UNDERSTANDING CROP DOMESTICATION - RESPONSES OF LETTUCE ROOTS UNDER DIFFERENTIAL PHOSPHORUS CONDITIONS

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ABSTRACT

Root system architecture (RSA) is a dynamic system of root network capable of adapting to changes in soil environment such as decline in soil moisture and nutrient deficiency. RSA responses to different nutrient concentration levels provide a unique system to study interactions between plants and their soil environment and the effect of different nutrient levels on root development. Wild (*Lactuca serriola*) and domesticated (*Lactuca sativa*) lettuce root systems have contrasting RSA and therefore populations developed from these two contrasting parents provide unique resources to explore root traits between cultivated and domesticated crop species.

Wild lettuce has a deeper root system with capability to exploit deeper soil horizons for nutrients and water while the domesticated lettuce possesses a shallow root system capable of acquiring resources mostly from the topsoil. Although there are clear RSA differences between wild and domesticated lettuce grown under normal soil condition, an understanding of the effect of different phosphorus (P) levels on RSA is lacking. P is one of the most important macronutrients for most crops after nitrogen, especially used as one of the building blocks of nucleic acid, phospholipids and many metabolites. Furthermore, P is often immobilised in the soil, therefore understanding the optimal uptake of P through RSA is important.

The present study aims to provide a better understanding of the effect of crop domestication on root traits by evaluating lettuce RSA, specifically the contrasting features of wild and domesticated lettuce, in response to a wide array of P levels. Specifically, the root systems of lettuce parental lines, wild lettuce (*Lactuca serriola* acc. UC96US23) and domesticated lettuce (*Lactuca sativa* cv. Salinas) were evaluated using agar-based and paper-based root phenotyping methods. This was followed by the QTL analysis of the lettuce parental lines and an recombinant inbred lines (RIL) mapping population derived from the cross of the two lettuce parental lines. The present study also explored the use of x-ray microcomputed tomography (μCT) to visualise the undisturbed lettuce RSA in 3D.

The agar-based root phenotyping method utilised vertical agar-filled petri dishes at five different P levels (0, 6, 312, 625 and 1250 μM P) and images of the roots were obtained through a flatbed scanner and analysed *in silico*. Seven RSA traits showed significant difference ($P \leq 0.049$) between lettuce parental genotypes × P levels interaction. The subsequent multiple comparison tests implied that the wild lettuce showed significant enhanced primary root (PR) growth ($P < 0.001$) while domesticated lettuce significant showed enhanced lateral roots (LR) formation ($P < 0.001$), especially at very low and high P levels.

The paper-based root phenotyping method utilised vertical paper pouches and images were obtained through simple DSLR camera setup, and then analysed *in silico*. The results showed significant mean differences ($P \leq 0.006$) between the parental genotypes in most of the measured traits. The trait means of domesticated lettuce were consistently higher than the wild. Additionally, the correlation tests revealed strongest significant correlation ($r \geq 0.82$, $P < 0.001$) of similar trait classes (i.e. lateral-lateral, primary-primary and global-global root traits), suggesting similar growth mechanisms between highly related traits.

The confirmation of significant genotypic differences in previous experiments led to the QTL mapping of the traits using an F_8 RIL mapping population. From multiple QTL mapping (MQM) analysis, six QTLs and a putative QTL were obtained, mostly clustered in a hotspot in linkage group (LG) 1. The traits were mainly of the primary and global root traits. The primary root length (PRL) in this hotspot was driven by wild lettuce, which may imply association of domestication QTL in lettuce rooting depth as opposed to interval mapping (IM) or multiple QTL mapping (MQM) analysis. Using a non-parametric Kruskal-Wallis (KW) QTL analysis, 48 QTLs were identified, in which some clustered at hotspots (i.e. LG1, LG4, LG5 and LG8) dominated by lateral root traits. These clusters of trait loci may imply similar mechanisms control similar growth-related traits.

The overall differences seen between wild and domesticated lettuce RSA have provided an understanding of the effects of domestication on RSA traits. The present study showed some deviation in P adaptation between the parental lines, suggesting the novel domestication QTL identified particularly in LG1 which relates to the PRL. The outcome of this study could potentially be applied in identifying RSA traits that should be maintained or selected in other species, particularly the underutilised crops, during improvement process. Development of improved varieties with superior root traits such as deep rooting system, may pave the way for more sustainable agricultural practice thereby reducing dependency of crops on inputs such as chemical fertilisers and excess water.

The results obtained from the present study were obtained from 2D images, which may not entirely represent the 3D architecture of the roots in the soil. The utilisation of x-ray microcomputed tomography (μCT) in visualising the lettuce RSA in a preliminary study have shown interesting 'umbrella-shaped' root architecture, which cannot be clearly identified in experiments using 2D images. The study could be potentially expanded to explore more traits, especially using 3D-related root traits, to better understand the lettuce RSA, particularly responses towards different P levels.

