1, HT-2 & HT-3) and recovery days (R-0 to R-25).

Statistical analysis-4: Two factor ANOVA was performed on the over all podding data across all the time points, in bambara groundnut genotypes (IITA-686, S19-3, AHM-753, Uniswa red, Getso and DipC), using the 'statistical analysis tool' (Microsoft excel version 15.16). The genotype and time were considered as two factors during this analysis. Line graphs were plotted using the time scale on x-axis (measured in DAF) against total number of flowers tagged at each time point. The results are presented in the **section 4.3.3.3**.

4.3. Results

4.3.1. Improved pollen germination medium and finalized methodology for bambara groundnut pollen studies

Two kinds of pollen germination media liquid (l-PGM) and solid (s-PGM), were prepared using varying concentrations of sucrose (10, 15 and 20%), and the pollen germination percentage was determined in the bambara groundnut genotype, IITA-686. The data was analyzed by two-factor ANOVA, **table 14** shows the result of this statistical analysis. There was a statistically significant ($p<0.05$) interaction between PGM media and sucrose concentrations. This shows that the varying concentrations of

sucrose and type of PGM both effected the pollen germination percentages in the bambara groundnut genotype. There was an exponential increase in the pollen germination percentage with increasing sucrose concentrations with s-PGM, whereas, no similar effect was observed in the interaction with l-PGM (**Figure 21**). Therefore, the combination of solid-pollen germination medium (s-PGM) with 20% sucrose concentrations was determined to be ideal for pollen germination assays in future experiments.

Table 14: Two- factor Analysis of Variance (ANOVA) table of pollen germination in liquid and solid PGM medium, and at different sucrose concentrations, showing significance at 0.05 level; s.s. (square root of squares), d.f. (degrees of freedom), m.s (mean of squares) and F. pr (Probability).

Source of Variation	***s.s***	***d.f***	***m.s***	***F***	***p-value***
PGM media	7804.2	1	7804.2	3515.4	<0.05
Sucrose concentration	3613.0	2	1806.5	813.7	<0.05
Interaction (PGM x Sucrose)	3128.3	2	1564.1	704.6	<0.05
Residual	26.64	12	2.22		
Total	14572.10	17			

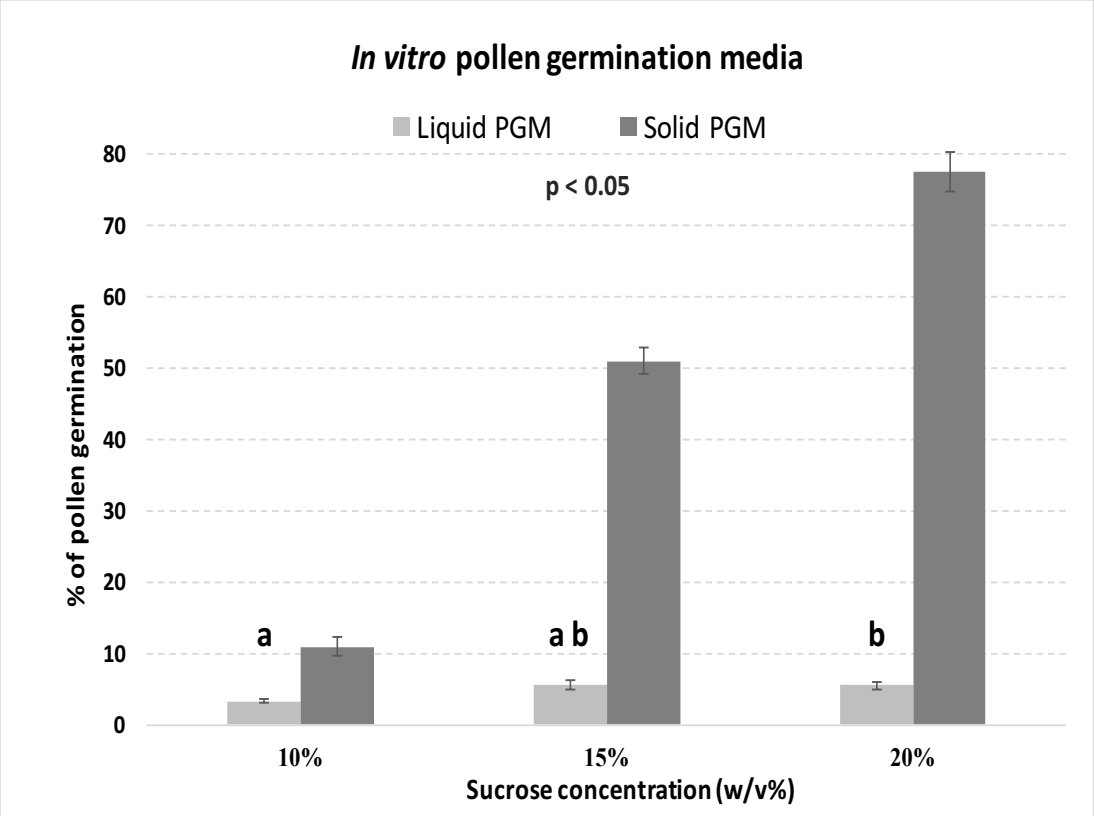


Figure 21: Pollen germination percentages of bambara groundnut (genotype IITA-686), at 10, 15 and 20% sucrose concentrations, and, liquid and solid PGM media. Briefly; the Bonferroni post-hoc t-test multiple comparison showed significant difference at the 0.05% level, between PGM media and sucrose concentrations and their interactions, except between l-PGM at 10% & 15% (a), and l-PGM at 15% & 20% (b).

4.3.2. *In vitro* pollen germination of seven bambara groundnut genotypes

The percentage of pollen germination during the control experiment was determined for all seven genotypes IITA-686, S19-3, AHM-753, Uniswa Red, Gresik, Getso and DipC (**Figure 22**). Genotype S19-3 showed the highest pollen germination percentage (≅ 81 %), followed by IITA-686 (≅ 66%), Uniswa red (≅ 64%), AHM-753 (≅ 63%), Getso (≅ 49%) and Gresik (≅45%), respectively. However, DipC showed the lowest *in-vitro* pollen germination percentage among the genotypes (≅3%), therefore DipC was eliminated from the one-way ANOVA analysis, since it appears that DipC pollen is not responding to the standard medium and it would lead to biased analysis.

The one-way ANOVA output is presented in the **table 15**, it shows that the difference in pollen germination was significant between genotypes ($p<0.05$). The multiple comparison Bonferroni post-hoc t-test shows that the difference in pollen germination was significant in between genotype combinations as indicated in the box-whisker plot (**Figure 22**). The significant groupings were IITA-686 & S19-3 (a), IITA-686 & Gresik (b), S19-3 & AHM-753 (c), S19-3 & Uniswa red (d), S19-3 & Gresik (e), S19-3 & Getso (f), AHM-753 & Gresik (g).

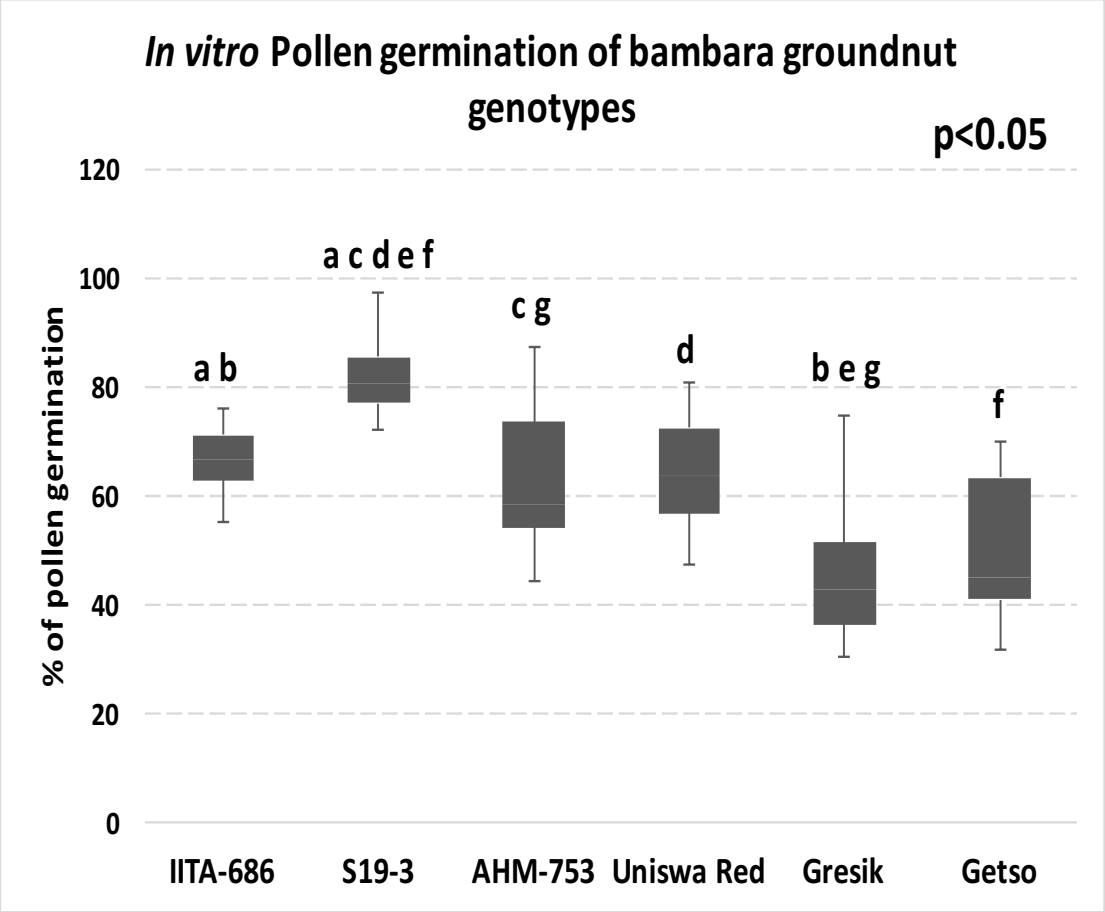


Figure 22: The box-whisker plot represents the pollen germination percentage of seven genotypes of Bambara groundnut. The Bonferroni post-hoc t-test multiple comparison show significant difference at 0.05% level, between (a) IITA-686 & S19-3; (b) IITA-686 & Gresik; (c) S19-3 & AHM-753; (d) S19-3 & Uniswa red; (e) S19-3 & Gresik; (f) S19-3 & Getso; (g) AHM-753 & Gresik, genotype combinations.

Table 15: One-way ANOVA output of pollen germination between genotypes of bambara groundnut. (* statistical significance at the 0.05 level). SS-sum of squares, d.f- degrees of freedom, MS-mean squares, p- probability).

Source of Variation	SS	d.f	MS	F	p-value
Between Genotypes	2437.015	5	487.40	66.46	<0.05*
Within Genotypes	88.01	12	7.33		
Total	2525.02	17			

4.3.3. Short-duration of high temperature stress

A short duration (3 days) of high temperature stress was applied two weeks after the first flower had opened, the high temperature of 36°C (day/night) was set up digitally using the CE-room controller unit. Due to technical aspects, on how reliable and controllable the CE room was, the actual temperatures were ≈ 36/30°C (day/night) during three days of heat stress duration, and the 28/22°C (day/night) during the recovery period. The observations and assays such as, flower morphology and anthesis, pollen tube growth and germination, podding and seed set, was recorded and performed, respectively, during the three days of high temperature stress duration (HT-1, HT-2 & HT-3) and recovery periods (R-0, R-1 & so on.).

4.3.3.1. Effect on flower morphology and flowering pattern

The Bambara groundnut flowers were considered completely open when the Standard petal and wing petals open (The flower opening was observed usually in the morning hours of 8-10 am). During both short duration temperature stress treatments, it was observed that the standard petal failed to open all day long (**Figure 23**). During temperature stress treatment-1 and treatment-2, the flowers failed to open during the three days of stress treatment (HT-1, HT-2, HT-3). During the recovery period the flowers eventually opened in all the genotypes. In IITA-686, S19-3, Gresik and Getso, some flowers opened 7 days after recovery. In AHM-753 and DipC, some flowers

opened at 6 and 8 days, respectively. All the genotypes showed complete recovery in flower opening by 12 days of recovery.

Figure 23: Flower morphology and behaviour observed in control and heat stressed (HT day-1) plants of six genotypes of bambara groundnut observed during treatment-1 and 2. Briefly; all control plants show completely opened flowers, whereas heat stressed plant showed only unopened flowers.

Effect on flowering trend

The flowering trend/pattern observations by comparing the control and temperature stress treatment-1, shows different patterns in each genotype (**Figure 24, 25 & 26)**. The bambara groundnut genotypes IITA-686 (**Figure 24[a]**), S19-3(**Figure 24[b]**), AHM-753 (**Figure 25[a]**), Uniswa red (**Figure 25[b]**), Getso (**Figure 26[a]**) and DipC (**Figure 26[b]**), shows that there was increase in the number of flowers during heat stress treatment-1 when compared with control. The flowering trend of bambara groundnut genotypes during treatment-2 follows a similar pattern as treatment-1 (**Figure 27, 28 & 29**). The bambara groundnut genotypes IITA-686 (**Figure 27[a]**), S19-3(**Figure 27[b]**), AHM-753 (**Figure 28[a]**), Uniswa red (**Figure 28[b]**), Getso (**Figure 29[a]**) and DipC (**Figure 29[b]**), shows that there was increase in number of flowers during heat stress treatment-2, in comparison with control experimental data. Few genotypes exhibited an increase in the number of flowers after heat stress (after HT-1 to HT-3 days), such as IITA-686 (**Figure 24[a] & Figure 27[a]**), AHM-753 (**Figure 24[a] & Figure 27[a]),** Uniswa red (**Figure 25[b] & Figure 28[b]**), Getso (**Figure 26[a] & Figure 29[a]**) and DipC (**Figure 29[b]**).

Anthesis (anther opening to shed pollen grains), was observed in all seven genotypes (**Figure 30**), S19-3 (**Figure 30 [c]**), AHM-753 (**Figure 30[e]**), Uniswa red (**Figure 30[g]**) & DipC (**Figure 30[k]**), and unopened anthers were seen in genotypes IITA-686 (**Figure 30[a]**) and Getso (**Figure 30[i]**), during heat stress (HT-1). The Alexander staining showed viable pollen grains in all genotypes irrespective of dehisced and in-dehisced anthesis {**IITA-686 (Figure 30[b]), S19-3 (Figure 30[d]), AHM-753 (Figure 30[f]), Uniswa red (Figure 30[h]), Getso (Figure 30[j]) and DipC (Figure 30[l])**}. The Alexander staining only shows only the presence of cytoplasm in pollen cell, irrespective of control or treatment the pollen grains showed positive for the Alexander staining, but these pollen grains did not show any *in vitro* germination.

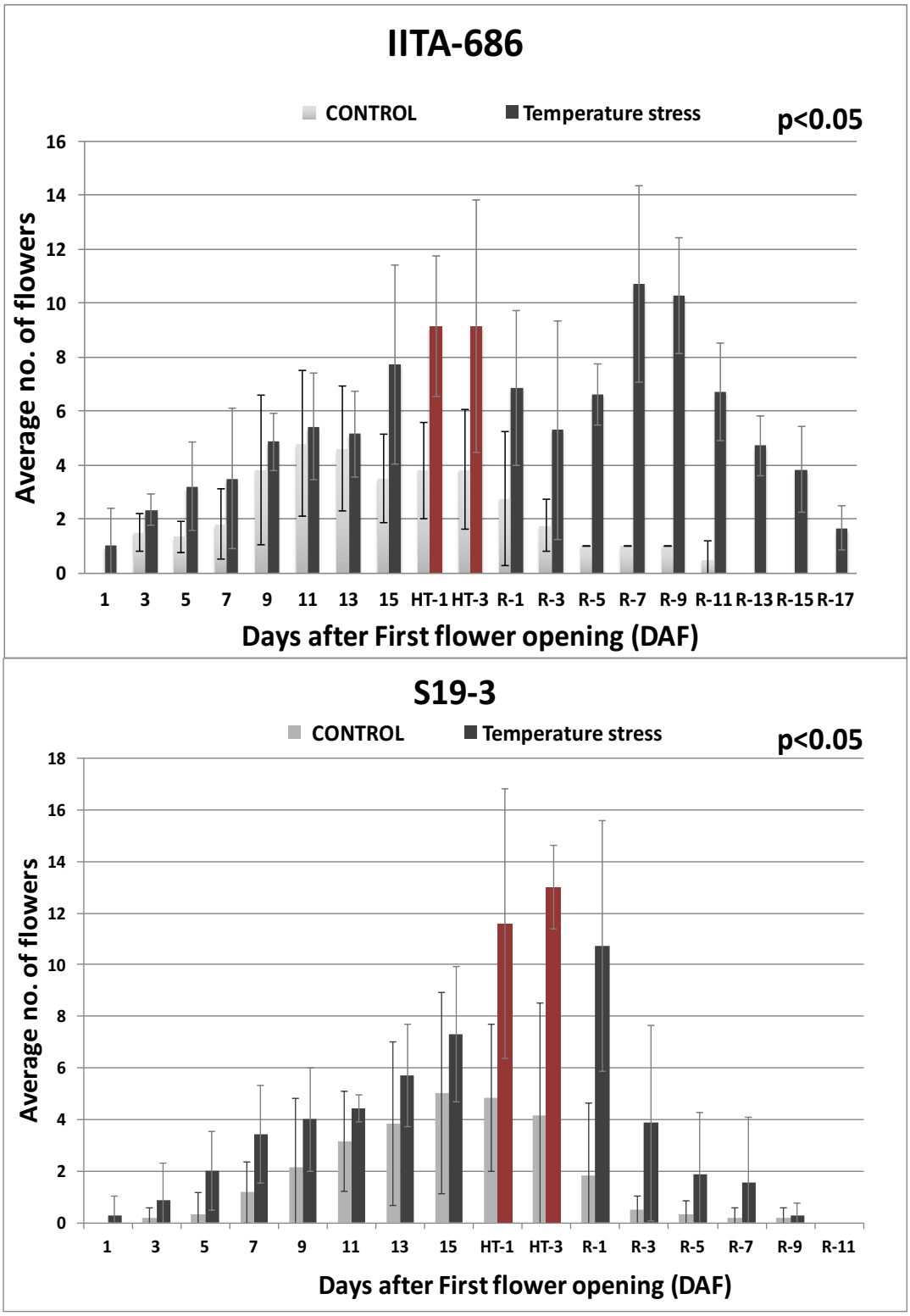


Figure 24: Comparison of control experiment (■) and temperature stress treatment-1 (■) flowering trend of bambara groundnut genotypes. Briefly; (a) IITA-686, (b) S19-3, genotypes show different flowering trends following heat stress (■). (error bars = standard deviation)

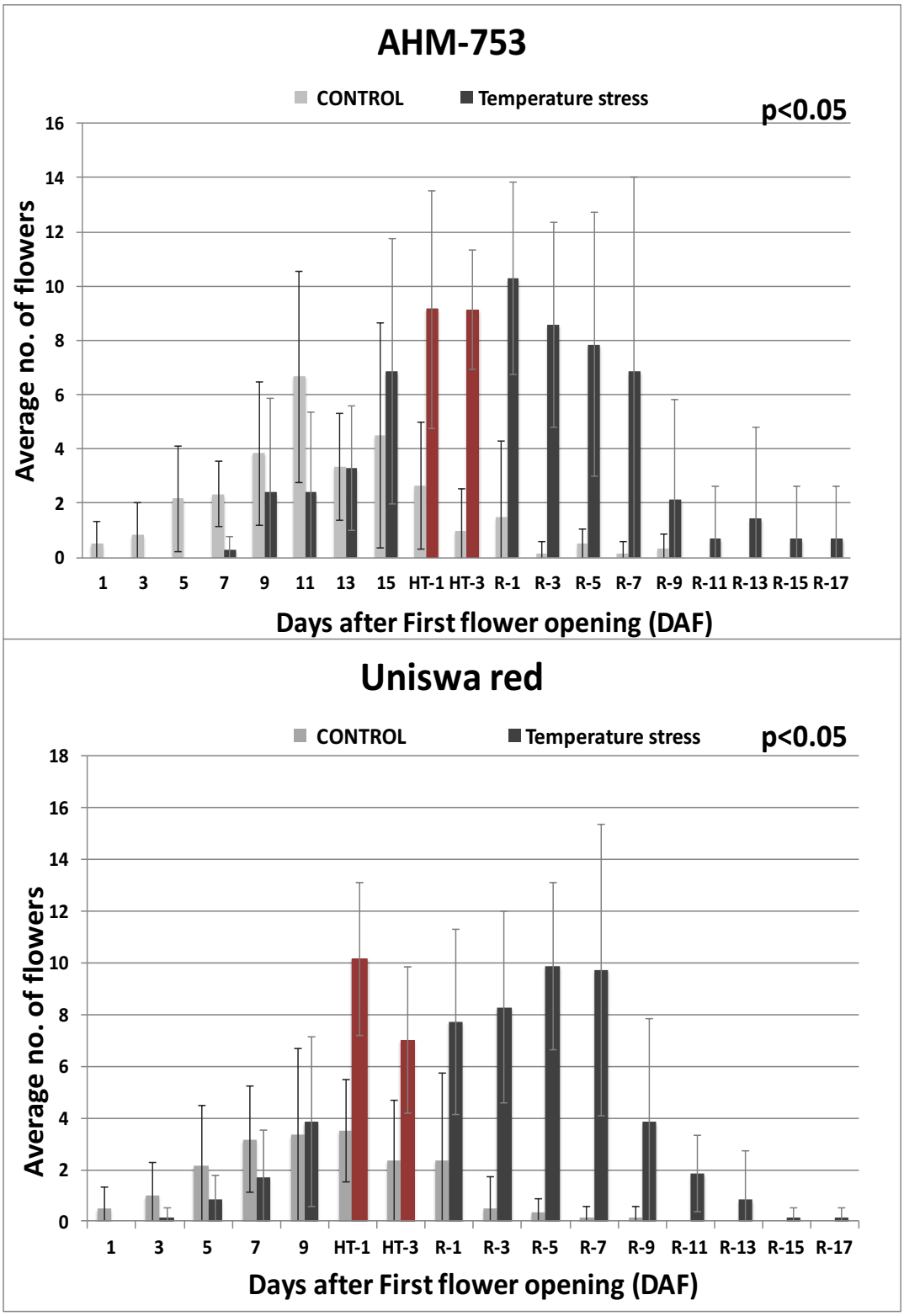


Figure 25: Comparison of control experiment (■) and temperature stress treatment-1 (■) flowering trend of bambara groundnut genotypes. Briefly; (a) AHM-753 (b) Uniswa red, genotypes show different flowering trends following heat stress (■). (error bars = standard deviation)