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LIST OF ABBREVIATIONS

2D - 2-dimensions
3D - 3-dimensions
ABA - abscisic acid
AFM - Arbuscular mycorrhizal fungi
AD - Average root diameter
ANOVA - Analysis of variance
CFF - Crops For the Future
CRISPR/Cas - Clustered regularly interspaced short palindromic repeat/Cas
CRW - crop wild relatives
D - diffusion coefficient
DNA - deoxyribonucleic acid
DSB - double strand breaks
DSLR - digital single-lens reflex
F_8 - Filial 8
FDR - false discovery rates
GBS - genotyping-by-sequencing
GEBV - genomic estimated breeding values
GM - genetically modified
gRNA - guiding RNA
GWS - genomic-wide selection
h - hour
ICP-MS - inductively coupled plasma mass spectrometry
IM - interval mapping
KW - Kruskal-Wallis
LD - lateral root diameter
LG - linkage group
LOD - logarithm of odd
LR - lateral roots
LRBD - lateral root branching density
LRD - lateral root density
LRL - lateral root length
LRN - lateral root number
LSA - lateral root surface area
LSD - least significant difference

LV - lateral root volume
MAS - marker-assisted breeding
MQM - multiple - QTL mapping
MRI - magnetic resonance imaging
MS - Murashige and Skoog (1962)
N - nitrogen
n - number of sample/replicates
NGS - next generation sequencing
P - phosphorus
PCA - Principal Component Analysis
PD - primary root diameter
P_i - inorganic phosphorus
PR - primary roots
PRL - primary root length
PSA - primary root surface area
p.s.i - pound per inch
PSI - phosphorus starvation induced
PT - permutation test
PV - primary root volume
PVE% - phenotypic variation explained percentage
QTL - quantitative trait locus
RCBD - randomised complete block design
REML - reduced maximum likelihood
RIL - recombinant inbred lines
RNA - ribonucleic acid
r_p - Pearson's product-moment correlation *r*-value
r_s - Spearman's rank correlation *r*-value
RSA - root system architecture
RV - Total root volume
SA - Total root surface area
Sal - *Lactuca sativa* cv. Salinas
SD% - seed dormancy percentage
SDW - sterilised deionised water
SED - standard error of difference
SEM - standard error of means
Ser - *Lactuca serriola* acc. UC96US23
SG% - seed germination percentage
SOM - soil organic matter
SR - SmartRoot
SV% - seed viability percentage
TRL - Total root length
TZ - 2,3,5-triphenyl tetrazolium chloride
µCT - microcomputed tomography

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CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Root systems are vital in plants as they play important roles such as nutrients and water uptake, food storage, and the site of interaction between plants and the soil environment. Roots also provide mechanical support for the plants. They are highly plastic and able to adapt to a wide array of environmental conditions (Hodge, 2004; Eapen *et al*., 2005), of which directly affect the spatial arrangement of the three dimensional (3D) root pattern in soil space, collectively referred to as root system architecture (RSA).

RSA can be classified in two levels. At macroscale level, RSA represents the organisation of the primary roots (PR), lateral roots (LR) and some other accessory roots (which can be seen in some cereals) such as crown, and seminal roots, within the soil space. On the other hand, RSA at microscale level relates to the finer roots including the root hairs (Gregory, 2006; Smith and De Smet, 2012). Resources are mostly heterogeneously distributed in the soil (Robinson, 1994), thus dynamic respond at macroscale RSA is an important key determinant of nutrient- and water-use efficiency in plants, with microscale RSA helping to further

aid the resource uptake by the plants (Lynch, 1995; Smith and De Smet, 2012; Paez-Garcia *et al*., 2015).

Over time, RSA changes have been driven by domestication and breeding, leading to different spatial arrangement of the roots (de Dorlodot *et al*., 2007). However, domestication and breeding programme have mainly concentrated on the above-ground organs such as grain size and tiller numbers (Waines and Ehdaie, 2007), due to its direct economic value in term of productivity and yield, almost neglecting the other hidden half, the root systems. This focus is also possibly due to technical difficulties in carrying out accurate root and soil studies. Such studies are time-, and labour-intensive. An impact study was carried out to examine the relationship between domestication and root sizes of pre- and post-Green Revolution period wheat by Waines and Ehdaie (2007). The study showed a reduction in size for modern wheat roots, in comparison to wheat landraces. Small root size is not a favourable characteristic for a crop as it may be too small for efficient uptake of nutrients and water hence affecting the grain yield, particularly in lower input agricultural systems. Thus, roots need to be highlighted as part of the main characteristics for selection in a crop improvement programme.