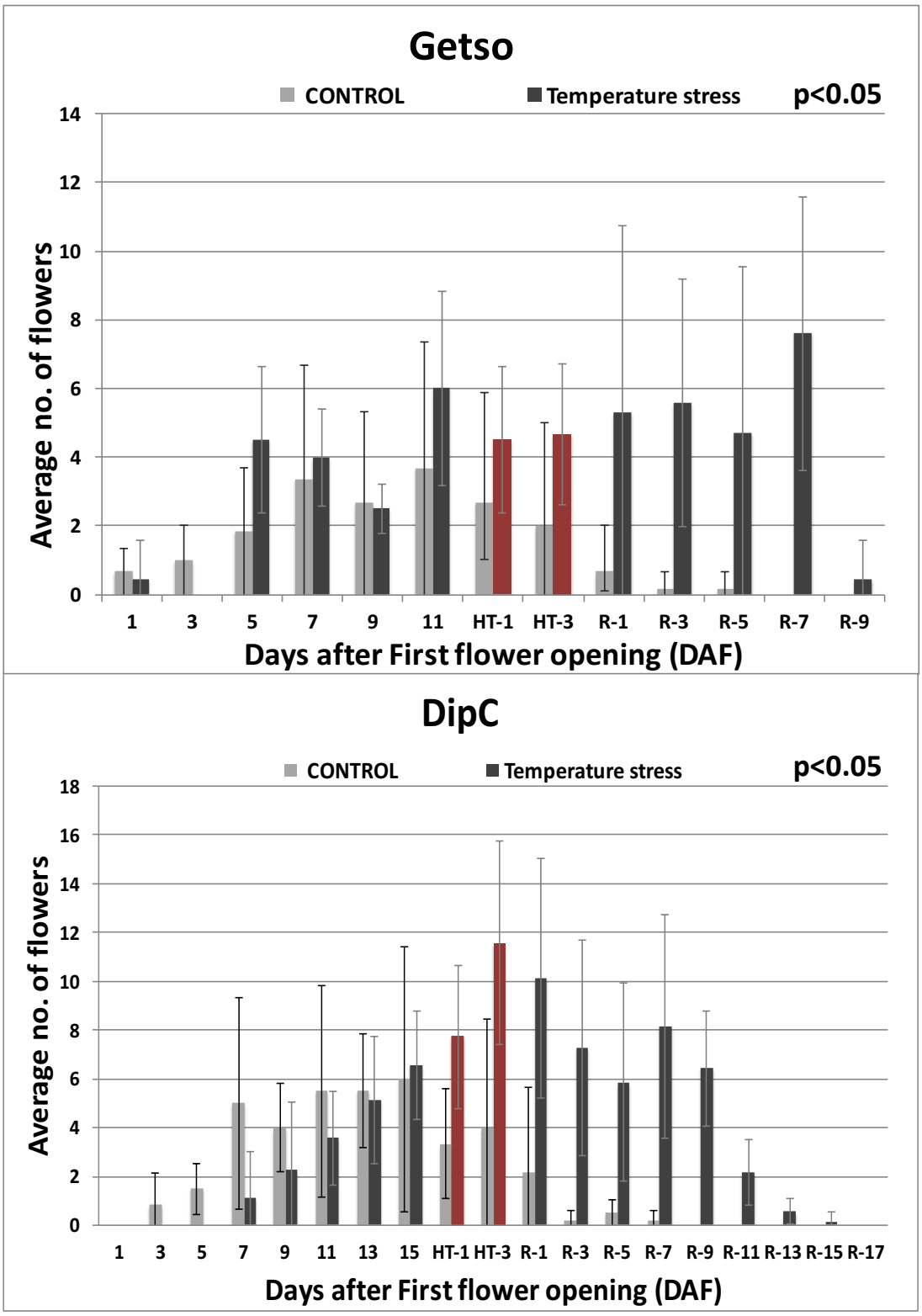


Figure 26: Comparison of control experiment (■) and temperature stress treatment-1 (■) flowering trend of bambara groundnut genotypes. Briefly; (a) Getso and (b) DipC, genotypes show different flowering trends following heat stress (■). (error bars = standard deviation)

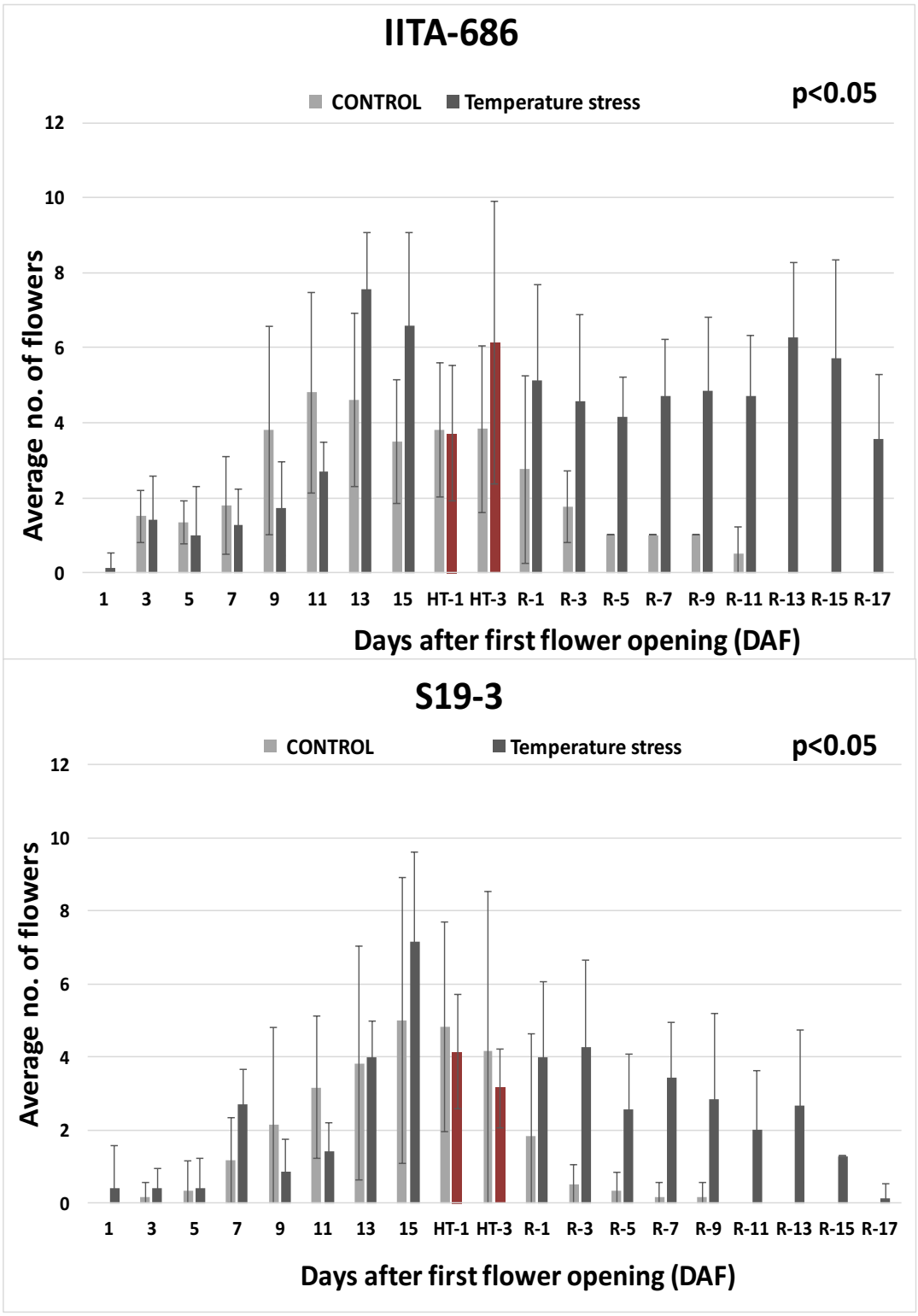


Figure 27: Comparison of control experiment (■) and temperature stress treatment-2 (■) flowering trend of bambara groundnut genotypes. Briefly; (a) IITA-686 and (b) S19-3, genotypes show different flowering trends following heat stress (■). (error bars = standard deviation)

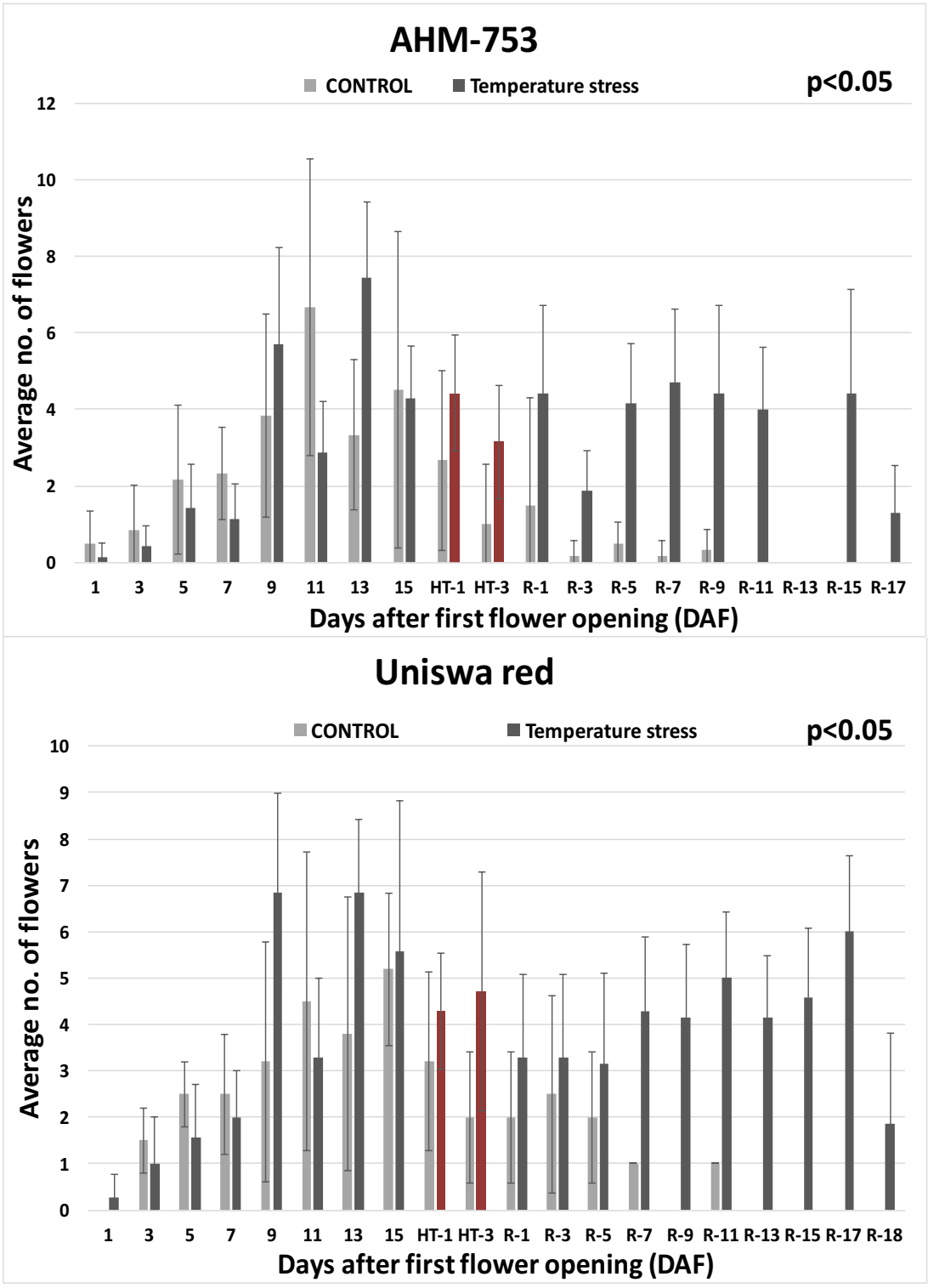
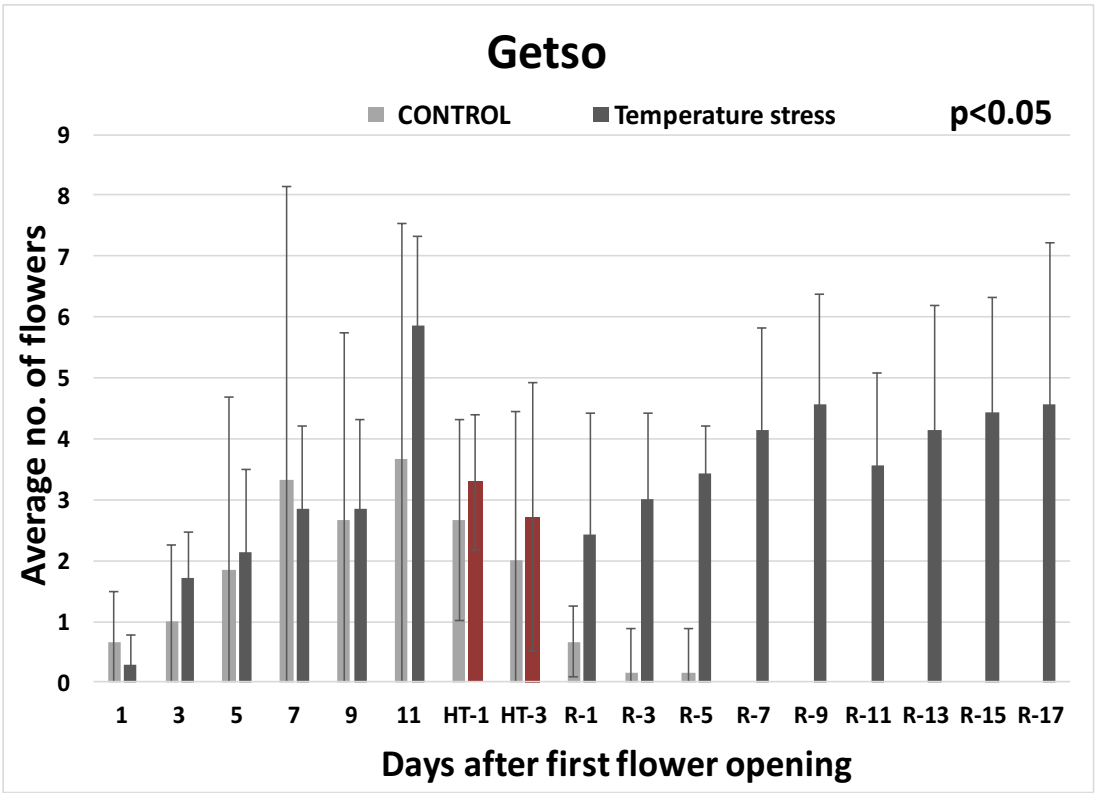


Figure 28: Comparison of control experiment (■) and temperature stress treatment-2 (■) flowering trend of bambara groundnut genotypes. Briefly; (a) AHM-753 and (b) Uniswa red, genotypes show different flowering trends following heat stress (■). (error bars = standard deviation)



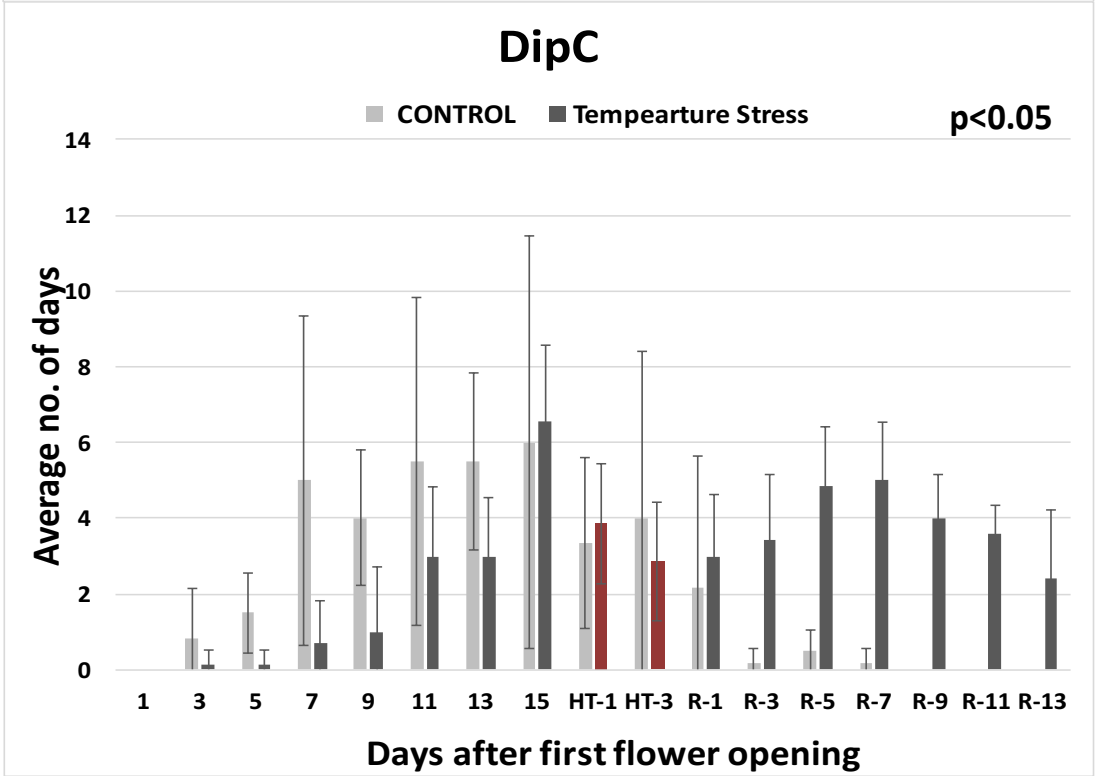


Figure 29: Comparison of control experiment (■) and temperature stress treatment-2 (■) flowering trend of bambara groundnut genotypes. Briefly; (a) Getso and (b) DipC, genotypes show different flowering trends following heat stress (■). (error bars = standard deviation)

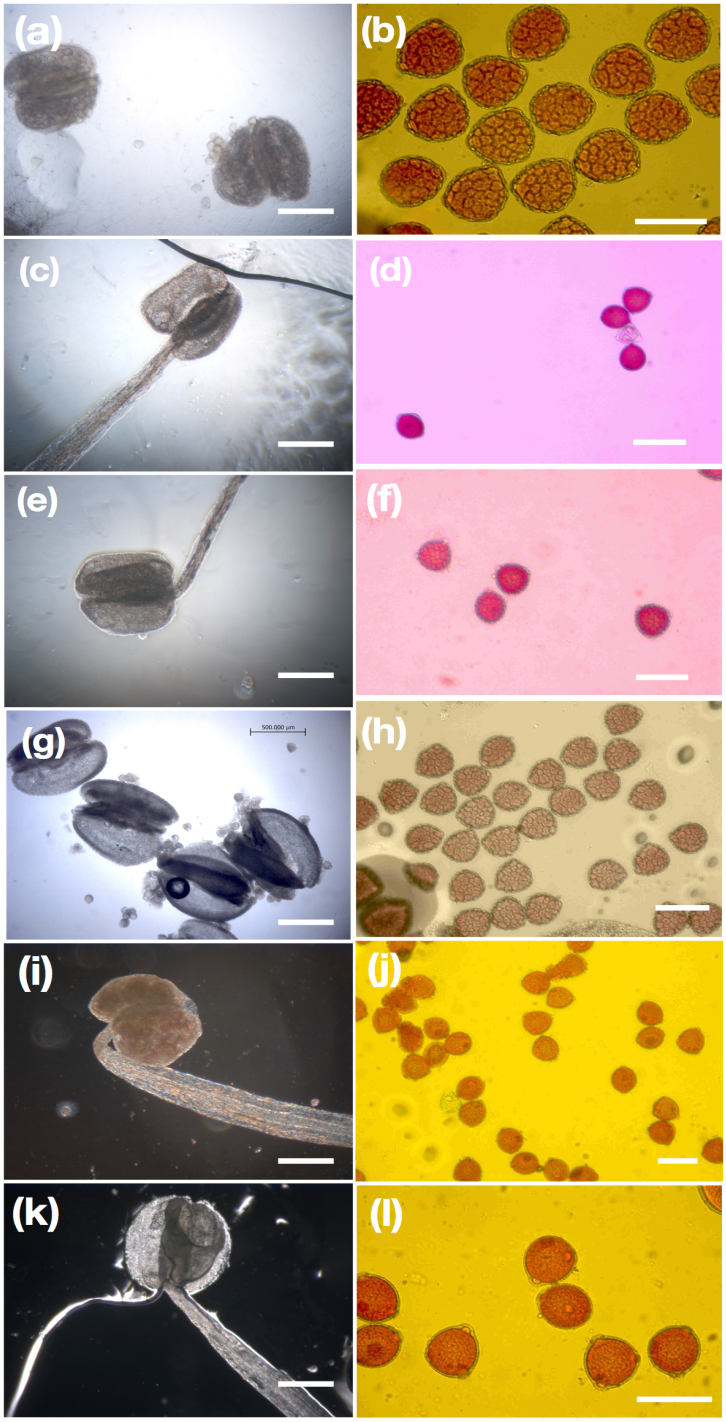


Figure 30: Anthesis (anther opening) and pollen viability during the temperature stress treatment-2 (HT-1) in all bambara groundnut genotypes. Briefly; The anthers were completely open during heat stress day (HT-1) in genotypes, S19-3 (c), AHM-753 (e), Uniswa red (g) & DipC (k), and unopened anthers were seen in genotypes IITA-686 (a) and Getso (i). The pollen grains were viable in all genotypes according to Alexander stain tests, IITA-686 (b), S19-3 (d), AHM-753 (f), Uniswa red (h), Getso (j) and DipC (l). (scale bar = 50 µm)

4.3.3.2. Effect on *in vitro* pollen germination

The observations during heat stress treatment-2 of bambara groundnut pollen tubes from control and heat stressed plants are collectively presented in **figure 31**. As observed there was no pollen tube growth/germination in most of the heat stressed plants on HT-1, the few germinated pollen grains showed short length and a zigzag pattern of growth, whereas the control pollen tubes showed smooth growth and long tube growth. The similar effects were observed on HT-1 during heat-stress treatment-1.

The pollen germination percentage was determined during experiment-1one-day prior to heat-stress [here considered as control (C)], on the day of heat stress (HT-1) and during the recovery periods (R-3, R-8 and R-12). A simple bar graph was plotted with the percentage of pollen germination on the y-axis against genotypes on the x-axis (**Figure 32**). Different color-coded bars represent the timescale during heat stress and recovery period, during which pollen germination of bambara groundnut genotypes was determined. *In vitro* pollen germination between genotypes and across time points (control, heat stress and recovery period) show significant difference at 0.05, level (**Table 16**).

Likewise, the pollen germination percentage between control and heat stressed plants on HT-1, R-0, R-3 and R-8 was determined during treatment-2. An interactive line-graph was plotted separately for each genotype, with the percentage of pollen germination on the y-axis and timescale on the x-axis (**Figure 33)**. The 24 hours (HT-1) of high-temperature stress ($36/30^0$C) reduced pollen germination (Graph 5 and 6). The *in vitro* pollen germination was significantly ($p<0.05$) different during heat stress and recovery period, in all genotypes (**Table 17**).

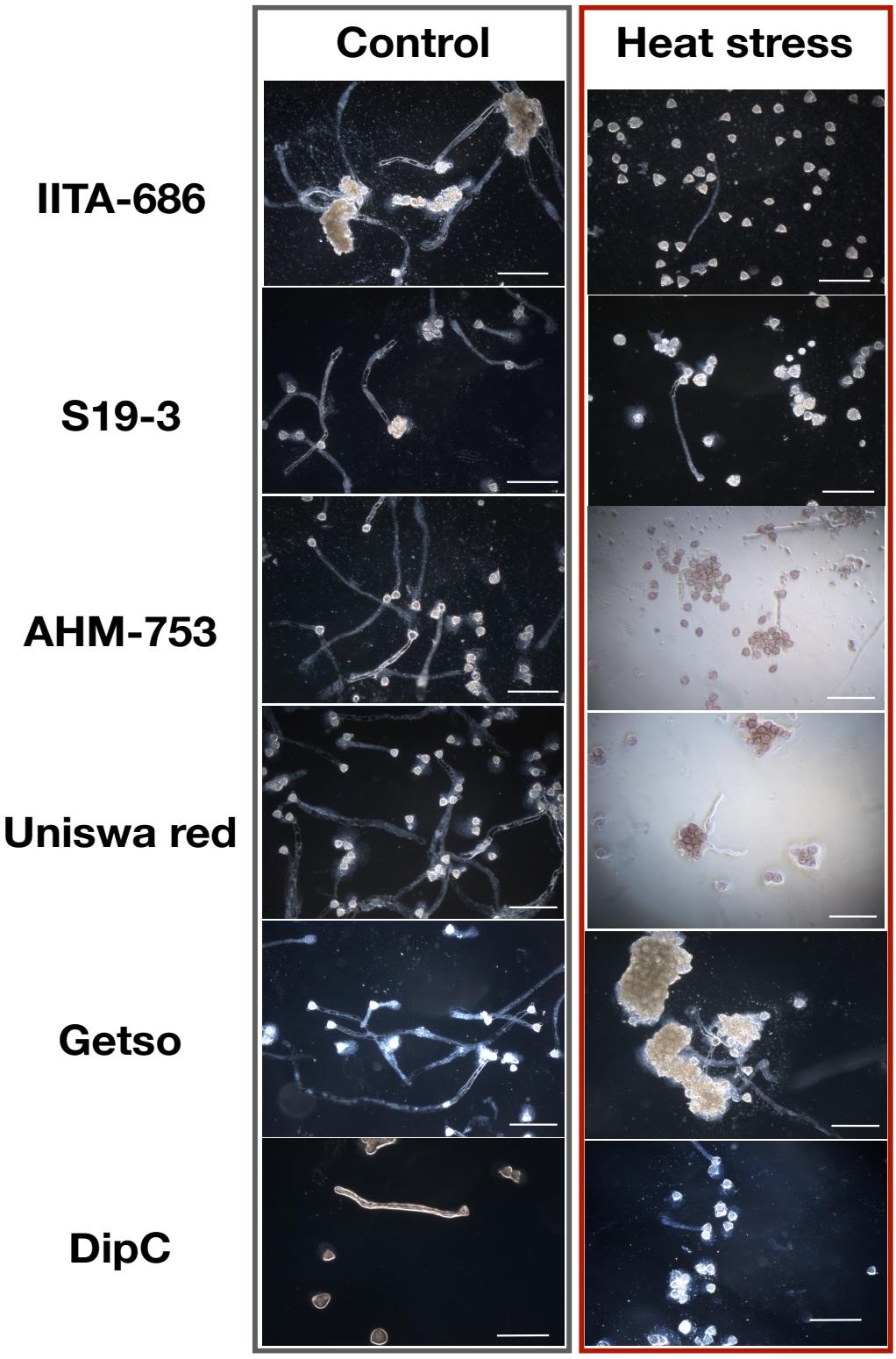


Figure 31: Pollen tube growth of six genotypes of bambara groundnut under control and heat stress (HT-day 1) conditions. Briefly; the control pollen grains show smooth and long pollen tubes, whereas heat stress pollen grains show only few germinated pollen grains, some of the pollen tubes were short and zigzag. (scale bar= 50 µm).

During both the high temperature stress treatment (-1 & -2), pollen germination was reduced to less than 10% in all genotypes ($p<0.05$). In temperature stress experiment-1 the pollen germination remained low (<15%) even after 3 and 8 days of recovery, gradually increasing after 12 days of recovery in genotypes such as S19-3 (≈34%), IITA-686 (≈30%), Uniswa red (≈23%) and AHM-753 (≈20) but not up to the point of full recovery within the observed period (**Figure 32**). During temperature stress treatment-2, pollen germination remained low (<20%) during recovery days, R-0 and R-3, in genotypes IITA-686, S19-3, Uniswa red and Getso. However, in genotype AHM-753, the percentage of pollen germination increased to ≈40% in three days of recovery ($p<0.05$). Other landraces showed an increase in pollen germination only after 8 days of the recovery period (**Figure 33**). The temperature at the time of recovery period during experiment-1 and experiment-2, was 28±1°C and 22±1°C, day and night respectively.

Table 16: Two- factor Analysis of Variance (ANOVA) table of pollen germination different genotypes and time points, during treatment-1. *showing significance at 0.05 level; d.f. (degrees of freedom), s.s. (square root of squares), m.s (mean of squares) and F. pr (Probability).

Source of Variation	SS	df	MS	F	P-value
Genotype	5,331.41	5	1,066.28	104.23	<0.05*
time period	23,687.04	4	5,921.76	578.87	<0.05*
Genotype x time period	6,866.05	20	343.30	33.56	<0.05*
residual	613.79	60	10.23		
Total	36,498.29	89			

Table 17: Two- factor Analysis of Variance (ANOVA) table of pollen germination different genotypes and time points, during treatment-2. *showing significance at 0.05 level; d.f. (degrees of freedom), s.s. (square root of squares), m.s (mean of squares), v.r. (variance) and F. pr (Probability).

Source of Variation	SS	df	MS	F	P-value
Genotype	68295.44	11	6208.68	114.03	5.03019E-50
Time point	6095.32	3	2031.77	37.31	4.49555E-16
Genotype x time point	6773.54	33	205.26	3.76	2.15192E-07
Residual	5226.92	96	54.45		
Total	86391.21	143			

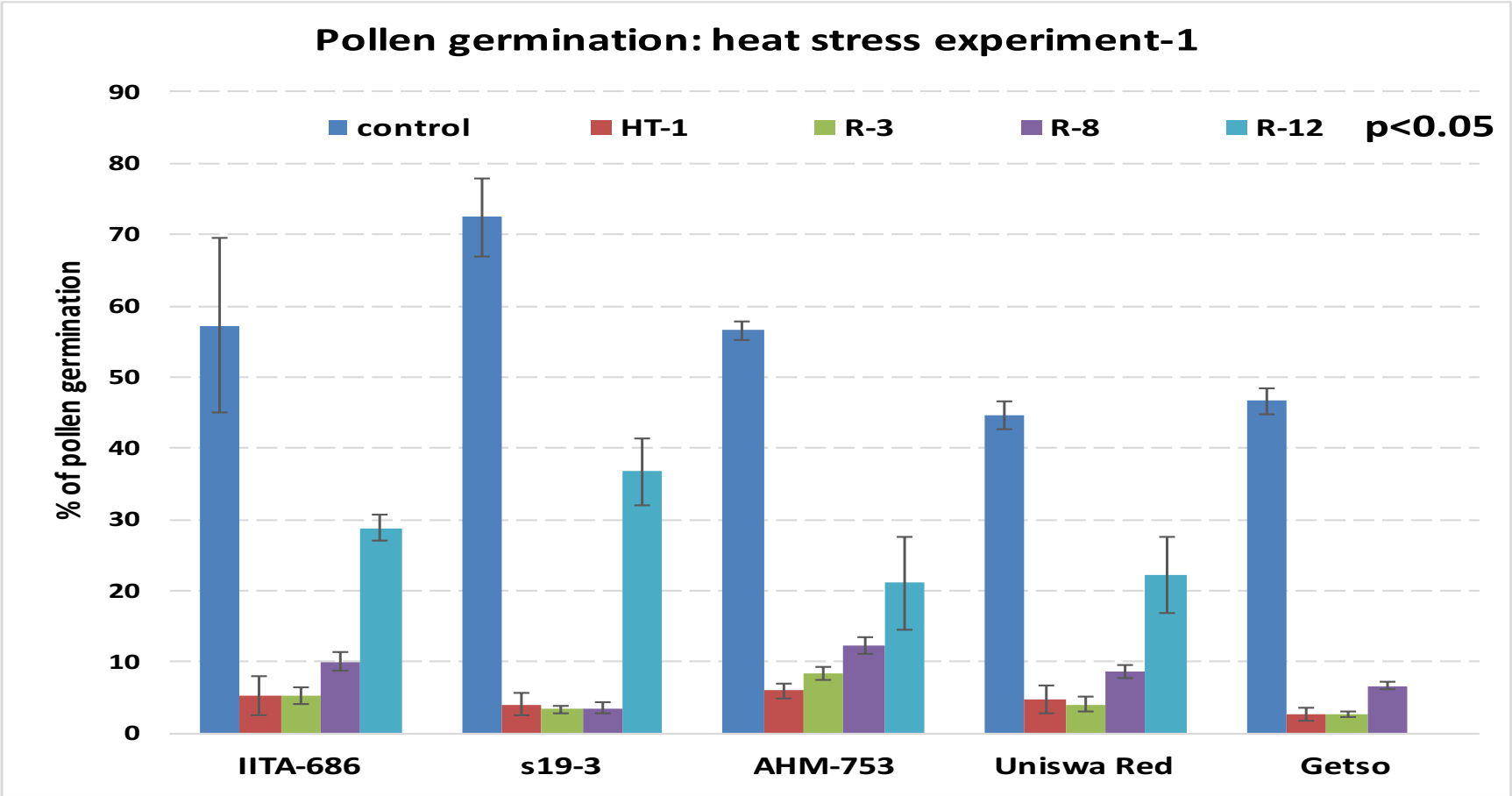


Figure 32: The percentage of in vitro pollen germination during high temperature stress treatment-1, showing significant difference between control, heat stress and recovery days. Briefly; control (represents one day prior to heat stress); HT-1 (heat stress day-1); R-3 (recovery day 1); R-8 (recovery day 8); R-12 recovery day). Error bars=standard deviation.

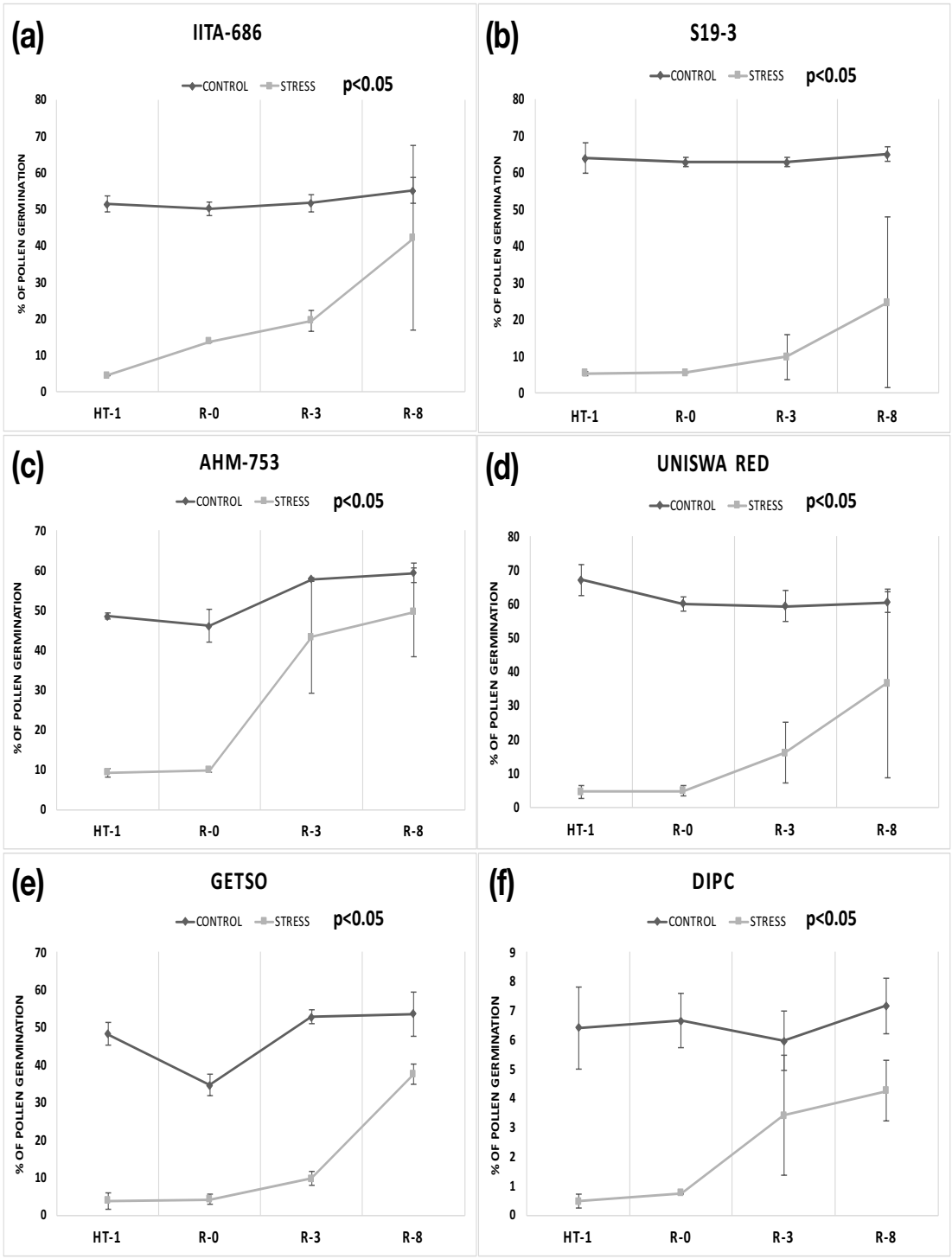


Figure 33: Percentage of in vitro pollen germination during high temperature stress experiment-2 at heat stress day (HT-1) and recovery days (R-0, R-3 & R-8), compared with control pollen germination of the respective genotypes; (a) IITA-686; (b) S19-3; (c) AHM-753; (d) Uniswa red; (e) Getso and (f) DipC. Briefly; All genotypes show significant ($p<0.005$) decrease in pollen germination on HT-1 and different rates of recovery during R-0 to R-8.

4.3.3.3. Effects on pod and seed set

During temperature stress treatment-1, a total of seven flowers/genotype was tagged each day, before heat stress (10 to 15 DAF), during heat stress (HT-1, HT-2 & HT-3) and during the recovery period (R-0 to R-23). Successful podding was observed in all genotypes on days before heat stress measured in DAF (days after first flower opening) **(Figure 34)**. There was no podding observed during the heat stress period (HT-1, HT-2 & HT-3) in all the genotypes **(Figure 34)**. Podding resumed in most of the genotypes during the recovery period, in IITA-686 (at R-13), S19-3 (at R-12), AHM-753 (at R-15), Uniswa red (at R-13) and DipC (at R-14), but Getso failed to produce any pods after heat stress. Two-factor ANOVA shows that there was difference in the number of successful pods formed which was significant across the time period at p=0.05 level, in all 6 genotypes (**Table 18**).

Similarly, during heat stress treatment-2, a total of 10 flowers/genotype was tagged each day before heat stress (9 to 15 DAF), during heat stress (HT-1, HT-2 & HT-3) and in the recovery period (R-0 to R-25). All the genotypes showed successful pod set before heat stress; however, pod-set was absent during the heat stress period except for a few pods from Uniswa red, S19-3 and Getso. Pod set was resumed in genotypes IITA-686, S19-3, DipC, Uniswa red and AHM-753 during the recovery period at R-10, R-5, R-5, R-10 and R-5 days, respectively, but Getso did not produce any filled pods after heat stress (**Figure 35**). Two-factor ANOVA analysis shows that there was statistically significant ($p<0.05$) differences in the number of successful pods formed across time points during which the flowers were tagged to observe pod formation, and the difference was significant in all genotypes at the p=0.05 level (**Table 19**).

During temperature stress treatment-1 and -2, there were aborted pods with undeveloped seeds in heat stressed plants, whereas the control plants showed normal healthy podding and seed set (**Figure [36] & [37]**). The genotypes, IITA-686, S19-3, AHM-753, Uniswa red, Getso and DipC showed pod abortion after heat stress, period (day HT-1). This might be an indication that the temperature stress might had an effect on successful seed development.