Recently, such RSA research has gained attention in the field, with much more evidence linking RSA to plant fitness, crop performance and also grain yield (Rogers and Benfey, 2015; Khan *et al*., 2016). This is also

strengthened by the emergence of simpler root phenotyping techniques such as agar-based and paper-based assessment methods, and also more advanced and accurate 3D root phenotyping methods including X-ray microcomputed tomography (μCT; Mooney *et al*., 2012) and magnetic resonance images (MRI; Schulz *et al*., 2013) technology.

Furthermore, quantitative trait locus (QTL) analysis has been an important part in understanding the genetic variation of RSA of many species. The species includes major cereal crops such as rice (Steele *et al*., 2013) and maize (Li *et al*., 2015; Pestsova *et al*., 2016) and also model plants such as *Arabidopsis thaliana* (Gerald *et al*., 2006) and *Brachypodium distachyon* (Ingram *et al*., 2013). The QTL identification faced challenges as underlying plasticity nature of the RSA derived by the interaction between environmental factors, genotypic heritability and genetic interactions (i.e. epistasis and pleiotropy) (de Dorlodot *et al*., 2007).

Furthermore, important QTLs can be driven by lost alleles in wild crop progenitor or the one in underutilised plants. The utilisation of these gene pools are pivotal in improving crops for tackling climate change, abiotic and biotic stresses while at the same time increasing the productivity of a crop and securing food production (Mayes *et al*., 2012; Brozynska *et al*., 2015; Zhang et al., 2017). For instance, allelic contribution of wild lettuce (*Lactuca serriola*) was linked to deeper root traits which can improve the uptake of nitrogen and water in deeper soil profile (Johnson *et al*. 2000).

Besides, introgression of wild barley (*Hordeum vulgare* ssp. *spontaneum* accession ISR42-8) into domesticated barley (*H. vulgare* ssp. *vulgare*) background has improved root and shoot traits, with 15 chromosomal regions identified with co-localised QTLs originating from the wild barley (Naz *et al*., 2014). These surrounds the philosophy of super-domestication; processes that refer to a domesticate with improved traits driven by its genomic understanding and the use of advanced technology (Vaughan *et al*., 2007).

This chapter reviews RSA responses to nutrient uptake especially nitrogen and phosphorus. The review also highlights progress on genotyping and phenotyping platforms for the identification of relevant RSA for target environment. The literature review was followed by research motivation, aim, objectives and the thesis overview.

1.2 ROOT RESPONSES TO NITROGEN AND PHOSPHORUS AND ITS IMPACT TO GLOBAL ACQUISITION IN SOIL

RSA changes are influenced by the environmental resources, including nutrients and water (Gruber *et al*., 2013). This relationship between RSA and the environment has led to the identification of RSA under certain target environments such as drought and nutrient deficiency. This is often related to root ideotype, specifically 'designed' for particular environment to optimise the resource acquisition (Kong *et al*., 2014). Root ideotype refers to ideal phenotype of the roots in target environments and general

root ideotypes suggested by breeders and researchers are mainly based around these three ideas: 1) to exploit large volume of soil horizontally and vertically; 2) less metabolic cost to grow and maintain these extensive network of roots and 3) traits that can increase efficiency of uptake and hydraulic conductivity of the roots. The identification of root ideotypes is done by recognising the root phenes (i.e. phene of phenotype as for gene for genotype; Lynch and Brown, 2012) associated with specific root functions.

The dynamics of mineral nutrient mobility in the soil is complex and depends on the diffusion coefficient (D) that is affected by many factors such as particle surface charge, soil pH and soil organic matter (SOM), leading to adaptation of RSA to reach these nutrient sources. Mineral nutrients are generally known to be heterogeneously distributed in soil (Robinson, 1994). However, these nutrients ions in the soil are relatively positioned in a vertical series of which are mainly influenced by leaching, weathering, dissolution and atmospheric deposition (Jobbágy and Jackson, 2001). This has been one of the main attributes to consider in RSA selection for improving crops, especially one that adapted to dynamic nitrogen (N) and phosphorus (P) levels in soil, as N and P are considered to be major limitations in crop production as compared to other nutrients.

1.2.1 Nitrogen

1.2.1.1 *Nitrogen in soil*

Nitrogen (N) exists in soil system in many forms and can transform (changes) to other form easily. The transformation was very much influenced by biological factors, which collectively called the nitrogen cycle. The cycle consists of processes which include the atmospheric nitrogen (N_2) fixation to organic N, mineralisation or ammonification of organic N into ammonia (NH_3) and immobilisation or assimilation of ammonia and nitrate (NH_4^-) into organic N (Schulten and Schnitzer, 1998; Lamb *et al*., 2014).