Table 18: Two-factor ANOVA output of podding during temperature stress treatment-1. Briefly; the podding was significantly different in all genotypes (*p<0.05) and across the time points (**p<0.05), during which the observations were made. [d.f. (degrees of freedom), s.s. (square root of squares), m.s (mean of squares), F (factor) and p-value (Probability)]

Source of Variation	s.s	d.f	m.s.	F	p-value
Genotype	31.94	5	6.39	6.385	1.95401E-05*
Time scale	621.82	32	19.43	19.424	2.90711E-40**
Residual	160.1	160	1.01		
Total	813.82	197			

Table 19: Two-factor ANOVA output of successful podding during temperature stress experiment-2. Briefly; the podding was significantly different in all genotypes (*p<0.05) and across the time points (**p<0.05), during which the observations were made. d.f. (degrees of freedom), s.s. (square root of squares), m.s (mean of squares) and p-value (Probability)

Source of Variation	SS	df	m.s.	F	P-value
Genotypes	21.34	5.00	4.27	2.97	0.02*
Time scale	43.24	15.00	2.88	2.01	0.03**
Error	107.82	75.00	1.44		
Total	172.41	95.00			

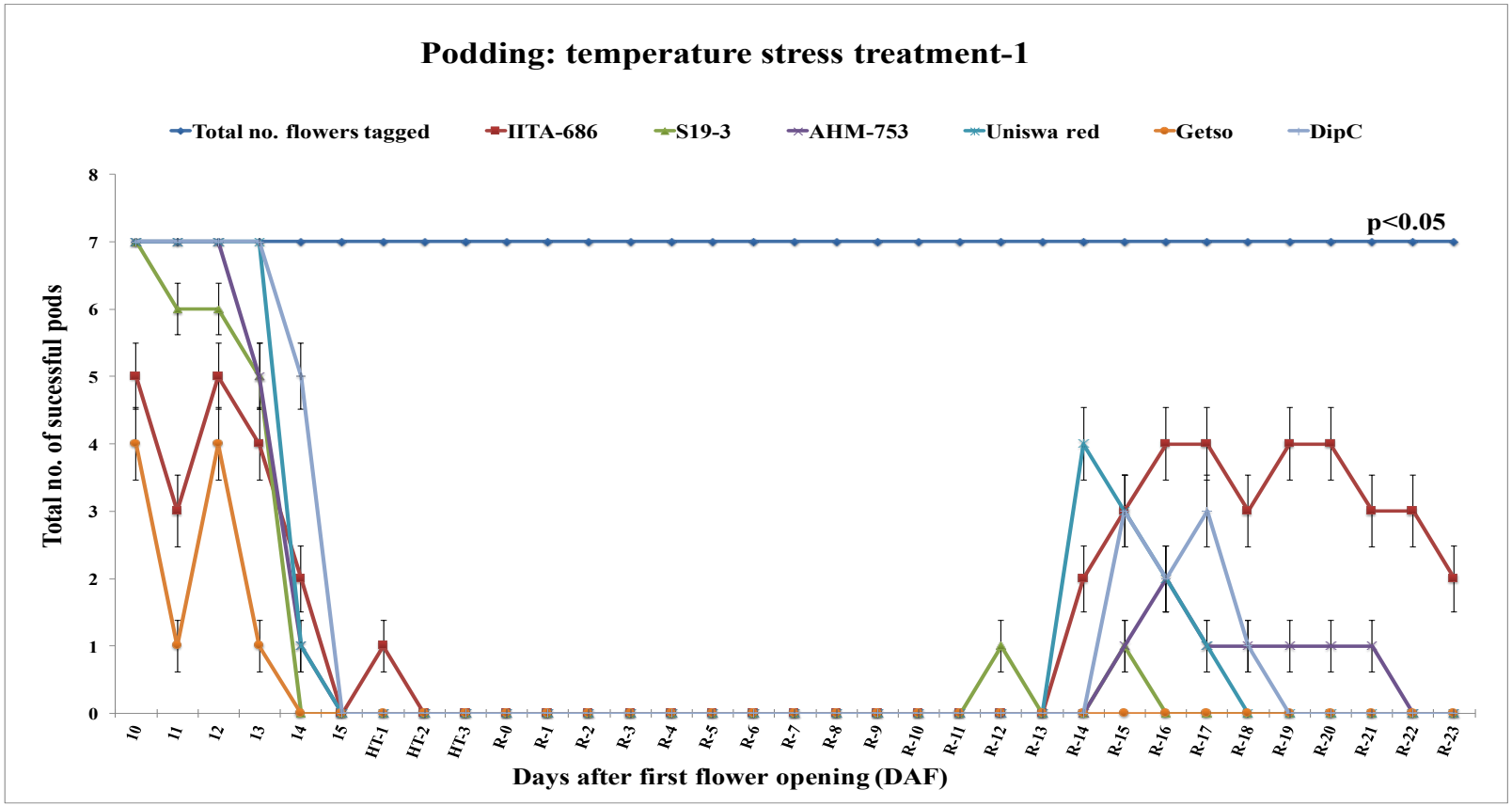


Figure 34: Podding data of bambara groundnut genotypes (IITA-686, S19-3, AHM-753, Uniswa red, Getso and DipC), obtained by tagging opened flowers during heat stress treatment -1. Briefly; There were significant differences ($p<0.05$) in the total no. of pods formed prior to heat stress (10-14), during heat stress (HT-1, HT-2 & HT-3) and the recovery period (R-0 to R-23), in all genotypes. (Error bar = standard deviation)

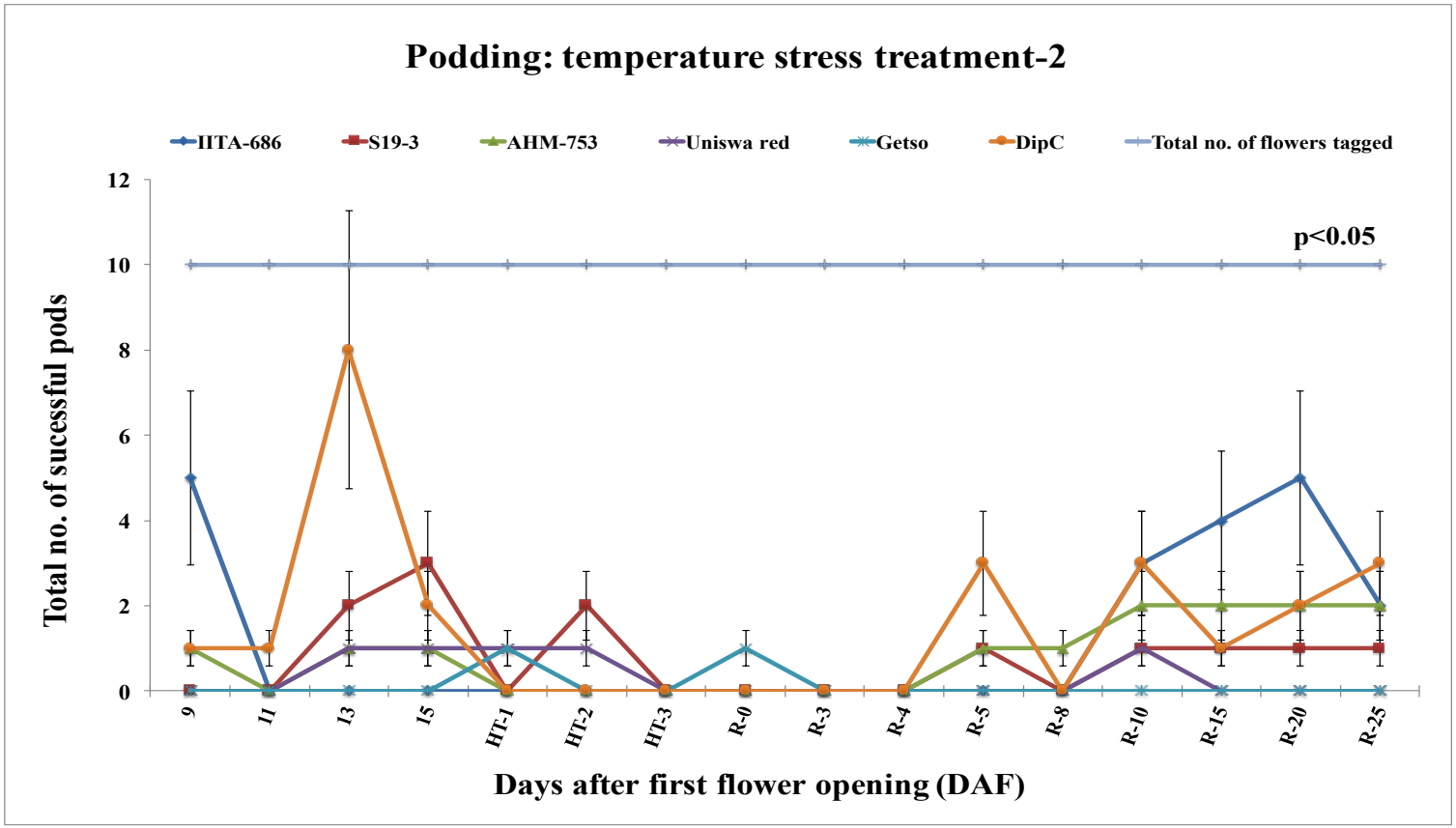


Figure 35: Podding in bambara groundnut genotypes (IITA-686, S19-3, AHM-753, Uniswa red, Getso and DipC), obtained by tagging opened flowers during heat stress treatment-2. Briefly; There were significant differences ($p<0.05$) in the total no. of pods formed prior to heat stress (10-14), during heat stress (HT-1, HT-2 & HT-3) and recovery period (R-0 to R-23), in all genotypes ($p<0.05$). (Error bar = standard deviation)

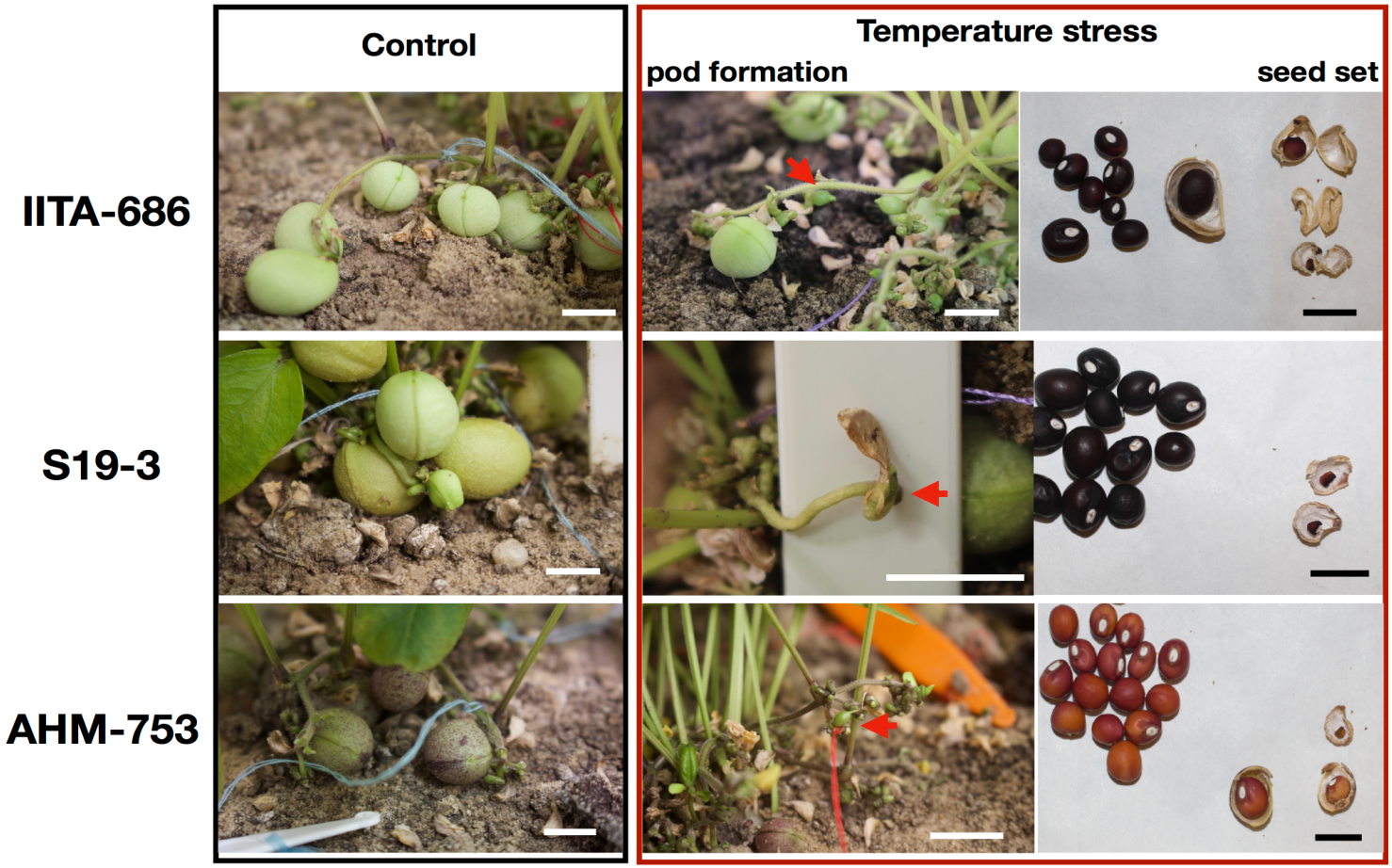


Figure 36: Pods of control and heat stress plants during temperature stress treatment-2, of bambara groundnut genotypes IITA-686, S19-3 and AHM-753. Successful podding was observed in control plants, pod abortion (red arrows) and incomplete seed development was spotted during temperature stress in all three genotypes. (Scale bar = 1 cm)

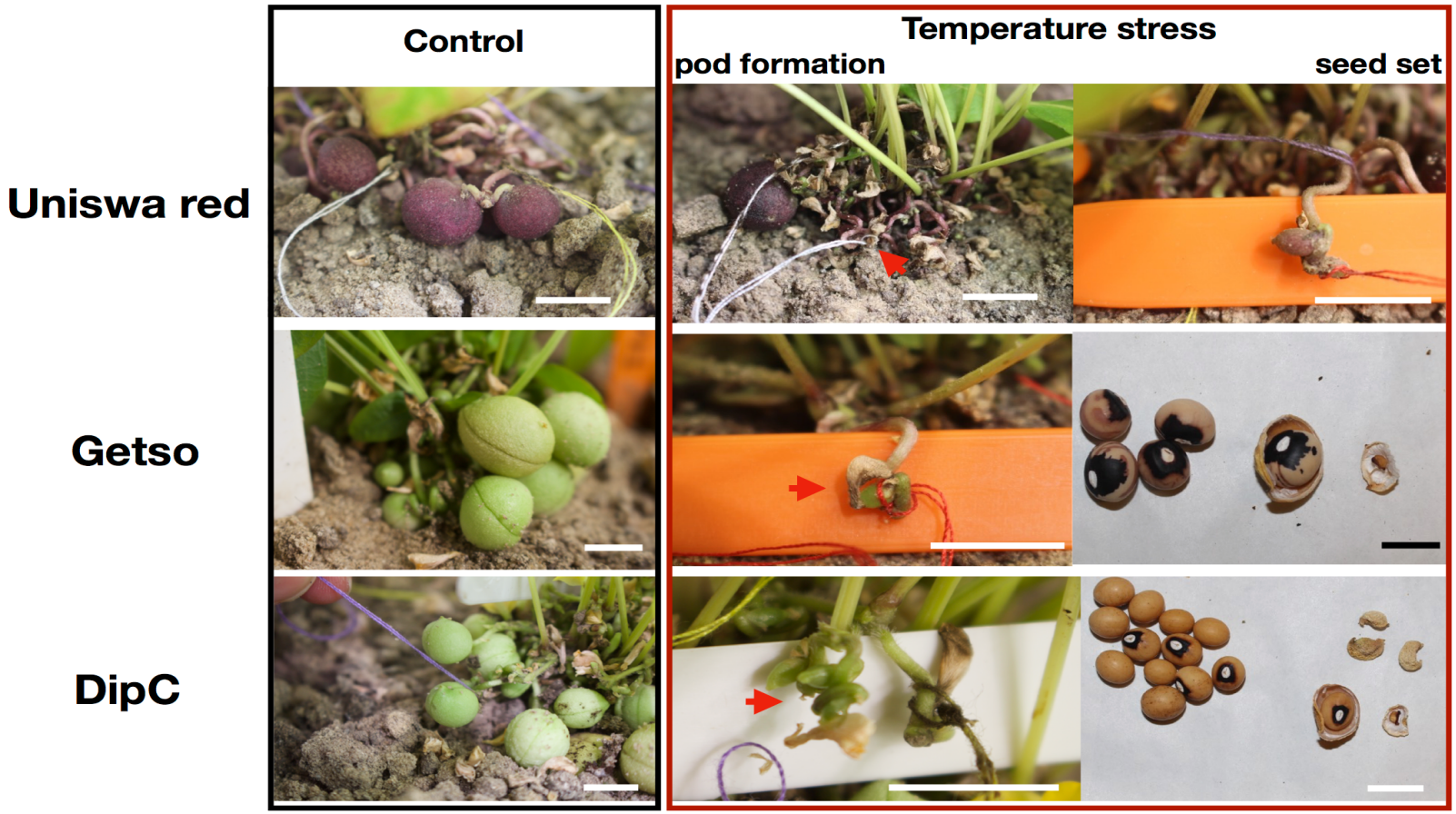


Figure 37: Pods of control and heat stress plants during temperature stress treatment-2, of bambara groundnut genotypes Uniswa red, Getso and DipC. Successful podding was observed in control plants, pod abortion (red arrows) incomplete seed development was spotted during temperature stress in all three genotypes. The pod-set (with seed development) was observed during recovery period. (Scale bar = 1 cm)

4.4. Discussion

This study demonstrated that a three days of short duration high temperature 36/30°C (day/night) stress can cause damage to bambara groundnut genotypes, such as partial or lack of anther dehiscence, low pollen germination rates and a decrease in pod set and filling.

4.4.1. Flower morphology and anthesis

During temperature stress treatment-1 and treatment-2, the flowers failed to open during the three days of high stress (HT-1, HT-2, HT-3). During the recovery period the flowers eventually opened in all the genotypes (figure 23). These findings show that high temperature stress may affect flower opening in bambara groundnut, the same phenomenon was also observed in green houses where bambara groundnut plants had been grown, when the temperatures reached beyond 39/40°C most of the flowers failed to open and whether off the next day. In our study, genotypes IITA-686, S19-3 and Getso, exhibited partial to complete anther indehiscence (**Figure 23**). Similar, studies show that heat stress before anthesis can influence anthers causing indehiscence in other legumes (Gross and Kigel 1994b; Devasirvatham et al. 2013). The same may also be true in green houses and even in field conditions, the heat wave might cause anther indehiscence and incomplete flower opening which could lead to severe yield loss.

4.4.2. Flowering trend during high temperature stress

From the observations in the results **section 4.3.3.1**., the number of flowers produced after heat stress increased exponentially, in all genotypes. This suggests that bambara groundnut has the ability to reflush (re-flowering) after heat shock. This was graphically indicated in genotypes, IITA-686, S19-3, AHM-753, Uniswa red, Getso and DipC, the difference was apparent between heat stress and control treatments for each genotype. For most cereals, if a heat shock occurs at anthesis, then it would mean the loss of yields, whereas bambara groundnut is plastic enough to recover and produce new flowers/pods. The effect of high temperature stress was prominent and would delay maturity, but is a lot better than a complete loss of yield. Bambara

groundnut being indefinite flowering type could also be an evolutionary adaptation, since both the region of origin and cultivation show high temperature regimes, the crop might have acquired the ability to reflush. The crop is able to produce minimal yield in the regions where most of the other crops fail (Massawe et al. 2005a), may also be attributed to its ability to reflush in the regions where high temperature heat waves are common.

4.4.3. Pollen germination under heat stress

Determining pollen tube germination gives a direct indication of the effect of high temperature stress on male reproductive tissues, especially pollen viability. In both heat stress experiments, the influence of high temperature on pollen viability was strong and reduced pollen germination percentages within 24 h (HT-1) of the application of the stress (**Figure 31**). This may be due to the effect of high temperature stress on transient accumulation of starch and sugars in mature pollen grains, as these seem to have an unusually high demand for energy due to high number of mitochondria in their cells (Müller and Rieu 2016). The same effect was seen in maturing pollen grains of tomato, when they were subjected to short duration of mild heat stress at 32/26°C (day/night). All bambara groundnut genotypes showed drastic reductions (to <10%) in pollen germination during temperature stress treatment-1 and -2, on heat stress day (HT-1) ($p<0.05$), but AHM-753 (R-3) recovered relatively quickly compared to other genotypes (**Figure 32 and 33**).

The high temperature beyond 40°C is known to affect sporophytic or vegetative cell division by causing defects in microtubules, cyto-skeleton and spindle orientation, whereas 35°C for 3 h was more than enough to affect pollen tube growth, and the severity of the effect increased with increasing temperature (Müller and Rieu 2016). Similarly, the results of our experiments may be an indication of a critical temperature (≈36°C) in bambara groundnut beyond which the rate of pollen germination, successful fertilization and pod set is reduced. Similarly in other legumes such as common bean, soybean and groundnut, a short duration of high temperature reduced pollen viability when stressed at 35°C or above for a 24 h period (Prasad et al. 2001; Koti et al. 2005).

Both short term high and long term mild temperature stress can negatively affect pollen development depending on the stage during which stress was applied (Chen et al. 2016; Müller and Rieu 2016). From the previous studies on bambara groundnut pollen germination, it was evident that the pollen exposed to ambient conditions beyond five minutes after harvest did not show any germination (Oyiga et al. 2010a). This suggests that bambara groundnut pollens have a short lifespan due to rapid loss of water at high temperatures under tropical conditions. The cell that needs to gr through a series of developmental steps within a defined period has limited opportunity to respond to environmental stress which leads to complete failure at high temperature (Rieu et al. 2017), this might be the reason why there was drastic decrease in pollen germination in all bambara groundnut genotypes within 24 h of heat stress. This also can be attributed to weak heat shock response (HSR), heat shock transcription factors (HSFs), heat shock proteins (HSPs) in pollen grains, which greatly impairs its thermo tolerance.

4.4.4. Pod set and recovery

Pollen heat sensitivity can be an adaptation in itself, since subsequent processes of embryo and fruit development are adversely affected by high temperature, preventing investment in reproduction at too high temperatures through regulated reduction in fertility might be beneficial for plant fitness to conserve for future when the ideal climatic conditions return (Driedonks et al. 2016). Our findings supports the same hypothesis, as this could be advantageous in bambara groundnut plant adaptation. The short-duration (3 days) of temperature stress (36/33°C) impacted podding ($p<0.05$) and pollen germination ($p<0.05$) in all bambara groundnut genotypes, IITA-686, S19-3, AHM-753, Uniswa red, Getso and DipC, during temperature stress experiment-1 and experiment-2 (refer to **section 4.3.3.2 and 4.3.3.3.**). Genotypes such as IITA-686, S19-3, AHM-753 and DipC, successfully recovered pollen germination after the heat stress and during the recovery period (**Figure 32 and 33**) and, podding was also recovered during the recovery period after temperature stress ended (**Figure 34 and 35**).

The earliest heat induced defect occurs at meiotic division stage during which pollen mother cell divide to form tetrads/microspores, in our current study we have already

shown that 12 to 14 days before anthesis corresponds to tetrad/microspore stage in bambara groundnut flower development (refer to chapter 3, **section 3.4.**). The flowers at tetrad or microspore stage (12 to 14 days to anthesis), when under heat stress did not produce any pods ever after 12 to 14 days' of recovery period. This may be due to the fact that during this stage, high temperature stress increases frequency of chromosome crossover, aberrant spindle orientation and recombination events, this might lead to unbalanced chromosome separation and formation of dyads (Müller and Rieu 2016). This could be the cause for low pod set due to defects in pollen development caused by high temperature stress in all the genotypes of bambara groundnut, and variability in recovery periods. However, the lowest number of filled pods/plant was observed in Getso and Uniswa red during the short duration of heat stress (HT-1 to HT-3) even though pollen germination recovered after R-8 days, possibly suggesting additional effect of the heat treatment, perhaps on the female reproductive tissues. The flower buds initiated after heat stress show podding in IITA-686, S19-3, DipC and AHM-753 genotypes, which corresponds to 14±2 days after heat stress (**Figure 34 and 35**).

Similarly a study showed a decline in pod yield in Uniswa red at 33°C, whereas S19-3 produced more pods at 33^0C (Al Shareef *et al.* 2013). AHM-753, IITA-686, DipC and S19-3 showed significant podding during recovery ($p<0.05$), which indicates that these genotypes recover faster after a short duration heat stress period. This also suggests genetic variability for heat stress recovery within species, which could be used to select for more tolerant varieties. It is evident that the flowers that were open during and for a few days following the short duration of heat stress did not produce any pods. However, the difference in pod set between genotypes is not statistically significant (in other words, all the genotypes were affected the same way by the introduction of stress or the effect was similar on all the genotypes.). The flower development scale can be used to determine the stages of flower and pollen within which was affected by high temperature stress. This methodology could be improved and implemented to find variation in the effect of high temperature stress on different bambara groundnut genotypes when stress was applied on different developmental stages.

4.4.5. Significance of temperature stress on bambara groundnut plant reproduction

With global warming plants have to face severe and more frequently occurring high temperature stress, while this affects the whole plant, development of male gametophyte (pollen grain) seems to be the most sensitive (Chen et al. 2016; Müller and Rieu 2016). From the data obtained from this study, we could conclude that like most of the legumes, bambara groundnut pollen is sensitive to high temperature stress, with the temperatures 36/33°C producing significant effects on viability and pod-set and filling. Genotypes were grouped into two categories based on their performance at high temperature stress. First category consists of genotypes AHM-753, IITA-686, DipC and S19-3, which showed significant recovery in pollen germination ($p<0.05$) and podding after heat stress. And second category with genotypes such as Getso and Uniswa red, which showed a significant reduction in pod yield after heat stress. The variation in tolerance between genotypes could be an evolutionary adaptation itself, as high temperature stress promotes outcrossing through increase homologous recombination frequencies which in turn result in higher genetic variation and chance of genetic adaptation to adverse conditions. This provides scope to use genotypes from different categories to obtain gene expression profiles during heat stress using RNA seq or Microarray analysis. This shows potential to detect transcripts which are important to the species-specific response (if it exists) of bambara groundnut to heat stress which could be beneficial in future agriculture.

That high temperature stress has a clear effect on reproductive development is well documented in many species (Rieu et al. 2017). Pollen is considered as hypersensitive to heat stress relative to vegetative tissue, therefore analysing pollen grains would give direct indication of the effect of high temperature stress on pod yield. Therefore, pollen could be used as a screening tool for heat tolerance since there is enough evidence to suggest that pollen development and fertilization are often the most affected weak links under heat stress (Hedhly et al. 2009b; Zinn et al. 2010; Hedhly 2011; Kazan and Lyons 2016) . Pollen heat sensitivity can be beneficial for hybridization trials, as plants select either to kill the male gametes or keep the female

gametophyte, this hypothesis needs to be investigated thoroughly in bambara groundnut.

Currently some genome projects have been completed, especially for the major legume crops (Smýkal et al. 2014), while other remain in progress, including that for bambara groundnut by the African Orphan Crops Consortium, although completion may still be a number of years away. Several post genomic analysis technologies such as microarray, cDNA-AFLP and RNA-seq, which can be used to investigate thousands of genes within a single treatment/experiment are now available. Especially new and improving NGS platforms, RNA-seq enables the detection of flower and pollen specific genes in crops and plants that lack genomic data and which is generally expensive to generate by full genome sequencing. Such technologies could be used in bambara groundnut to discover novel transcripts and potential genes with respect to environmental stress responses (heat, drought and water stress) and stress tolerance. The current research shows that the pollen is highly affected at relatively short durations (HT-1, 24h) of high temperature stress (36/33°C). A prospective well-designed RNA seq experiment using the genotypes mentioned in the present study could be used to generate gene expression profiles with respect to temperature stress. There is also potential for developing screening techniques at male gamete level for selection of heat tolerance in bambara groundnut that can be used in breeding novel varieties.

CHAPTER 5

RNA sequencing

5.1. Introduction

Advances in crop genomics have begun to enable a new cohesive understanding of the biology of many crop species and this has also resulted in the development of a powerful set of molecular and bioinformatic tools. Genome sequencing is one such method which can be used to generate data, interpret and improve the genetics of crops. Application of advanced genomic tools has led to the construction of whole genome sequences of many legume species such as lotus (*Lotus japonicas*)(Sato et al. 2008), soybean (*Glycine max*)(Schmutz et al. 2010), alfalfa (*Medicago truncatula*) (Young et al. 2011), pigeon pea (*Cajanus cajan*) (Varshney et al. 2012a) and chickpea (*Cicer arietinum*) (Varshney et al. 2013). In the case of underutilized crops like bambara groundnut, which serves mainly as a regional food source, there has been limited investment in genetic and genomic tools.

With whole genome sequence available from the reference genomes, soybean (*Glycine max*), *Medicago truncatula* and *Lotus japonicas*, it may be possible to use the reference genomes to facilitate research in related species (Varshney et al. 2012b). Such approaches have been used in common bean, groundnut, barrel medic and some wild relatives of major crops (Deokar et al. 2011; Ford-Lloyd et al. 2011; Prabhu Dhanapal 2012; Varshney et al. 2012b). Recently, a Gene Expression Markers (GEM) map for bambara groundnut was constructed using the Affymetrix Soybean Genome GeneChip (Chai et al. 2017). Identifying synteny between major crops and model crop genomes and underutilised species is an important step towards translation of information and resources from major to minor crop species (Ho et al. 2017). Ho *et al.* (2017), demonstrated the possibility to identify candidate genes through the underlying conserved syntenic location of QTLs in bambara groundnut, by comparing with already sequenced closely related genomes such as common bean (*Phaseolus vulgaris*) and the partially annotated genomes of adzuki beans (*Vigna angularis*) and mung bean (*Vigna radiata*).

Gene expression profiling is widely used to study plant-stress regulatory mechanisms that control cellular processes (Van Verk et al. 2013). RNA-seq extends

the possibility to analysing previously unknown genes through transcriptome analysis and it also offers a dynamic range of quantification at reduced cost, which is particularly helpful in the species (orphan/underutilized crop species such as bambara groundnut) without reference genomes (Chapman 2015). A typical RNA seq experiment starts with a research question and a good experimental design to address the latter. Followed by isolation of RNA and subsequently conversion of this RNA into cDNA to form an RNA-seq library. By sequencing the millions of DNA fragments (also known as reads) in the library using NGS platforms, an accurate measure of relative abundance of each transcript and splice variants can be obtained. The final step – and perhaps the most challenging one of all - is to process the sequence data and generate information on the gene expression levels using bioinformatic tools (Metzker 2010; Cresko Lab 2012).

Some studies report an alteration in gene expression under environmental stress during reproductive development. Expression profiling studies in *Arabidopsis*, indicate more than 14,000 genes (approximately half the genome) are involved during pollen development (Borg et al. 2009; Borg and Twell 2010) and almost half of the predicted transcription factors can be detected at the microspore, bicellular pollen, tricellular pollen and mature stages of pollen development (Honys and Twell 2004; Honys et al. 2006). These studies report that the development of functional male and female gametes are highly complex processes and are considered to be sensitive to environmental factors. Still a great deal of work remains; to develop mapping populations for bambara groundnut to study traits of interest such as temperature stress, and to characterize more traits and genes in model crops and to extend new genomic tools to underutilised crops. Our current research is one step closer to achieving the goal, by implementing advanced technologies and resources available in bambara groundnut research.

5.2. Aim

The main aim of our research was to generate differential gene expression data at high temperature stress (36°C, day/night) for short duration of 24 hr. in two contrasting genotypes (IITA-686 & Uniswa red) of bambara groundnut (*Vigna subterraenea* L. Verdc.) using flower and leaf derived total RNA.

And, to identify and annotate differentially expressed transcripts by comparative analysis of expression data of control and temperature stressed samples, obtained at different time points during temperature stress.

5.3. Materials and Methodology

5.3.1. Experimental design

From the previous short duration heat stress experiments using six genotypes (**IITA-686, S-19-3, DipC, AHM-753, Uniswa red & Getso**) where plants were stressed at 36/30°C for short durations of 3 days (UoN, UK), it was evident that within 24h of heat stress imposition, *in vitro* pollen germination of all the genotypes was reduced to less than 5%, and any flowers which were open on the day of heat stress and following recovery did not produce any filled pods (refer to chapter 4). The six genotypes were categorized into two sets based on the observational data on time taken by each genotype to recover pollen germination and successful podding. One set consists of IITA-686, S19-3, AHM-753 and DipC, which recover after heat stress, the other set consisted of Uniswa red and Getso, which performed rather poorly with no pods after heat stress. From these two categories, the genotypes IITA-686 and Uniswa red were selected for the current experiment to obtain differential gene expression under heat stress. Since there is an obvious effect on pollen germination, the mature flowers and flower buds, corresponding to the tri-cellular stage and the tetrad/microspore stage of pollen development, respectively (refer chapter-3), were sampled to extract total RNA for the RNA-seq experiment.

5.3.2. Plant materials

Seeds of bambara groundnut (*Vigna subterranea* (L.) Verdc) genotypes 'IITA-686' and 'Uniswa red' were germinated in modules with universal potting mix (VAM, Potgrond) in Controlled Experiment chambers (A1000, Conviron, Crops For the Future Research Center, Malaysia) maintained at 28/22°C as day/night temperatures and 12 hours of photoperiod. 14 days after seed germination seedlings were transplanted into 5L pots with the mixture of organic soil and river sand in 3:2 ratios. These potted plants were arranged in a randomized complete block design (RCBD) in two control environment rooms (BDW40, Conviron, Crops For the Future Research

Center, Malaysia), which were maintained at 28/22°C (day/night) temperatures, 12 hours (photoperiod) and 60-65% humidity. Manual watering of plants was carried out until 30 DAG (days after germination) and an irrigation system installed for automated watering (200mL) every alternative day at 6 am throughout the experiment. The temperature and humidity was maintained with constant monitoring to avoid any fluctuations. Phenological measurements such as seed germination, 4-leaf stage and flowering time was noted in all plants.

5.3.3. Temperature stress treatment

The outline of the method of heat stress is given in the **Figure 38**. At 100% flowering (14 days after first flower opening), three replicates of both the genotypes with 4 plants/replicate were selected and heat stress of 36°C day and night was applied for 24h period. The control plants (2 replicates/genotype) with 4 plants/replicate were maintained at control conditions (28/22°C, 60% humidity and 12 hr. photoperiod).

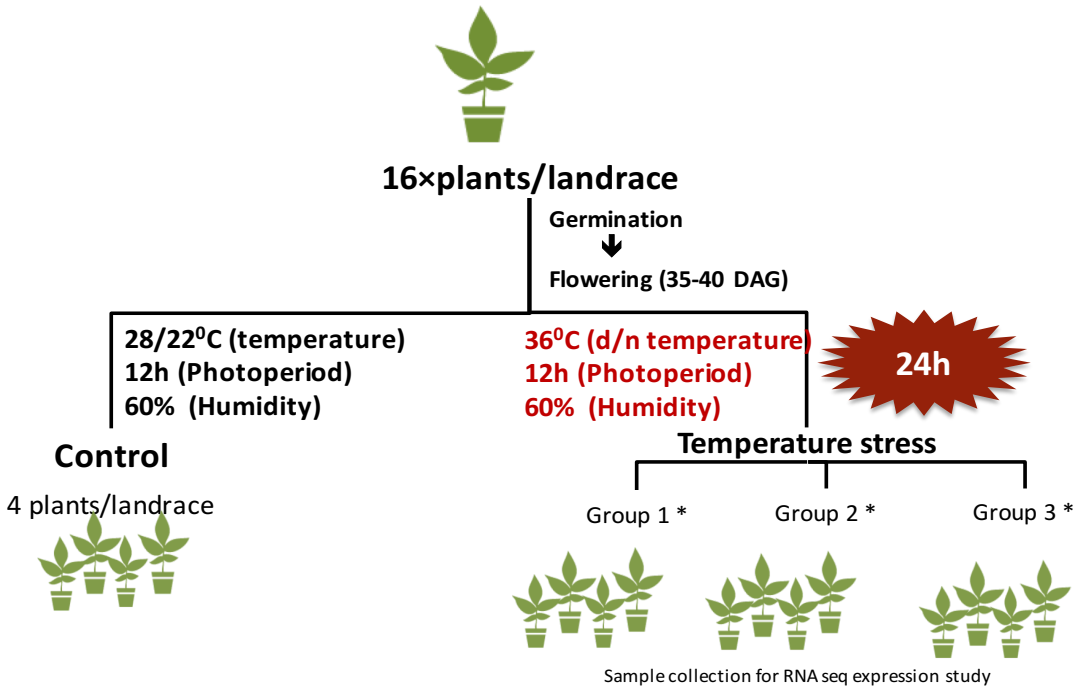


Figure 38: The outline of heat stress experiment carried out with distribution of number of plants/replicate. Briefly, bambara groundnut plants were grown under 28/22°C (day/night) conditions until flowering, at 100% flowering, temperature stress of 36°C (day/night) was applied, whereas control plants were kept at ideal 28/22°C (day/night) conditions.

5.3.4. *In vitro* pollen germination

In vitro pollen germination was determined at the end of the heat stress (at 24h time point, HT-1) in the heat stressed plants and at the same time point in control plants growing under ideal conditions. Pollen germination medium (PGM) described in Boavida and McCormick (2007), was used in the present study. 1X PGM medium with 20% sucrose was prepared and the pH was adjusted to 7.5, the solid PGM (sPGM; 0.5%) media was prepared by microwaving the medium with lab quality grade agarose and about 400µL sPGM was pipetted onto the slide (rectangular shape 40mm×20mm). The pollen from mature flowers on the day of anthesis were dusted onto the medium and incubated in an inverted position in a moisture chamber for 4-5 hours at room temperature (RT) in the dark. Two flowers from the same plant were used per replicate and three biological replicates per genotype was used to determine the pollen germination. The germinated and total pollen grains were counted under a light microscope; pollen grains were counted as germinated when the observed pollen tube was longer than the diameter of the pollen grain. The percentage of pollen germination was determined for both control and heat stress (24h time point) for both the genotypes. **Statistical analysis**: *in vitro* pollen germination between control and heat stressed plants (HT-1) of IITA-686 and Uniswa red genotypes, was analyzed by a Two-factor ANOVA (Microsoft excel, version.15.16). The results are presented in the **section 5.4.1.**

5.3.5. RNA-seq sample collection

The outline of sample collection is illustrated in the (**Figure 39**). Samples were collected at 0 h (before heat stress), 6, 10 and 24 h during the heat stress period. Three biological replicates/time point/genotype consisting of three combined mature flowers/replicate (≅50mg) and five combined tetrad stage flower buds/ replicate (≅50mg) were collected and snap frozen in liquid Nitrogen (liq. N_2). Similarly, three biological replicates/time point/genotype of leaf sample (two leaves/replicate) (≅200mg) were collected in Aluminum foil and snap frozen in liq. N_2. All the snap frozen samples were stored immediately in a -80°C (Block C and BRC, at UNMC, Malaysia). Extra samples were collected at each time point/genotype as a backup sample.

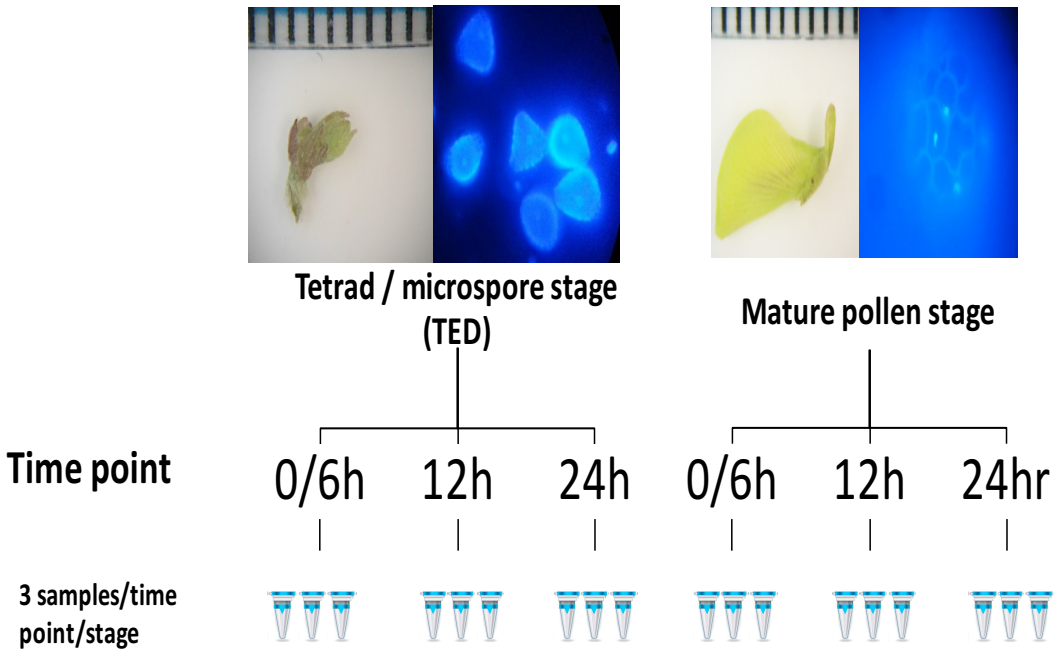


Figure 39: Schematic representation of sampling in RNA-seq experiment (original illustration). Briefly, samples were collected at 0, 6, 12 and 24 h time period from flower buds at tetrad/microspore stage and mature opened flowers.

5.3.6. Total RNA extraction protocols

From the RNA preparation to the final data, each step, including sample test, library preparation, and sequencing, influences the quality of the data, and data quality directly impacts the analysis results. To guarantee the reliability of the data, quality control (QC) is performed at each step of the procedure. Therefore, after each extraction total RNA samples quality was analysed by a Bioanalyzer (RNA 6000 Nano-chip, NeoScience, Malaysia). As described in the following section, different RNA extraction protocols were used and modified, to get good quality and quantity of RNA from flower and leaf tissues. Before extraction of RNA from temperature stress samples, several protocols to extract RNA from flower and leaf samples were tested. The three methods are presented in this section.

5.3.6.1. RNA extraction Method-1: Qiagen method

The total RNA was extracted from flower and bud samples of the bambara groundnut genotypes IITA-686 and Uniswa red, following 'RNeasy Plant Mini kit' (Qiagen, cat. No. 74904) according to the manufactures instructions. The starting material was

50mg of flower and bud tissues. The final elution was done with 50µL RNase-free water and stored at -80°C. The quality of total RNA samples was tested through RNA 6000 Nano-chip Bioanalyzer (service provided by NeoScience). Bioanalyzer results are described in the results **section 5.4.2.1**.

5.3.6.2. RNA extraction method-2: Trizol method

Trizol method according to Ambion|RNA, by *Life* technologies (manual: MAN0001271 and Part no. 15596026.PPS) was used to extract RNA from mature flowers, buds and leaves of IITA-686 and Uniswa red genotypes. The starting material was ≈50, ≈50 and ≈100mg of flower, bud and leaf tissues, respectively. The RNA concentration and quality was visualized by gel electrophoresis (1% agarose gel with TAE [Tris-acetate-EDTA]). RiboRuler High range RNA ladder (Thermo Scientific^{TM}), was used for reference band sizes and quantity measurement. The observations are presented in the results section **5.4.2.2**.

5.3.6.3. RNA extraction method-3: modified Qiagen Method

The method-1 (Qiagen) and method-2 (Trizol) did not yield high concentrations of RNA from flower and bud tissues (refer results **section 5.4.2.1. & 5.4.2.2.**). This may be due to presence of the polysaccharides or phenols in flower tissues. Hence following suggestions from Qiagen technical service, 1% (w/v) PVP- 40 (polyvinylpyrrolidone) was added to RLT buffer. This helps RNA extraction from phenol- and polysaccharide-rich plant tissue (Qiagen customer support, December 2016). Total RNA was extracted using the 'RNeasy Plant Mini kit' (Qiagen, cat. No. 74904) following the manufacturer instructions, from flower, bud and leaf samples freshly collected from IITA-686 genotype and snap frozen in liquid N_2 at the time of collection. With the following additional step. The results are presented in the **section 5.4.2.3**.

Additional step: add 1% (w/v) PVP- 40 (polyvinylpyrrolidone) into the RLT buffer and dissolve before adding β-mercaptoethanol. After homogenising the plant tissues with liq. N_2, add 400µL of RLT buffer (with PVP- 40 and β-mercaptoethanol) and leave at RT for 5 min. and centrifuge at full speed (14,000 rpm) for 30 sec. to pellet

out cell debris and precipitates of phenol & polysaccharide-rich samples. The final first elution was done with 30 µL RNase-free water (maintained at 65°C). Second final elution was completed with 20 µL RNase-free water same elution tube. The samples were stored at -80°C (block C, UNMC). The RNA integrity was first determined by gel electrophoresis using 1% gel with TAE buffer (Tris-acetate-EDTA).

5.3.7. Total RNA extraction from temperature stress samples and quality control

As described earlier in the section 'RNA-seq sample collection' (5.3.5.), The tissue samples during short duration (24h) heat stress were collected at 0h, 6h, 12h and 24h time points. The RNA extracted from these samples was to be sent for RNA-sequencing, but before that quality and integrity of RNA was tested using Bioanalyzer 6000 Nano-chip (service provided by NeoScience, Malaysia).

5.3.7.1. 0 h-time point samples

The RNA from 12 samples which includes temperature stress (6 samples, at 0h) and control (6 samples at 0 h) were extracted using RNA extraction method-3 (modified Qiagen method). Samples were sent for Bioanalyzer analysis on ice. The sample log and Bioanalyzer report are presented in the section 5.4.3.1.

5.3.7.2. 6 h-time point samples

The RNA from the 6 h time point was extracted from leaf and flower tissues of IITA-686 and Uniswa red. Freshly collected leaf and flower samples were used as controls to compare the RNA quality. The sample log and Bioanalyzer results are presented in the section 5.4.3.2.