The ability of plants to tap large reservoir of atmospheric N_2 is limited to certain microorganisms only, such as rhizobia species, which form N-fixing nodules at the roots of leguminous plants (Zahran, 1999). Therefore, most of other plants requiring other readily forms of N especially inorganic N of ammonium (NH_4^+) and nitrate (NH_4^-) for uptake (López-Bucio *et al*., 2003; Nibau *et al*., 2008). Plus, the physical and chemical properties of certain soils may also influenced the availability of N in the soil system too. Generally, N is very soluble and mobile in the soil, therefore exposed to the potential of N leaching with excess water below the root zone. This however can be effectively captured by plants having deeper root system. Other mechanism would include denitrification of NO_3^- and volatilisation of

NH_3 gas into the atmosphere. Below is Figure 1.1 showing the transfer of N within the soil system.

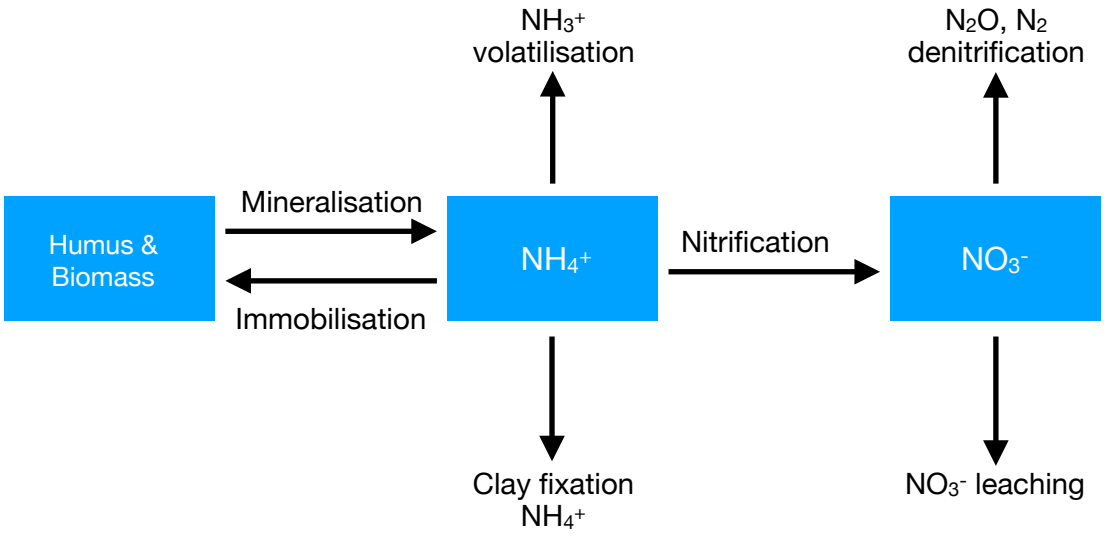


Figure 1.1 Transfer of N within the soil system.

Furthermore, the N distribution may vary according to different climatic conditions and geographical area from which the soils being sampled. Generally, it was estimated that the distribution of nitrogen consists of ca. 40% proteinaceous materials (proteins, peptides, and amino acids); 5-6% amino sugars; ca. 35% heterocyclic N compounds (including purines and pyramidines); 19% ammonia (NH_3) with 1/4 of it constitutes fixed nitrate (NH_4^+) (Schulten and Schnitzer, 1998). Soil organic matter, especially humic substances, provides almost 95% source of nitrogen (N) (Schulten and Schnitzer, 1998), especially in low input systems (Lynch, 2013). External inputs such as fertilisers and manures are rich in ammonium (NH_4^+) and nitrate (NH_4^-), added into the agricultural system for better soil N fertility.

1.2.1.2 *Morphophysiological response of roots to N*

The response of roots towards N in the soil depends on its abundance and spatial location in the soil. Two distinct morphological adaptations lead to either local or global responses. In the case of low nitrate levels in the soil, the area that contains high concentration of N acts as local stimulators to induce proliferation of lateral roots towards the patch (Zhang and Forde, 1998; Kerbirou *et al*., 2013). The lateral roots formed are also relatively longer, except in extremely low N conditions where the length decreased (Gruber *et al*., 2013). Auxin may have a role in regulating this behaviour, for example the *axr4* mutant of *Arabidopsis* failed to respond to localised nitrate (Zhang *et al*., 1999). However, lateral root formation is inhibited when N is high in the soil (Zhang *et al*., 1999; Zhang and Forde. 2000). This is known as a systemic inhibitory effect, thought to be regulated by ABA (Signora *et al*., 2001).