5.3.7.3. 24 h-time point

The RNA from 24 h time point was extracted from leaf and flower tissues of IITA-686 and Uniswa red. The RNA extracted from freshly harvested flower and leaf tissues were used as control. The sample log and Bioanalyzer results are presented in the section 5.4.3.3. To find whether there was any component which promotes degradation in the heat stress samples, equal volume of RNA from fresh and heat stress samples

were also mixed and sent for Bioanalyzer analysis.

5.4. Results

5.4.1. *In vitro* Pollen germination under heat stress

In-vitro pollen germination in bambara groundnut genotypes, IITA-686 and Uniswa red show a significant ($p<0.05$) decrease during heat stress when compared to controls (**Figure 40**). The two-factor ANOVA output is presented in **table 20**; the effect of heat stress was significant on the pollen germination of both the genotypes at the $p = 0.05$ level. This drastic decrease in pollen germination shows a direct effect of heat stress on pollen viability and pollen tube growth.

Table 20: Two-factor ANOVA output of *in vitro* pollen germination between control and heat stress plants of bambara groundnut genotypes, IITA-686 and Uniswa red. Briefly; the effect of heat stress on pollen germination was significant at the p=0.05 level. . SS-sum of squares, d.f- degrees of freedom, m,s-mean squares, p- probability)

Source of variation	**s.s.**	**d.f**	**m.s.**	**F**	**p-value**
Genotype	66.48	1	66.48	18.76	<0.05
Heat stress	10,627.41	1	10,627.41	2,999.06	<0.05
Heat stress x Genotype	64.20	1	64.20	18.12	<0.05
Residual	28.35	8	3.24		
Total	10.786.44	11			

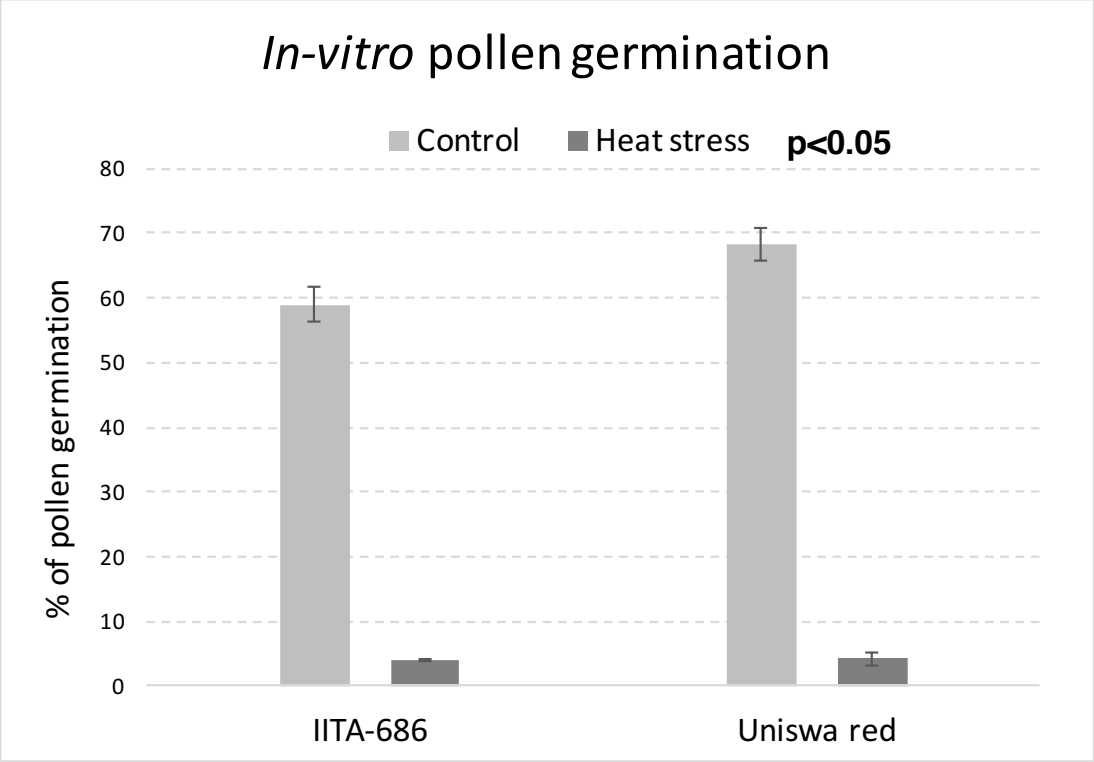


Figure 40: *In vitro* pollen germination of bambara groundnut genotypes IITA-686 and Uniswa red, during Control and heat stress, day-1. Briefly; The pollen germination was significantly reduced ($p<0.05$) in heat stressed plants when compared with the control.

5.4.2. Improved method of RNA extraction from bambara groundnut flower and leaf tissues

The quality of total RNA extracted by method-1 (Qiagen method) (section 5.3.6.1.), was tested by Bioanalyzer (NeoScience, Malaysia). The RNA samples with RIN (RNA integration number) less than 4 were considered as low quality, unfit for downstream processing and RNA sequencing. Total RNA with RIN value of 4 to 7 was considered as intermediate quality and yet not good enough for RNA sequencing. RNA samples with RIN value of 7 to 10 was considered good quality for downstream applications during RNA-sequencing.

5.4.2.1.Method-1

The Bioanalyzer results of RNA samples extracted using method-1, are presented in **table 21 and figure 41**. The first drawback of method-1 was low total RNA

concentrations in flower samples (sample 1 to 6) and variable concentrations from bud samples (sample 7 to 12). Secondly the RNA samples were either degraded or of intermediate quality. The RIN value was lower than 7 in all the samples. **Figure 41** also shows that all the samples were degraded. Therefore, there was need to develop/improvise the methods to get uniform concentration and good quality RNA, from flower and bud tissues.

Table 21: Sample log showing tissue type (source) and RNA quality of RNA samples extracted using method-1. (N/A – not applicable due to not enough data)

Sample no.	**Tissue type**	**RNA concentration (ng/μL)**	**RIN value**	**RNA quality**
1	Flower (IITA-686)	4	N/A	N/A
2	Flower (IITA-686)	16	1.1	degraded
3	Flower (IITA-686)	3.23	N/A	N/A
4	Flower (Uniswa red)	17	1.1	degraded
5	Flower (Uniswa red)	16	2	degraded
6	Flower (Uniswa red)	8	N/A	N/A
7	Buds (IITA-686)	362	5.2	intermediate
8	Buds (IITA-686)	36	6.2	intermediate
9	Buds (IITA-686)	169.5	5.2	intermediate
10	Buds (Uniswa red)	99	5.9	intermediate
11	Buds (Uniswa red)	53	5.7	intermediate
12	Buds (Uniswa red)	480	2.2	degraded

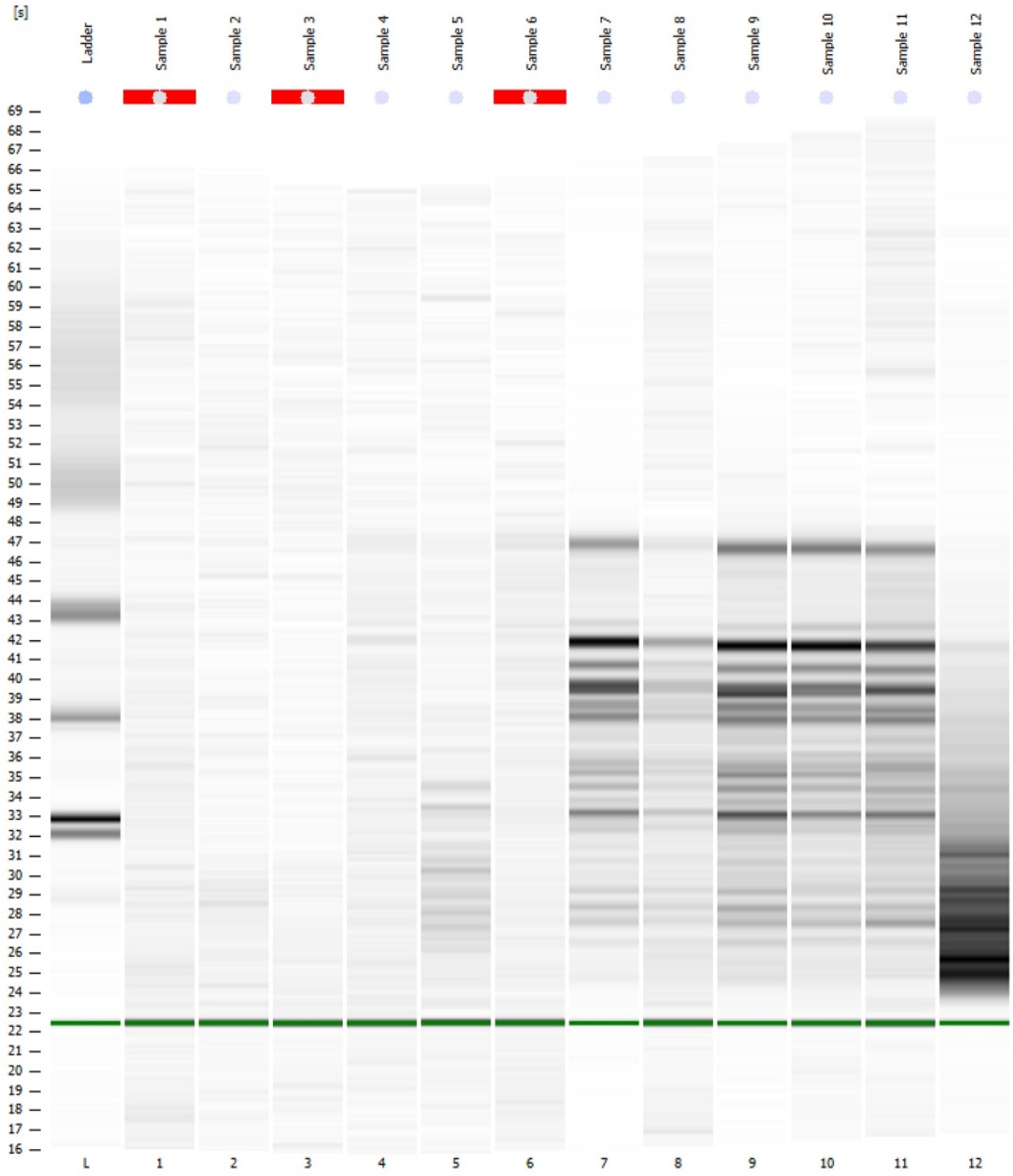


Figure 41: Electropherogram image from the Bioanalyzer results and the sample details are listed in the **table-21**. Briefly; all the RNA samples extracted from flower and bud tissues using method-1, show degradation (RIN < 7). [©2003-2005 Agilent Technologies. Inc.]

5.4.2.2. Method-2

The gel image from RNA samples (sample log **table 22**) extracted using method-2 (Trizol method) is presented in **figure 42**. The RNA concentration of leaf sample (gel lane 1 and 8) was high and does not show any degradation. Whereas no visible traces

of flower (gel lane 2, 4 & 6) and bud (gel lane 3, 5 & 7) RNA were observed. Therefore, it can be concluded that Trizol method is suitable for leaf RNA extraction but flower and bud tissues does not yield RNA using the standard protocol. Future methods need to be tried and optimized for the flower/bud samples of bambara groundnut genotype IITA-686.

Table 22: Sample log showing tissue type (source) and RNA quality of RNA samples extracted using method-2. (*good quality and quantity)

Lane number	Tissue type (Genotype)	RNA quality
1	Leaf (Uniswa red)	Good*
2	Flower (IITA-686)	none
3	Bud (IITA-686)	none
4	Flower (Uniswa red)	none
5	Bud (Uniswa red)	none
6	Flower (IITA-686)	none
7	Bud (IITA-686)	none
8	Leaf (IITA-686)	Good *

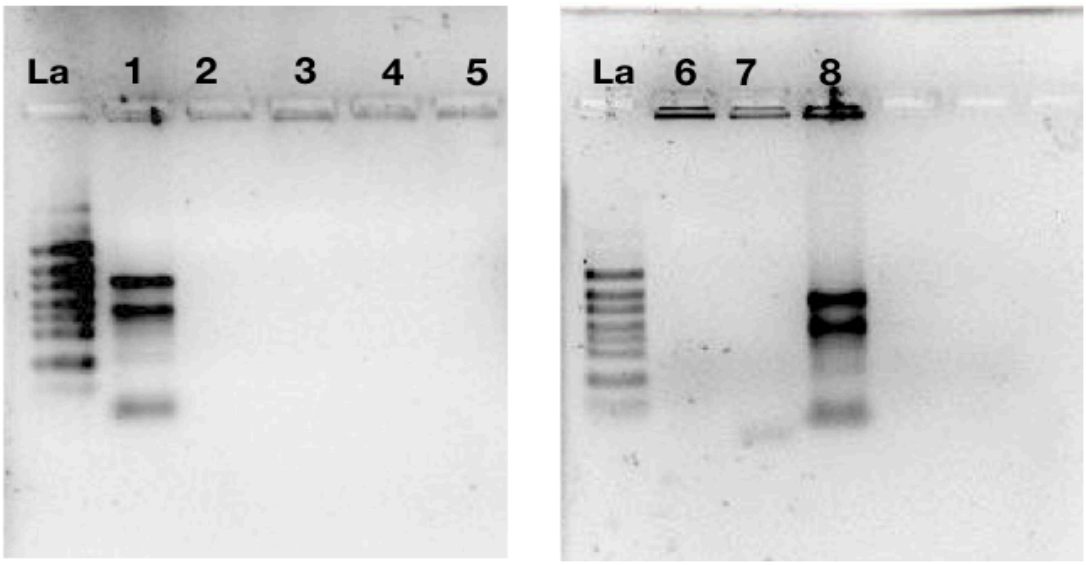


Figure 42: Gel image of RNA samples extracted by method-2 (Trizol protocol). Briefly; lane 1 and 8 show, good RNA concentrations. No traces of RNA were observed from flower (lane 2, 4 & 6) and bud (3, 5 & 7) tissues. [RiboRNA high ladder (La)].

5.4.2.3. Method-3

The description of samples used in the RNA extraction method-3 are listed in **table 23**. To determine the best protocol for RNA extraction from flower and bud samples, some samples were extracted using method-3 and some with method-1.

The additional step using PVP in method-3, was used to extract Samples 1 & 3, and method -1 was used in sample 2 and 4 for RNA extraction. RNA integrity and presence was determined using Gel electrophoresis. As seen in **figure 43**, there were clear bands of RNA from flower sample (1), which is brighter than sample 2. This suggests that PVP helps in precipitation of phenol/polysaccharides from the flower tissues. Therefore, the new method works better for flower samples. There was not much change in the leaf sample 3 and 4. The method also seems to works even with low starting material (50mg). Therefore, method-3 was used in all future RNA extraction from flower tissues.

Table 23: Sample log of flower and leaf samples used with RNA extraction method-3 and method-1. Briefly; the RNA extracted using method-3 shows higher concentration of RNA in the flower and bud tissues. Leaf tissue yielded the same amount of RNA with both the methods. (* extracted with method-3)

Lane no.	Tissue type	Source Genotype	Method of RNA extraction	RNA quality
1	Flower	IITA-686	Method-3*	Good*
2	Flower	Uniswa red	Method-1	-
3	Leaf	Uniswa red	Method-3*	Good*
4	Leaf	Uniswa red	Method-1	Good
5	Flower	IITA-686	Method-1	Good*
6	Flower	IITA-686	Method-1	-
7	Bud	IITA-686	Method-1	Good*
8	Bud	IITA-686	Method-1	-

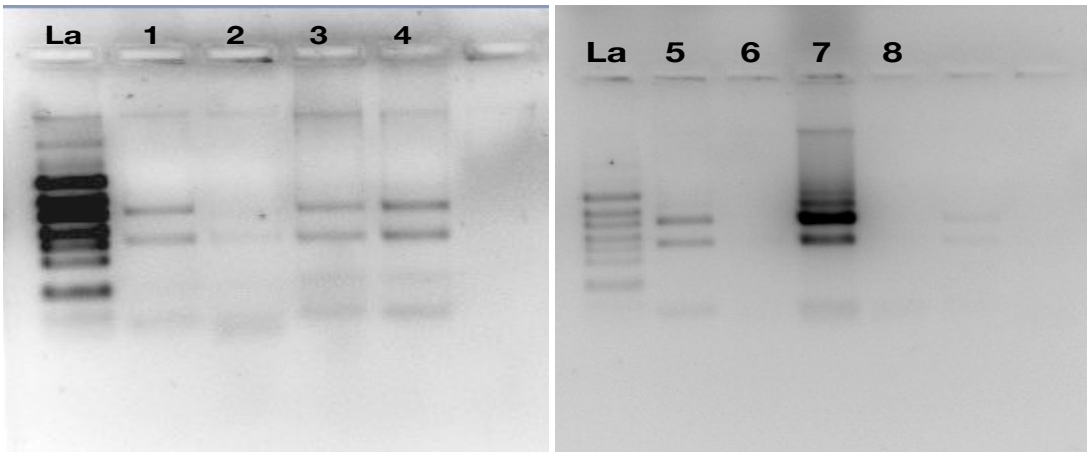


Figure 43: Gel image of RNA samples extracted by method-3 and method 1. Briefly; lane 1, 3 and 5 show, RNA extracted from method-3 with relatively high concentrations, compared to RNA extracted from method 1 (lane 2, 8). [La = RiboRNA high ladder]

One set of samples extracted from freshly collected tissues (flower, bud and leaf) were sent for RNA-quality check by Bioanalyzer 6000 Nano (service provided by NeoScience). The sample log and the report are presented in **table 24** and **figure 44**. These results show the quality of RNA extracted using method-3, in flower samples (lane 1, 5 and 6), bud samples (7, 8, 11 and 12) and leaf samples (3, 4) show RIN value greater than 7, which indicates that the RNA samples were of good quality. This quality RNA therefore can be used in library preparation and RNA-seq.

Table 24: RNA sample log of flower and leaf samples extracted by method-3, and RNA concentration and RIN values from Bioanalyzer reports. Briefly; The samples with RNA showing RIN value greater than 7 are considered good quality.

Lane no.	Sample name	Tissue type (/source genotype)	RNA concentration (ng/µL)	RIN value
1	1F	Flower (IITA-686)	43	8.3
2	2F	Flower (IITA-686)	7	-
3	3L	Leaf (IITA-686)	43	7.9
4	4L	Leaf (IITA-686)	53	7.8
5	5F	Flower (Uniswa red)	122	9.2
6	6F	Flower (Uniswa red)	15	7.6
7	7B	Bud (Uniswa red)	501	9.3
8	8B	Bud (Uniswa red)	18	8.1
9	9F	Flower (IITA-686)	6	-
10	10F	Flower (Uniswa red)	5	-
11	11B	Bud (IITA-686)	21	8.2
12	12B	Bud (IITA-686)	19	8.3

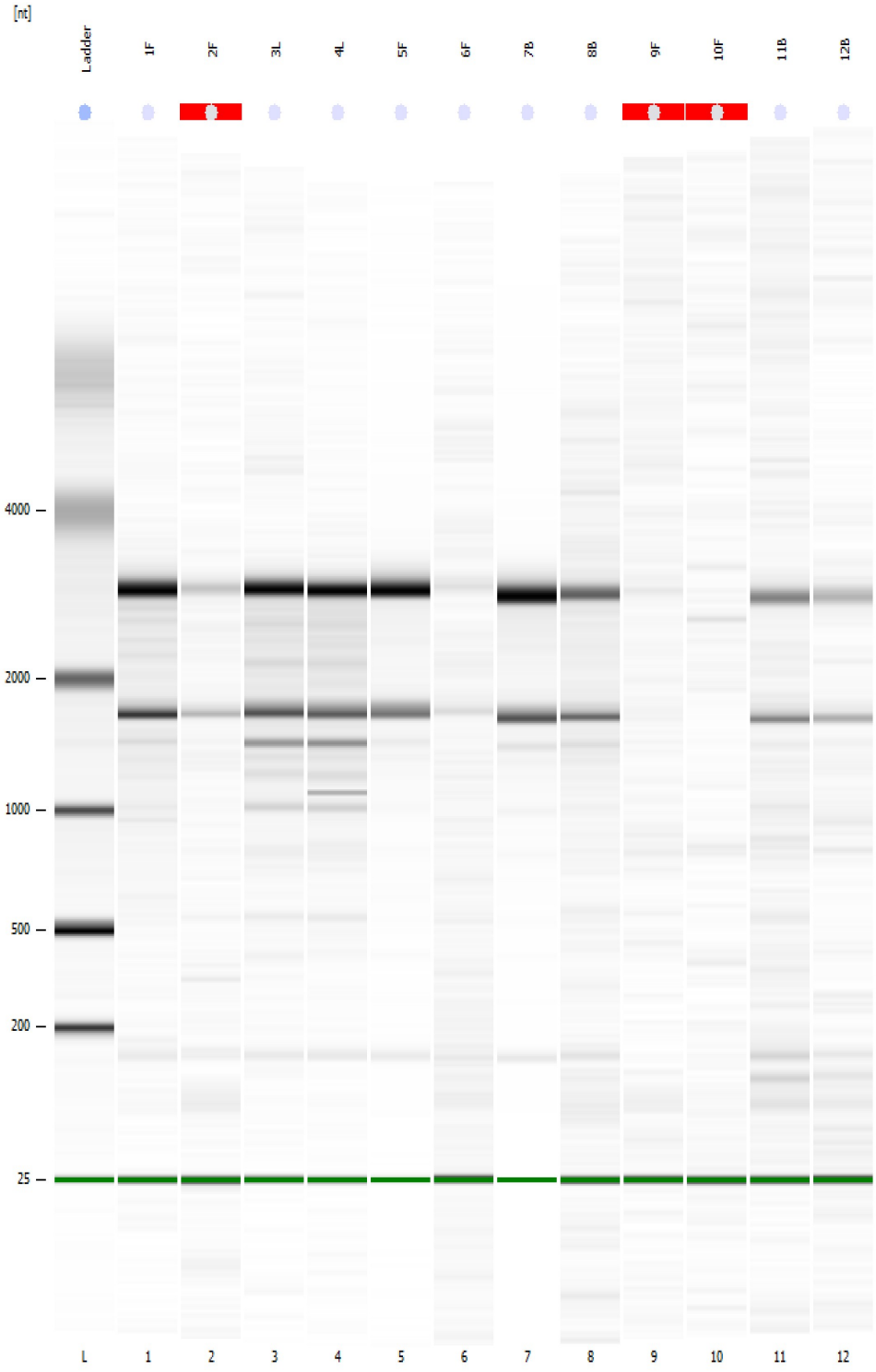


Figure 44: Electropherogram image from Bioanalyzer results and the sample details are listed in the table-24. Briefly; the RNA extracted from flower (1 & 5), leaf (3 & 4) and bud (7, 8, 11 & 12) tissues, show good quality RNA (RIN>7). (©2003-2005 Agilent Technologies. Inc.)

5.4.3. The RNA quality control before RNA-sequencing

5.4.3.1. Quality control of RNA samples at 0 h time point

The 0 h time point samples extracted for RNA-quality control, are listed in **table 25 and figure 45**. The table also provides the details of Bioanalyzer results for RNA Integrity Value (RIN) of each Flower (F) and tetrad buds (B) samples. The RNA quality was assessed using the RIN value (RIN value 7-10: good quality, RIN value 4-7: intermediate quality, RIN value <4: degraded RNA). The heat stress samples and control samples collected at 0h time point show degraded RNA (RIN<4). This quality was unfit for RNA-sequencing and downstream processing; hence we lost 0h time point samples.

Table 25: RNA sample log of flower and leaf samples collected at 0 h time point during heat stress. Briefly; The table lists the RNA concentration and RIN values from Bioanalyzer reports (RIN<4 = degraded RNA)

Lane no.	Sample name	Time point of collection	Tissue type (Genotype)	RIN value	RNA quality
1	1 I F	0h Heat stress	Flower (IITA-686)	2.4	Degraded
2	2 I F	0h Heat stress	Flower (IITA-686)	3.10	Degraded
3	3 I F	0h Heat stress	Flower (IITA-686)	2.4	Degraded
4	1 I B	0h Heat stress	Bud (IITA-686)	1.60	Degraded
5	2 I B	0h Heat stress	Bud (IITA-686)	1.50	Degraded
6	3 I B	0h Heat stress	Bud (IITA-686)	1.50	Degraded
7	C1 I F	0h Control	Flower (IITA-686)	2.20	Degraded
8	C2 I F	0h Control	Flower (IITA-686)	2.20	Degraded
9	C3 I F	0h Control	Flower (IITA-686)	2.80	Degraded
10	C1 I B	0h Control	Bud (IITA-686)	1.60	Degraded
11	C2 I B	0h Control	Bud (IITA-686)	1.60	Degraded
12	C3 I B	0h Control	Bud (IITA-686)	1.50	Degraded

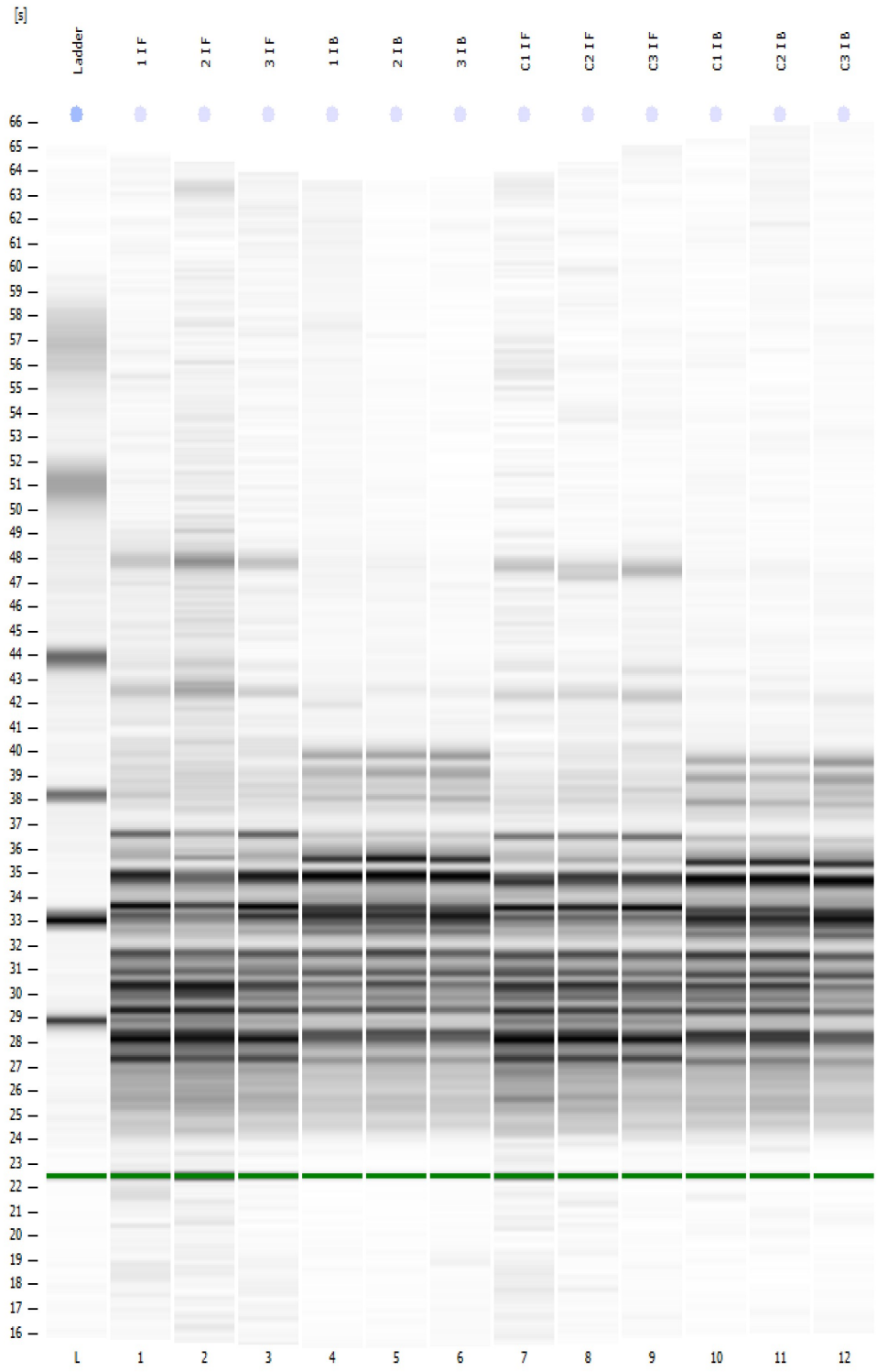


Figure 45: Electropherogram image referenced from Bioanalyzer results and the sample details are listed in the table-25. Briefly; the control and heat stress samples collected at the 0 h time point shows RNA degradation (RIN<4). (©2003-2005 Agilent Technologies. Inc.)

5.4.3.2. Quality control of RNA samples at 6 h-time point

The description of the RNA samples collected at the 6 h time point during heat stress, and RNA quality results from Bioanalyzer are presented in **table 26** and **figure 46.** The report shows that the RIN value of all heat stress samples (in lane no. 1, 2, 3, 5, 6, 7, 8, 9 and 10) irrespective of tissues type (leaf/flowers) or genotypes (IITA-686/ Uniswa red), was less than 4 with the exception of samples 10,11 and 12. This was an indication of completely degraded RNA. The RNA extracted from freshly collected control samples (8, 11 and 12) show good quality RNA (RIN>7). The extraction was carried out in same manner hence there was no technical bias during RNA extraction. All the samples collected at 6h period show degraded RNA, hence not fit for further processing and downstream application during RNA-seq.

Table 26: RNA sample log of leaf and flower samples collected at 6 h time point during heat stress. Briefly; The table lists the RIN values from Bioanalyzer reports (RIN<4 = degraded RNA; RIN<7 = intermediate; RIN≈7-10 = good quality), heat stress samples show degradation of RNA and control samples show good quality.

Lane no.	Sample name	Tissue type (source)	RIN value	RNA quality
1	1 I L (heat stress)	Leaf (IITA-686)	2	Degraded
2	2 I L (heat stress)	Leaf (IITA-686)	1.9	Degraded
3	3 I L (heat stress)	Leaf (IITA-686)	2.10	Degraded
4	C I L (Control)	Leaf (IITA-686)	2.10	Degraded
5	1 U L (heat stress)	Leaf (Uniswa red)	2.20	Degraded
6	2 U L (heat stress)	Leaf (Uniswa red)	1.9	Degraded
7	3 U L (heat stress)	Leaf (Uniswa red)	2.30	Degraded
8	2 U F (heat stress)	Leaf (Uniswa red)	2.20	Degraded
9	1 U F (heat stress)	Flower (Uniswa red)	2.20	Degraded
10	C U L (Control)	Flower (Uniswa red)	7.40*	Good quality
11	1 C L (Control)	Leaf (IITA-686)	7.10*	Good quality
12	2 C L (Control)	Leaf (Uniswa red)	7.60*	Good quality

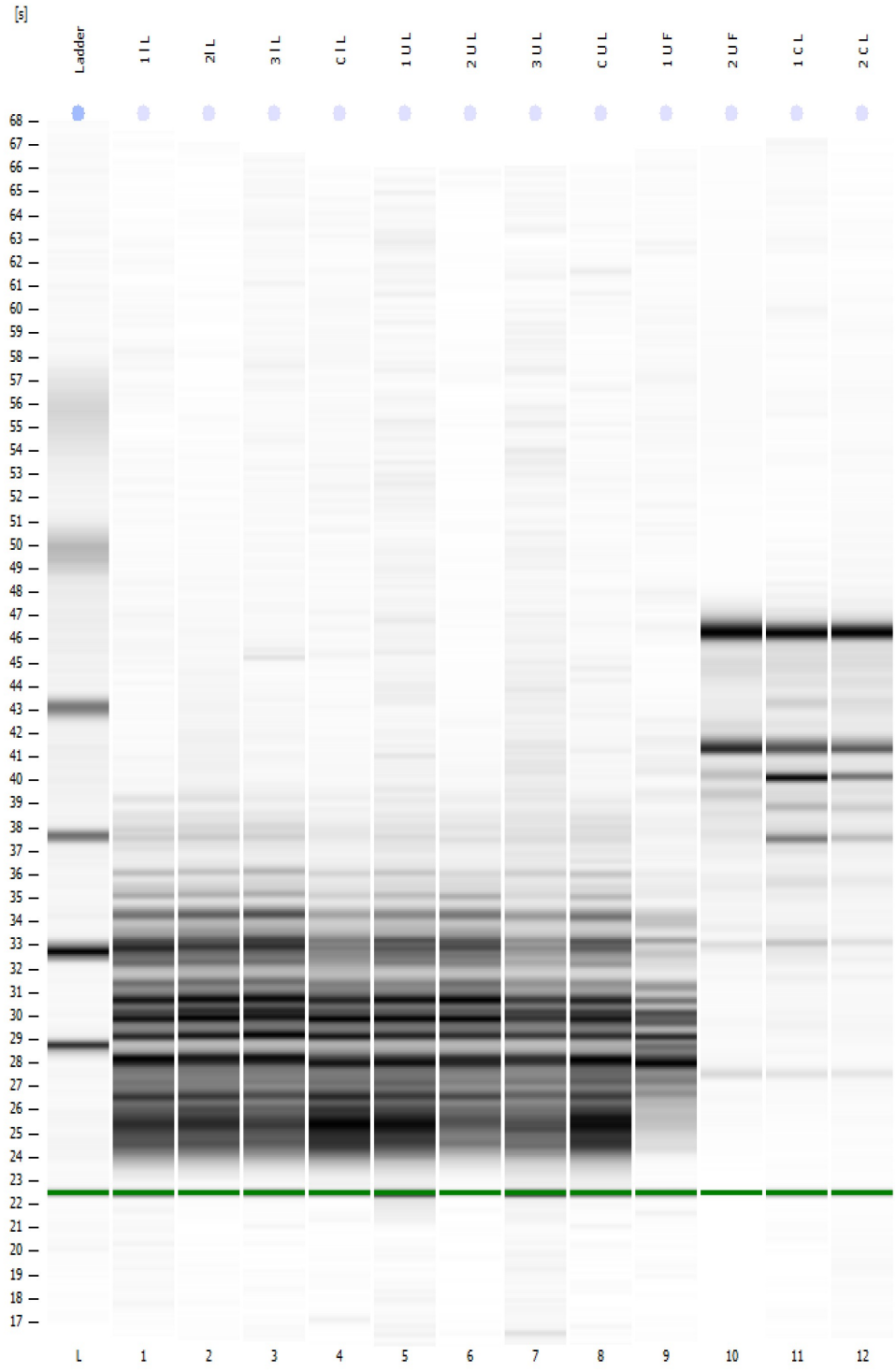


Figure 46: Electropherogram image referenced from Bioanalyzer results and the sample details are listed in the **table-26**. Briefly; the heat stress samples collected at 6h time period shows complete RNA degradation (RIN<4), whereas the RIN value of freshly harvested leaf and flower samples was greater than 7 (good quality RNA). (©2003-2005 Agilent technologies.Inc.)

5.4.3.3. Quality control of RNA samples at 24 h-time point

The list of RNA samples extracted from leaf and flower tissues collected at the 24h time point is presented in **table 27** and **figure 47**. The table also contains RIN values and RNA quality information obtained from reports of Bioanalyzer analysis (NeoScience).

The RNA extracted from leaf and flower tissues (lane no.1, 2, 3, 4, 5 & 6), collected during short duration of temperature stress experiment, from heat stressed (36°C) and control plants (at 28/22°C) of the same experiment, at 24 h time period show low RIN values (<4). This is indicative of the fact that, irrespective of whether the samples were heat stressed or from control tissues collected during heat stress experiment, both show RNA at completely degraded stage. When the RNA from heat stressed sample (lane 6) was mixed with RNA extracted from fresh tissues (lane 9 &10), it reduced the quality of RNA (RIN<7). These Bioanalyzer results showed that the RNA extracted from 24h time period tissue samples were completely degraded and unfit for upstream processing and transcriptome sequencing.

There was technical malfunction in the -80°C freezer where the 0 h, 6 h and 24 h time point tissue samples were stored, even though the samples were shifted before the freezer temperature reached -20°C, there seems to have an effect on RNA quality.

Table 27: RNA sample log of leaf and flower samples collected at 24h-time point during heat stress. Briefly; The table lists the RIN values from Bioanalyzer reports (RIN<4 = degraded RNA; RIN<7 = intermediate; RIN≈7-10 = good quality), heat stress samples show degradation of RNA and control samples show good quality.

Lane no.	Sample name	Description	Tissue type (source)	RIN value	RNA quality
1	I L C5	Heat stress (24 H)	Leaf (IITA-686)	2.20	Degraded
2	U L C6	Heat stress (24 H)	Leaf (IITA-686)	2.20	Degraded
3	L I C2	Heat stress (24 H)	Leaf (IITA-686)	2.20	Degraded
4	L U	Heat stress (24 H)	Leaf (IITA-686)	2	Degraded
5	1 U B 7	Heat stress (24 H)	Bud (Uniswa red)	1.70	Degraded
6	1 I L C5	Heat stress (24 H)	Leaf (IITA-686)	2.20	Degraded
7	2 B	Control (fresh tissues)	Bud (Uniswa red)	7.30	Good quality
8	C L	Control (fresh tissues)	Leaf (Uniswa red)	7	Good quality
9	C L + 6	Fresh leaf sample + heat stress	Leaf (Uniswa red)	6.60	Medium quality
10	C L + 6	Fresh leaf sample + heat stress	Leaf (Uniswa red)	5.60	medium quality
11	C L 3	Control (fresh tissues)	Leaf (IITA-686)	6.90	Good quality
12	C L 4	Control (fresh tissues)	Leaf (Uniswa red)	7.50	Good quality

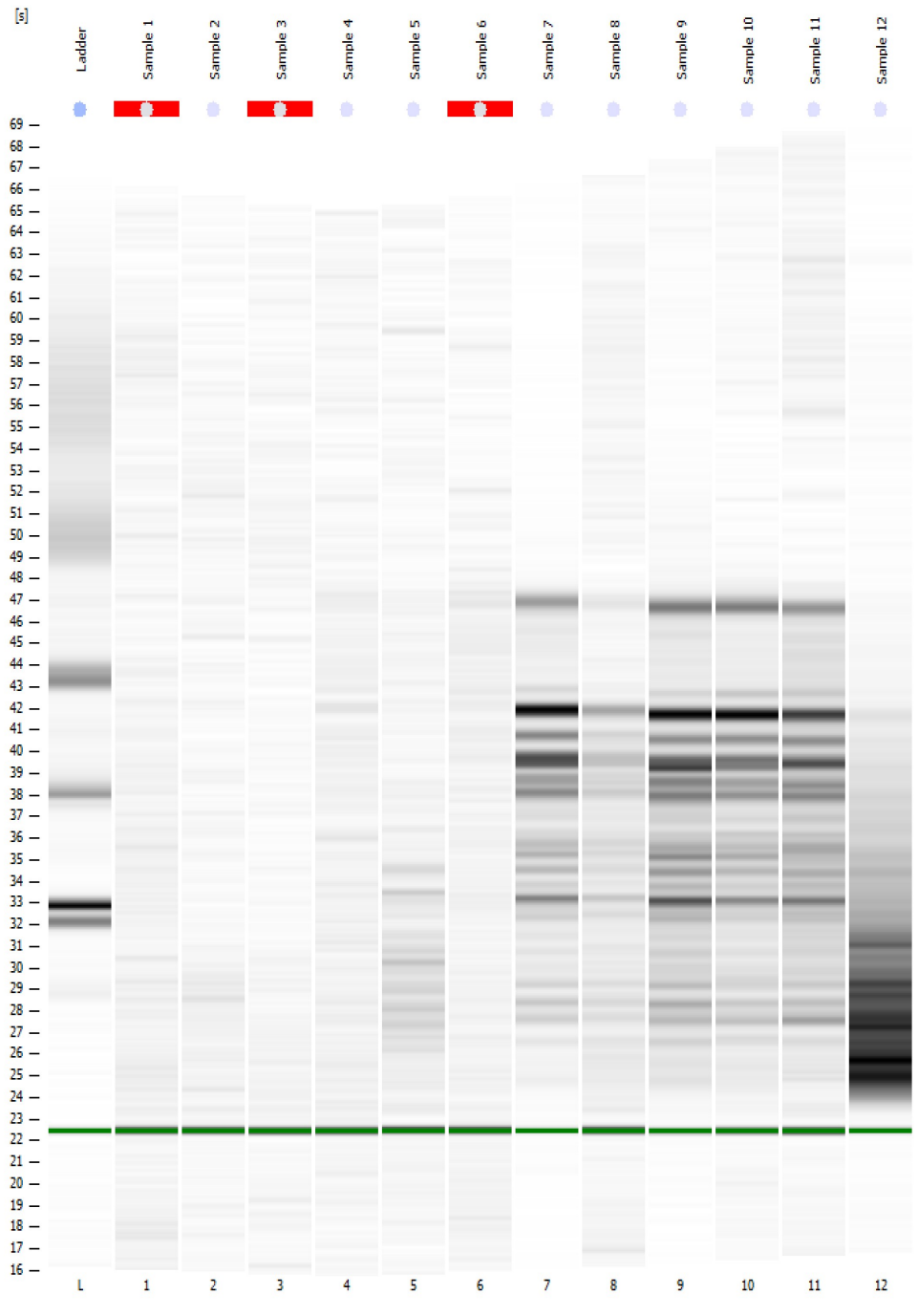


Figure 47: Electropherogram image referenced from Bioanalyzer results and the sample details are listed in the **table-27**. Briefly; the samples collected at 24h time period during heat stress shows complete RNA degradation (lane 1 to 6), whereas the RIN value of freshly harvested leaf (lane 8, 11 & 12) was greater than 7 (good quality RNA). The lanes 9 and 10, with equal volumes of RNA from heat stress sample (lane 6) and freshly collected tissues, show partial RNA degradation (RIN<7) (©2003-2005 Agilent technologies)

5.5. Discussion

The research on reproductive development and temperature stress cumulatively contributed to the design of the RNA-seq experiment. The main aim of the research was to generate differential gene expression data at high temperature stress (36/30°C, day/night) in two genotypes (IITA-686 & Uniswa red) of bambara groundnut (*Vigna subterranea* L. Verdc.) using floral and leaf derived total RNA. This would allow us to identify and annotate differentially expressed transcripts by comparative analysis of the expression data of control and temperature stressed samples, obtained at different time points during the temperature stress. The success of RNA-seq experiment can be greatly dependent upon recovering pure and intact RNA (Conesa et al. 2016). Since the goal of the experiment was to measure the transcriptome levels, the first step involves the isolation and purification of RNA from the selected tissue type. In our experiment it was from two flower developmental stages of bambara groundnut (tetrad stage flower buds and mature flowers). Careful consideration was made on reagents used and extraction protocols practiced, during RNA extraction. To obtain high concentrations of RNA from flower and bud tissues, different protocols (method-1, method-2 and method-3) were tried and tested. The final protocol which works best with bambara groundnut flower and bud tissue was method-3, during this method 'PVP' (poly-vinyl-propylene) was added to homogenized tissues to remove phenols and polysaccharides which usually present in flower tissues (refer to **section 5.4.2.3.**). The quality and quantity/integrity of RNA needs to be assessed before proceeding to the next step and some of the commonly used methods are UV absorbance and the Agilent Bioanalyzer. During our study, we used the Agilent Bioanalyzer (service provided by NeoScience, Malaysia) to check the quality and integrity.