Proliferation towards high N patches seems to be a good option for root phenes for N uptake, however, proliferation can cost more carbon expenditure for growth and maintenance in roots (Robinson, 2001). To compensate for the disadvantages of proliferation, a domesticated crops is possibly designed with better enhanced nitrate inflow into roots, although the models predict that inflow must increase proportionally more than root length density to achieve the same N capture in comparison to acquisition of N by proliferation alone (Robinson, 2001). However,

improvement of nitrate inflow in roots may be a challenge as it involves a detailed molecular dissection and domestication of molecular nutrient exchange components along the root region. Yield improvements from acquiring deep resources through larger investment in fine roots at deeper roots and less root proliferation at surface would be the best model (King *et al*., 2003).

Lynch (2013) has proposed the steep, cheap and deep (SCD) root ideotype for monocot models particularly maize (*Zea mays*) roots for better N and water acquisition. The premises behind the ideotype focussed on having steeper roots instead of shallow roots, hence a deeper root system while at the same time reducing the metabolic cost for soil foraging or known as rhizoeconomics (Lynch, 2013; 2015). Although this has been proposed for maize specifically, the premises could be suitably be adapted for many species, especially in exploiting deeper N and water resources.

For instance, *DEEPER ROOTING 1* (*DRO1*; Uga *et al*., 2011) has been one of the gene that was spliced into an elite lowland *indica* rice cultivar, IR64 from traditional Kinandong Patong *japonica* rice cultivar from upland Philippines, in which the former has a shallower root system than the later in which has a deeper rooting system. *DRO1* confers drought tolerance (Uga *et al*., 2013) and improve the N uptake (Arai-Sanoh *et al*., 2014) in rice and therefore increases the yield production. This is important as N

tend to be deeper in soil during the growing season, often leached with water, as deficiency of these resources often outpace the root growth for resource capture, causing major N and water deprivation (Lynch, 2013).

1.2.2 Phosphorus

1.2.2.1 *Phosphorus in soil*

Phosphorus (P) is generally known to be an immobile nutrient in the soil, usually abundantly available in the topsoil especially due to high fertilisation applied from above soil. In soil, P exists in 3 different forms, namely; soluble P, active P and fixed P, technically known as 'pools' (Busman *et al*., 2002). P especially in the form of orthophosphate or inorganic phosphate (P_i) from the soil solution P pool, is among the essential macronutrient needed by a plant, important for plant growth and development, and key component for nucleic acids and phospholipids (Péret *et al*., 2014).

Nevertheless, it is the least accessible element required by the plants for their optimal growth, with concentrations of soluble P_i in the soil often up to a 1000 times lower than those of other required ions (Raghothama, 2000; Vance *et al*., 2003). The soluble P_i availability in soil is highly influenced by the soil pH. P_i is insoluble in soil due to its formation into calcium salts at high pH or complexes with constituents such as iron and

aluminium oxide at low pH (Abel *et al*., 2002; Busman *et al*., 2002). Unlike nitrogen, which can be cycled in a rather closed system, depleted phosphorus needs to be substituted with external sources of phosphorus to increase the level of soil phosphorus (Abel *et al*., 2002).

Phosphorus deficiency in crop plants is truly a widespread issue around the globe especially in highly weathered acidic soils (Fageria and Baligar, 1997; Fageria and Baligar, 2001; Faye *et al*., 2006). This is often mitigated by applying a large amount of P fertilisers to the soil. However, demand of P fertiliser productivity is projected to be at 55-60 Tg in 2050, an increase of 27-36% from year 2000 data (Fess *et al*., 2011). This is clearly not a sustainable option especially in this so-called 'peak society' (refers to peak usage of resources) era as phosphorus ore, which needs to be mined, is one of main resources that is finite and non-renewable (Abel *et al*., 2002), and may face depletion by the end of the century (Runge-Metzger, 1995; Steen, 1998; Cordell *et al*., 2009). Plus, long-term high P fertilisation in agricultural soil may give negative impact on the development of arbuscular mycorrhizal fungi (AFM) colonies. AFM is essential to provide optimal rhizosphere conditions for P uptake by plants through root system (Grant *et al*., 2005).

Furthermore, P_i leaching from the heavily applied fertiliser field might threaten the surface water resources which include river stream and lake, due to the effect of chemical immobilisation of P_i and agricultural soil

sediment runoff (Abel *et al*., 2002; Busman *et al*., 2002). Such current issues are best tackled by having crops that are better in utilising and responding well to available soil phosphorus and also proper management of soil fertility.

1.2.2.2 *Topsoil foraging adaptation*

Under P deficiency, RSA responses is rather species-specific but may include general observation of primary root growth inhibition, induction of lateral root formation and some with formation of cluster or proteiod roots at an extreme P deficiency especially in white lupin (*Lupinus albus*) and harsh hakea (*Hakea prostrata*) (Williamson *et al*., 2001, Nibau *et al*., 2008; Péret *et al*., 2014; Rogers and Benfey, 2015). These adaptations for P deficiency are collectively termed as topsoil foraging as first described in common bean (*Phaseolus vulgaris*) (Lynch and Brown, 2001).