The reports on the quality of RNA extracted from flowers, buds and leaf samples of both control and heat stressed plants collected at 0, 6 and 24 h time-points, shows extensive degradation (RIN value < 7). As it was clearly shown that, the RNA quality from the freshly harvested flower and bud tissue was good (RIN > 7), when compared with the heat stress experiment samples which were stored at -80°C freezer. There was technical malfunction in the -80°C freezer where these samples were stored, even though the samples were shifted before the freezer temperature reached -20°C, there

seems to have an effect on RNA quality irrespective of sample origin (control or heat stress). Therefore, here we conclude that due to degraded RNA samples, we could not continue with RNA-sequencing, hence the research objective was not achieved; to sequence bambara groundnut transcriptome for differential gene expression under control and heat stress conditions.

But, "because learning what doesn't work is a necessary step to learning what does – Jonas Salk (American medical researcher and virologist)" (Jeffrey Kluger 2014), we could only learn from out setback.

5.6. Future considerations

The main setback during the experiment was storage of tissue samples which were affected by a technical malfunction. The next RNA-seq experiment which we need to plan should take into consideration of such scenarios. Therefore, the future RNA-seq experimental design follows the same outline as the described in the section (5.3.1.), except the change will be introduced during sample collection (refer to section 5.4.5.), during which instead of snap freezing tissues samples directly in liquid Nitrogen (Liq. N_2), the tissues will be added with RNA*later*™ Stabilization Solution (Invitrogen™, ThermoFisher Scientific). The RNA*later* is a non-toxic aqueous tissue storage solution, this stabilizes and protects cellular RNA in the tissue. The tissues with RNA*later* can then be stored at -20°C and -80°C freezer (indefinitely) before processing.

Once the extraction of good quality RNA is completed and quality checked with the Bioanalyzer, another major obstacle will be the transportation of RNA samples for RNA-sequencing. The traditional methods which are followed are transportation of samples on dry-ice, which is expensive. The best alternative would be to process the RNA samples with RNAstable® LD (Biometrica) (Ohgi et al. 2010). This liquid can stabilize RNA even at room temperatures (15 to 25°C), by simply adding the required amount of RNAstable® LD to RNA solution and drying. The dried samples can then be shipped in heat sealed moisture barrier bag with desiccant, even without ice.

The bambara groundnut genotypes show variation in recovery of pod-set and pod-filling, after subjecting them to short duration of heat stress. This gives a potential

genetic variation for recovery, further investigating whether there could be variation that can be bred from a wider selection of genotypes is a suggestion for future investigators. The same can be used to develop transcriptome data to analyse gene expression during recovery, this might lead to uncovering links as to how bambara groundnut show plasticity in terms of its flowering trend and pod set recovery under temperature stress conditions.

CHAPTER 6

Discussion

6. Overview

As an aid to the reader the concluding chapter of this thesis provides a brief overview of study, including major methodology followed, in the light of the main aims of our research and to discuss the pertinence of the results in bambara groundnut (*Vigna subterranea* (L.) Verdc,) research. In the present research, the work was aimed at interpreting the importance of plant reproduction within this drought tolerant species, especially at the gametophytic stage, from pollination to fertilization and at seed set/fruit formation. This is relevant with the expected increase in global temperatures. This thesis describes the main results of investigating flower development and pollen development (Chapter 3), effects of a short duration temperature stress on reproductive development (Chapter 4) and the progress and obstacles encountered during the proposed RNA-seq experiment and future considerations for the same (Chapter-5).

6.1. Reproductive biology of bambara groundnut

One of the main objectives of our research was to gain a deeper understanding of the reproductive biology of bambara groundnut. The concept of plant sexual reproduction and relation between pollen and the formation of seeds and/or fruits has long been recognised. Proper pollination and fertilization are extremely important for crop seed yields, for example, in the world's major crops such as rice, wheat, maize, soybean etc., where seed is the economic yield, the final yields are dependent on environmental conditions such as temperature and photoperiod. The reproductive phase of development is considered highly sensitive to environmental factors such as temperature and photoperiod. Bambara groundnut is considered a drought tolerant crop which can produce yields when many other crops fail to survive. As such, it potentially offers a crop to help to address the food security challenges of the future. There are still many knowledge gaps, especially a clear understanding of its reproductive biology.

Therefore, the study was undertaken to deepen the understanding of the reproductive biology of the bambara groundnut, with a particular emphasis on inflorescence, floral development and pollen formation. The research used advanced microscopy techniques, such as fluorescence microscopy (University of Nottingham, UK) and Scanning electron microscopy (SCM) (Queens Medical Centre, Nottingham, UK) to visualize flower and pollen development at the microscopic level. The method was used for the first time in bambara groundnut research.

6.1.1. Bambara groundnut floral taxonomic descriptors

The phenology of bambara groundnut inflorescence and flower development was documented with new information after a long gap in research since 1970 (Doku 1968; Doku and Karikari 1970). The main documented observations were, the bambara groundnut inflorescence was racemose (pseudoreceme), which terminates with two flowers at each nodal region (refer to **section 3.1.2.**). Floral formulae and floral diagram are the means in summarizing isolated and static floral morphologies (Prenner et al. 2010; Ronse de Craene et al. 2014), we also agree that floral formulae should be given greater weight in botanical research, as a valuable tool in comparative biology and developmental studies. Here we provide the first documented floral diagram and floral formulae of the bambara groundnut flower, which was designed in accordance with Unicode system (refer to **section 3.1.2.1.**), that is **B Bt_2 % ↓ ⚥ $K_{(5)}$ $C_{1+2+(2)}$ $A_{1+(9)}$ $\underline{G}_{(1)}$** , [briefly; the flower is bracteate (B), bracteolate (Bt) with two bracteoles and zygomorphic (%) with a single plane of symmetry in median direction (↓). The flower was pentamerous with alternating whorls of five sepals (K), five petals (C), two sets of stamen (A) whorls and a single carpel/gynoecium (G) with superior ovary].

International code of botanical nomenclature requires minimum levels of character-based information (including floral formulae) in any formal taxonomic description of the species, our research presents the first recorded floral formulae for bambara groundnut flower in recent literature. The floral formulae play an important role in comparative biology and developmental studies, the method is applicable within as well as between species, to compare wild type versus cultivated verities and different

ontogenetic stages (Prenner et al. 2010; Ronse de Craene et al. 2014). Prenner et al. (2010), also recommended that floral formulae can become a routine component of diagnoses in protologues and other formal taxonomic (re)descriptions, functioning as a logical phenotypic counterpart to the DNA barcode. The floral formulae have traditionally been used at species level, but they can also be used in comparison with model and mutant species. That is, through floral formulae, closely related species or model organism which shows conserved floral ontogeny can be identified and used as a reference at genomic and phenotypic level. In case of bambara groundnut floral formulae show conserved floral ontogeny across all the *Vigna* genera (Tucker 2003b; Prenner 2004), this gives the possibility to select across many major legumes such as Vigna unguiculata (cow pea) whose genome has been recently sequenced as genome models.

6.1.2. Flower and pollen development scale

The current bambara groundnut flower scale was developed based on the flower size as unit of measurement to identify different stages. This was because the system of categorising the flower based on size was developed along the way when we noticed, the time of initiation of flower buds was difficult to identify. This was because in semi spreading varieties (IITA-686 and S19-3) inflorescence was small and develops at nodal region of 9 or 10^{th} leaf, where as in bunchy type (DipC) the initiation of inflorescence was near to crown of leaves. In order to develop a universal scale of flower development for spreading, semi-spreading and bunchy type bambara groundnut genotypes, it was more comprehensible through size of flower buds as it was easy to identify the underlying stages. In our study flower development stages were determined for the flower buds ranging in size from ≈1 mm to 9 mm by SEM. We have provided a series of reference observations and a first guide to bambara groundnut flower development (refer to **section 3.1.**). The stages of flower development determined were from stage 8 to stage 13, which corresponds to floral organ differentiation and flower opening, respectively.

For plant breeders, the interval between flower initiation and anthesis can be of the interest. According to Onwubiko et al. (2011a), the average interval between flower initiation and anthesis in bambara groundnut was 4 days, as they have considered the

emergence of flower bud from the bracts covering inflorescence as the day of flower initiation. However, the term flower initiation refers to the initiation of flower primordia from the primary inflorescence (I_1). Therefore, our findings have shown that smallest flower buds that were identified and tagged was at stage 8, it took 14 to 16 days for this flower bud to reach anthesis and complete flower opening (refer to section **3.4.** and **Table 13**). The current bambara groundnut flower development scale can be applied in plant breeding trials to identify stamen and carpel development stages during which emasculation can be done.

Pollen formation (micro-sporogenesis) and development (micro-gametogenesis) processes were studied using fluorescence microscopy (refer to **section 3.3.2.1.**). This study is the first one to link flower phenology to the stages of pollen development through morphological data obtained from advanced microscopy studies (refer to section **3.4**.), i.e., the flower stage-8 corresponds to anther stage 4 in which Pollen Mother Cells (PMC) are present. These documented stages of flower development and corresponding stamen/pollen development are similar to that observed in *Arabidopsis thaliana* L (Heynh) (Scott et al. 2004). Similarly, stage 9 of flower development corresponds to anther stage 5 during which PMC undergo meiosis to produce tetrad of cells, which are enclosed in a thick callose wall. In *Arabidopsis thaliana,* at flower/anther stage 12, the anthers contain tricellular pollen by the division of generative cell through mitosis II, however in bambara groundnut, tricellular pollen grains were formed only at the end of stage-13 (on the day of anthesis). It takes total of ≈9 days for the flower bud at stage 12 to reach the end of stage 13, during which anthesis occur and tricellular pollen grains are formed. The timing of this second mitosis varies among the plant families, in most (higher angiosperms) it occurs during anthesis (pollen release) and pollen tube growth (McCormick 2004). During our study through independent experiments, we have confirmed that the bambara groundnut pollen grains are indeed "bicellular" and reach "tricellular" at the time of anthesis. The observation on the timing of tricellular pollen grain formation shows that they are indeed formed at the time of anthesis/flower opening, this gives plant breeders a solid proof to consider a window of time (1 or 2 days before flower opening) for emasculation of anthers. This increases the chance of successful fertilization and seed formation through manual cross pollination.

However, we could not able to determine whether the pollen grains become tricellular on the stigma surface or within pollen tubes, this would be interesting to uncover through future investigations.

The bambara groundnut pollen grain structure and architecture were also studied for the first time in the literature by SCM observations and the results are presented in the chapter 3 (refer **section 3.3.2.3.**). The outer layer of bambara groundnut pollen grain show reticulate or tectate pattern with ridges and furrows on the surface, apart from pure mechanical function it also contains microchannels, which are the sites of water egress and ingress during desiccation and hydration. This intern determines progressive desiccation limits, pollen viability and life expectancy. These findings can be extended in the future to further our understanding of reproductive development in bambara groundnut.

The pattern of flowering was also elucidated in a seven bambara groundnut genotypes (refer to **section 3.2.3.2.**). In this section, are documented flowering time and the different trends in seven bambara groundnut genotypes (IITA-686, S19-3, AHM-753, Uniswa red, Getso, Gresik and DipC). The flowering trend was assessed to determine the window of flowering during which all the plants were at 100% flowering. The observation concluded that 2 weeks after the first flower opening was the time when the plants produced the greatest number of flowers. This time window was used to determine an ideal time frame for a future short duration heat stress in subsequent experiments. In this study, it was also noted that only the fertilized flower remains stalked and the flowers with unsuccessful fertilization wither and fall off within 24 hours. This phenomenon can be used in field trials to get direct indication of success of fertilization, there is no need to wait until pod formation. This can be applied to study relative number of flowers formed which can produce successful pods.

6.2. Temperature stress: determining the week link in bambara groundnut plant reproduction

The second objective of our research and perhaps the most important was to experimentally evaluate the weakest link during reproductive phase (gametophytic development) during high temperature stress in bambara groundnut. Global

agriculture is driven by numerous factors, given its dependence on natural resources, agricultural production is at the mercy of uncertainties driven by climate change, including extreme temperature fluctuations, drought and flooding. In the current research, the main focus was on the effect of temperature stress as it is obvious that global warming is primarily associated with increasing temperatures (short episodes and longer term trends). Simulation studies on temperature and precipitation patterns in Arid, semi-Arid and tropical forest environments showed that bambara groundnut could be one of the resilient crops which can tolerate increased temperatures in semi-Arid regions (Singh et al. 2015).

However, there is very limited evidence on how bambara groundnut genotypes differ in their response to temperature stress with respect to plant reproduction. Therefore, we evaluated the effect of short duration temperature stress surrounding flowering time (pre- and post-anthesis) in different genotypes of bambara groundnut genotypes (IITA-686, S19-3, AHM-753, Uniswa red, Getso, and DipC). During which plants at 100% flowering were stressed with high temperature of 36/33°C (day/night) for three days before the temperature was returned to 28/22°C (control) which was maintained to the end of the plant life cycle.

The effect of short duration high temperature stress was evaluated on flower morphology, this showed that some of the genotyped showed anthers that were indehiscent and flower opening was also affected (refer **section 4.3.3.1**). These findings show that high temperature stress may affect flower opening in bambara groundnut, the same phenomenon was also observed in green houses where bambara groundnut plants had been grown, when the temperatures reached beyond 39/40°C most of the flowers failed to open and whether off the next day. In our study, genotypes IITA-686, S19-3 and Getso, exhibited partial to complete anther in-dehiscence. Similar, studies also shows that heat stress before anthesis can influence anthers causing indehiscence in other legumes (Gross and Kigel 1994b; Devasirvatham et al. 2013). The same may also be true in green houses and even in field conditions, the heat wave might cause anther indehiscence and incomplete flower opening which leads to severe yield loss.

In our study we have observed that the number of flowers produced after heat stress increased exponentially, in all genotypes. This suggests that bambara groundnut has the ability to reflush (re-flowering) after heat shock. This was graphically indicated in genotypes, IITA-686, S19-3, AHM-753, Uniswa red, Getso and DipC, the difference was apparent between heat stress and control treatments for each genotype. For most cereals, if a heat shock occurs at anthesis, then it would mean the loss of yields, whereas bambara groundnut is plastic enough to recover and produce new flowers/pods. The effect of high temperature stress was prominent and would delay maturity, but is a lot better than a complete loss of yield. Bambara groundnut being indefinite flowering type could also be an evolutionary adaptation, since both the region of origin and cultivation show high temperature regimes, the crop might have acquired the ability to reflush. The crop is able to produce minimal yield in the regions where most of the other crops fail (Massawe et al. 2005a), can also be attributed to its ability to reflush in the regions where high temperature heat waves are common.

Determining *in vitro* pollen germination would give a direct indication of the effect of temperature stress on male reproductive tissues, especially pollen viability and success of fertilization. The observations show that the effect of high temperature stress on pollen germination was significant ($p<0.05$) and reduction in germination was seen within 24 h of the application of the heat stress in all bambara groundnut genotypes. These conclude that bambara groundnut pollen grains are sensitive to sudden change in temperature (in this case high temperature) and podding was significantly ($p<0.05$) reduced during heat stress. Müller and Rieu (2016), hypothesize that pollen heat sensitivity could be an adaptation in itself, preventing investment in reproduction under adverse conditions until favourable conditions return, this may cause sudden reduction in pods under heat shock.

The pollen germination and podding was recovered in bambara groundnut genotypes, when the plants were maintained at control conditions after heat stress. However, the genotypes AHM-753, IITA-686, and S19-3 recovered relatively faster when compared to Uniswa red and Getso. If this is true bambara groundnut has the ability to heat acclimation or acquired thermo-tolerance, some of the genotypes may even posses' basal thermo-tolerance. From the data obtained from this study, we could

conclude that like most of the legumes bambara groundnut pollen is sensitive to high temperature stress, with the temperatures 36/33°C producing significant effects on viability and podding. These results indicate a critical temperature in bambara groundnut beyond which the rate of successful fertilization and pod set is reduced.

6.3. Future considerations

6.3.1. Development of complete early flower development scale

The flower is one of the complex structures of plants, which is critical for seed and subsequently crop yield, an ontogeny and morphological study of the same can contribute to the physiological and molecular understanding of the species itself. Although the research aim was to completely study flower initiation and flower development, we were only able to establish flower development from stage-8. One of the limitations was that the smallest flowers that could be collected from the inflorescence were 1-2 mm in size and it was practically difficult to collect and dissect flowers smaller than 1 mm during this study. However, our study can serve as a guide point for future researchers interested in establishing a complete flower development guide universal to spreading, semi-spreading and bunchy type bambara groundnut genotypes.

6.3.2. In depth evaluation of flowering trend

The bambara groundnut has the ability to reflush (re-flowering) after heat shock, this phenomenon to produce flowers in high numbers after heat shock may be an evolutionary adaptation in itself, this trait needs to be evaluated in-depth in other genotypes of bambara groundnut. This characteristic feature should also be used as one of the trait of interest in breeding programs in selection and evaluation for high temperature tolerant varieties.

6.3.3. Evaluation of pollen thermo-tolerance

Our work has established that male gametophyte/pollen grain is most sensitive to sudden change in short duration high temperature stress, but recover when ideal conditions return and the variation in recovery was different depending on bambara

groundnut genotypes. This suggests that selection for breeding should start at male gamete level, for example in tomato pollen viability reduced under mild temperature stress (32/26°C) due to loss of starch and sugar accumulation in pollen grains, the best solution was to breed for tolerant varieties which has high starch accumulation capacity under heat stress (Müller and Rieu 2016). Similar strategies should also be adapted into bambara groundnut breeding programs. Pollen viability screening through *in vitro* pollen germination, developed in our study can be used in identification of heat tolerant and sensitive varieties under heat stress, this could be adapted in breeding programs in the selection of parental lines.

Although, the phenomenon of pollen thermo-tolerance was not evaluated in the current work, our research provides enough evidence and prospect to explore whether the pollen of bambara groundnut has the ability of acquired or basal thermo tolerance. Müller and Rieu (2016), suggest that if a plant is subjected to mild temperature stress, it could induce higher tolerance of pollen grains towards subsequent high temperature changes, this may lead to acquired thermo-tolerance either within an individual or in the offspring. Future researchers could investigate ways in which pollen thermo-tolerance may be enhanced in bambara groundnut. The concept of acclimation or acquired tolerance is not limited to only temperature stress, it could also be adapted in other climatic changes such as water stress, drought and humidity. Investigation of other forms of stress tolerance could ultimately contribute to the development of future crop which could cope with future climatic anomalies.

6.3.4. Need for pollen specific transcriptome analysis

The heat stress response in plants to maintain cellular homeostasis is well studied in vegetative tissues, only little is known in pollen (Chen et al. 2016; Müller and Rieu 2016). Transcriptome analysis in *Arabidopsis* and maize pollen grains has shown that developing pollen is relatively unique when compared to vegetative tissues, this means that knowledge obtained from vegetative tissues is not necessarily applicable to developing pollen, hence it is necessary to perform pollen specific studies (Rieu et al. 2017). With unexpected turn of events, the research goal ‘to sequence bambara groundnut flower and leaf transcriptome for differential gene expression under control and heat stress conditions’, was not achieved. Therefore, considerations should be

made to achieve success in future experiments, one such future attempt would be to store the tissue samples in RNA*later*™ Stabilization Solution (Invitrogen™, Thermo Fisher Scientific), to protect and stabilize RNA in the tissues. The extraction of RNA should be carried out using the methodology described in our study and freshly harvested tissues should be immediately extracted.

Bambara groundnut genotypes show variation in recovery of pod-set and pod-filling, after subjecting them to short duration of heat stress. This gives a potential genetic variation for recovery, further investigating whether there could be variation that can be bred from a wider selection of genotypes is a suggestion for future investigators. The same can be used to develop transcriptome data to analyse gene expression during recovery, this might lead to uncovering its links as to how bambara groundnut show plasticity in terms of its flowering trend and pod set recovery under temperature stress conditions. We also recommend reproductive stage specific studies to investigate stress responses in bambara groundnut at molecular level.

6.4. Concluding remarks

Many research experts are considering bambara groundnut as a future crop based on its agro-ecological, cultural, genetic and nutritional importance (Azam-Ali 2010; Bamshaiye et al. 2011; Mayes et al. 2012; Massawe et al. 2015). There is need to promote uptake of underutilised indigenous and traditional crops in marginal agriculture. However, many do agree that there is still lack of knowledge about the basic aspects of their plant biology, production and agro-economic traits, hence still remains underutilised. This research is but one step in contributing to the knowledge of this future crop in terms of its reproductive biology and the effect of high temperature stress on the same.

The framework of this research was a systematic study to establish first guide to reproductive development in bambara groundnut. The emphasis was on documenting floral formulae and a diagram of bambara groundnut flower, for the first time and providing original illustrations to facilitate accurate staging and characterization. We were able to elucidate flower development and link it with corresponding pollen development events with the help of advanced microscopy studies which is unique in

underutilised crop research. The significant effect of short episodes of heat stress was documented on flower morphology, pollen germination and pod set, in IITA-686, S19-3, AHM-753, Uniswa red, Getso and DipC genotypes of bambara groundnut. These observations suggest that bambara groundnut was plastic enough to reflush or re-flower after heat shock. Even though the pollen grains show sensitive to sudden increase in temperatures, bambara groundnut pollen show recovery in its viability and its ability to produce pods, this could be attributed to pollen thermo-tolerance in bambara groundnut genotypes. These findings give enough evidence for future researchers to investigate thermo-tolerance in bambara groundnut genotypes.

The major setback before achieving transcriptome data under high temperature stress, was a great hindrance, But, “because learning what doesn't work is a necessary step to learning what does – Jonas Salk (American medical researcher and virologist)” (Jeffrey Kluger 2014), we could only learn from our setback. Therefore, careful considerations and recommendations for future researchers aiming to study the same has been presented.

Nevertheless, we believe that this thesis has provided considerable knowledge on bambara groundnut reproductive biology with respect to high temperature stress, facilitating effective study of this important trait in this underutilized crop.

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