Lateral root formation is induced under low P condition with influence on auxin-related and P perception pathway genes for example *TRANSPORT INHIBITOR RESPONSE1* (*TIR1*; Pérez-Torres *et al*., 2008), *PDR2* (Ticconi *et al*., 2004) and *PHT1;9* (Remy *et al*., 2012). The whole mechanism of reduced PR growth under low P, on the other hand, is not fully understood (Péret *et al*., 2014) and only few candidate genes have been identified which include *LOW PHOSPHATE ROOT* (*LPR*; Svistoonoff *et al*., 2007) and *ALTERED PHOSPHATE STARVATION RESPONSE1* (*ASPR1*; González-

Mendoza *et al*., 2013). Additionally, in some legumes, the reduction of gravitropic set-point angle can be seen in basal roots (Bonser *et al*., 1996; Liao *et al*., 2001), making the roots grow mainly in the top horizon of the soil. As the influx of P is virtually at the same rate for any root, spatial exploration is regarded as the main factor in determining the net influx of P into the plant (Rubio *et al*., 2004).

Enhanced topsoil foraging acquires phosphorus more efficiently than other architectures of equivalent root size. Adventitious rooting in bean has also been shown to increase aerenchyma abundance to reduce the rate of root respiration per unit of nutrient-absorbing surface area (Lynch and Brown, 2008). These architectural traits have been observed in other species such as rice (Panigrahy *et al*., 2009), maize (Li *et al*., 2012) and *Brassica* species (Shi *et al*., 2013).

Efficient topsoil foraging strategy alone may benefit crops in term of capturing the P concentrated at the topsoil strata or perhaps other immobile ion as well such as potassium, iron and manganese, especially under high input agricultural system. However, acquisition of nutrients near soil surface versus a deeper soil foraging creates biomass allocation tradeoff for the plants especially in multiple resources acquisition (Ho *et al*., 2005; Rogers and Banfey, 2015). In this case, RSA plasticity and root dimorphism are desirable traits for selection in order for plants to adapt and response to wider array of environmental conditions.

A dimorphic root system (i.e. both shallow and deep root systems) was observed when common bean were subjected to P and drought stresses, both representing differentially localised resources, topsoil and subsoil respectively (Ho *et al*., 2005). Furthermore, a study based on plant model *SimRoot*, optimal lateral root branching density (LRBD) for maize was shown to depend on the relative availability of both N and P (Postma *et al*., 2014). Most of the genotypes grown in the study have shown a level of LRBD that balances the uptake of both N and P. This confers RSA plasticity for multiple resource acquisition, worth targeted for breeding.

1.3 ALLELIC DIVERSIFICATION FOR CROP IMPROVEMENT

Crop domestication has been recognised since the beginning of recorded history which dates approximately 10,000 years ago, in which crops undergone modifications to suit human needs (McCouch and Tanksley, 1997; Doebley *et al*., 2006). Scientists' estimates show approximately 2500 species have undergone domestication, with over 160 families contributing one or more crop species (Zeven & de Wit, 1982; Dirzo & Raven, 2003). However, only a few species have been used as major food crops, mainly rice, wheat and maize, while neglecting many plant species biodiversity. These major staples have narrower genetic base, driven by modern plant breeding which in turn may jeopardise the ability of the crops to adapt to ever changing climate and also food and nutritional security (McCouch and Tanskely, 1997). In view of RSA improvement in

crops, the exploitation of gene pool of the crop wild progenitors and underutilised species germplasms may enhance adaptability of crops in wider array of environments and also improve agricultural productivity.

For instance, *Phosphorus uptake 1* (*Pup1*) QTL is an important major QTL associated with tolerance under P deficiency located on chromosome 12 in traditional rice *aus*-type Kasalath from India (Wissuwa *et al*., 1998, 2002). *Pup1* sequence has led to the identification of crown root *Pup1*-specific protein kinase gene, *PHOSPHORUS-STARVATION TOLERANCE 1* (*PSTOL1*), controlling root growth during low P, improving grain yield and P acquisition through RSA regulation (Heuer *et al*., 2009; Chin *et al*., 2011; Gamuyao *et al*., 2012; Kong *et al*., 2014; Rogers and Banfey, 2015). The gene is absent in modern rice varieties reference genome for P starvation intolerant traits (Chin *et al*., 2010; 2011), suggesting the importance of exploiting utilisation of traditional germplasm lines to improve the adaptation of modern cultivars. The gene homolog has also been successfully dissected and identified in maize (Azevedo *et al*., 2015) and sorghum (Hufnagel *et al*., 2014).

Furthermore, barley (*Hordeum vulgare*) is one of the best model systems to exploit exotic genes, as germplasm accessions are readily available at three distinct levels: wild forms, landraces and modern cultivars (Parzies *et al*., 2000; Wacker *et al*., 2002). A study on introgression lines population S42IL, derived from introgression of wild barley accession ISR42-8 in the

elite spring barley cultivar “Scarlett” has shown improvement of root performance under N stress, with increase effect of root length QTL as compared to Scarlett background (Hoffman *et al*., 2012). Separate studies have also shown root adaptation under drought condition by using the same population (Naz *et al*., 2012; 2014), indicating wild barley as sources for root improvement for modern barley cultivars.

Conversely, the root architecture of the lettuce wild progenitor (*Lactuca serriola*) and its domesticated relative (*Lactuca sativa*) are significantly different, although the overall allocation of the biomass is similar (Jackson, 1995). This indicates that the process of domestication has had a profound impact on RSA traits heritability and subsequent effects on roots to scavenge for nutrient and water. Domesticated lettuce plants produce a shallower root system with more laterals and external roots in the top 0-5 cm zone of the tap root, however, wild lettuce produces a deeper root system with more laterals at the tip of the tap root (Jackson, 1995), allowing for more efficient nutrient and water acquisition especially in deeper soil strata. In a series of experiments, 13 QTL responsible for this deep soil exploitation especially for nitrogen and water uptake, were detected in a study conducted by Johnson *et al*. (2000). As the differences did not require higher carbon expenditure for root growth and maintenance, deep rooting allele of wild lettuce germplasm can be utilised in modern lettuce cultivars, optimising the resource acquisition in the soil.

Moreover, the utilisation of species beyond major staple crops especially neglected, minor or underutilised crops has huge potential to improve food and nutritional security (Massawe *et al*., 2016). Exploiting plethora of crop diversity also reduce risks related to agronomic, ecological and economics due to heavy reliance on major crops, in view of global climate change (Ebert, 2014). Underutilised legumes such as bambara groundnut (*Vigna subterranea*) and hyacinth bean (*Lablab purpureus*), are important subsistence species for its protein content and have been proven to withstand drought conditions (Collinson *et al*., 1997; Ewansiha and Singh 2006) in comparison to common groundnut (Babekir, 1989). Amaranth (*Amaranthus spp.*) is an important leafy vegetable and grain crop, has shown quick recovery from wilting after being exposed to only small amount of rain as little as 2 mm (Myers, 1996), suggesting robustness in drier region.

Working with underutilised crops is however often associated with challenges in identification and collection of traditional germplasm that are scattered around the globe and also lacking in attention from research, policy and decision makers, donors, technology providers and also lack of demand from consumers itself (Padulosi *et al*., 2002; Warschefsky *et al*., 2014; Massawe *et al*., 2016). Major phenotypic improvement of the crops may not be a tempting approach for underutilised crops, as these species are simply adapted to the place they are grown, albeit, the understanding of the genetics may give an

insight of the beneficial alleles and its comparison with major crops (Mayes *et al*., 2011).

RSA characterisation of these underutilised crops is important in this case, nevertheless, with the advent of high throughput genomic tools such as next generation sequencing (NGS), the QTL or candidate genes and its orthologues may be identified and utilised if plants go through improvement programme. In spite of everything, the will of utilising underutilised plants needs collaborative effort from multidisciplinary field under a systematic research chain. Crops For the Future (CFF), for instance, has been the one of the epicentre with mandate for research and development of underutilised plants for food and non-food uses, focussing on the diversification of plant species especially for human needs (Mayes *et al*., 2011).

1.4 OVERVIEW OF MOLECULAR TOOLS IN CROP IMPROVEMENT AND APPLICATION IN RSA SELECTION AND IMPROVEMENT

In the past, crop breeding approaches have been solely emphasised on trait-based selection. The trait-based selection, which was practised by ancient farmers many years ago, was followed by a more systematic selection method by modern farmers and breeders. Trait-based selection has been successful in selecting useful traits for modern crops and human needs such as non-shattering seeds in cereals (Fuller and Allaby, 2009; Dong and Wang, 2015), loss of seed dormancy (Fuller and Allaby,

2009; Veasey *et al*., 2004) and modified morphology (Hufford *et al*., 2012), which can be directly linked to improved yield potential, stress avoidance, nutritional quality and agricultural practices. However, the selection of improved RSA traits has almost been totally neglected due to the hidden nature of the root system. As conventional plant breeding select traits based on Donald's (1968) high input ideotype framework, the RSA has been 'accidentally' chosen to fit this environment, making the crops vulnerable in more challenging agricultural environments such as drought prone regions and suboptimal soil nutrient regimes.

With the advent of molecular tools, molecular dissection related to beneficial traits can be carried out to improve selection efficiency in breeding programme (Brumlop and Finckh, 2011). Marker-assisted selection (MAS) is one of the tools used in identifying favourable traits, evolving from morphological- and allozyme-based markers, to the DNA markers in tagging the highest possible regions where the genes may affect the phenotypic variation, often in different locus of the genomes due to polygenic nature of the agronomic traits. These polygenic traits are quantitative traits including most of characterised RSA. Identification of quantitative trait locus (QTL) is the stepping stone in understanding complex genetic control mechanism underlying the phenotypic differences, enabling further identification and exploitation of beneficial alleles (de Dorlodot *et al*., 2007), especially in breeding process through MAS.

Identification of the QTL requires validation for confirmation of function of QTL or gene related to the phenotypic variation. These are often done through positional cloning or candidate gene approach, followed by integration of the genetic and functional information in routine breeding processes (de Dorlodot *et al*., 2007). However, this is practically challenging and require access to established genomic resources (e.g. large segregating populations and high-density marker maps) and logical informatics information (de Dorlodot *et al.*, 2007). Rice and maize are the main examples of crops with successful RSA gene characterisation through QTL analysis; well reviewed elsewhere by Mai *et al*. (2014) and Tuberosa and Salvi (2007) for rice and maize respectively.

Furthermore, reduction in time and costs of producing high quality next-generation sequencing (NGS) sequence data has widen the opportunity of improving traditional MAS methods, through genomic-wide selection. Genomic-wide selection (GWS) involves application of whole genome strategies (Xu *et al*., 2012), instead of only targeting specific association of marker to smaller region of the genome related to specific phenotype variation as practised in traditional MAS approach. GWS involves the usage of high density genome wide polymorphic markers that covers as much as possible of the whole genome, in which the effect of these markers are estimated through genomic estimated breeding values (GEBV).

GEBV is used as a selection criterion in contrast to rejection of markers below the level of statistic significance in traditional MAS (Xu *et al*., 2012) giving more advantages of selection to genes with small additive effect (Rutkoski *et al*., 2011). GWS through improvement in NGS and other high throughput technologies also enables exploitation of many species' genomic sequence data including underutilised plants, without or with little information on genetic information for possible understanding of genetic control and the interaction with environments hence selection for crop improvement. Several GWS studies on RSA has been reported by utilising Genotyping-by-Sequencing (GBS) platform for example drought avoidance RSA QTL characterisation in rice (Courtois *et al*., 2013) and seedling root development in maize (Pace *et al*., 2015).

Genomic editing method has received enormous attention recently due to its specific and targeted mutation of plant genome and ease of design (Khatodia *et al*., 2016). Clustered regularly interspaced short palindromic repeat/Cas (CRISPR/Cas) system is one the genetic editing tool, utilising the knowledge of adaptive immune system by bacteria in protecting itself against invading DNA such as bacteriophage (Jinek *et al*., 2012; Doudna and Charpentier, 2014; Khatodia *et al*., 2016). The system is able to target specifically the point of double strand breaks (DSB) through specifically designed artificial guiding RNA (gRNA), and the ability to produce mutation at the point by indels or insertion of any desirable genetic portion including the QTL or genes that has been characterised in QTL

analysis (Jinek *et al*., 2012). The method has been applied to few model plants such as *Arabidopsis* (Feng *et al*., 2013; Jiang *et al*., 2013; Ma *et al*., 2015), tomato (Brooks *et al*., 2014; Uluisik *et al*., 2016), and crops such as rice (Feng *et al*., 2013; Shan *et al*., 2013), maize (Svitashev *et al*., 2015) soybean (Jacobs *et al*., 2015) and wheat (Wang *et al*., 2014).

Application in RSA traits, for instance, involved studying mutation of soybean root hair of *GmFEI2* and *GmSHR*, with mutation rate detection of between 10-93.3%, with similar efficiency of exogenous transformation by using a relatively more difficult *A. tumefaciens*-mediated transformation method (Cai *et al*., 2015). However, improved crops through genetic editing tools such as CRISPR/Cas system may pose new challenges and social acceptance (Araki and Ishii, 2015; Ishii and Araki, 2016), although these do not involve any foreign DNA sources, non-random mutation process as opposed to genetically modified (GM) crops. Genetic editing provides opportunities to produce improved plants with transgene free genetically edited plants (Khatodia *et al*. 2016).

1.5 OVERVIEW OF ROOT PHENOTYPING PLATFORMS

For effective and efficient crop improvement, genotyping technologies, which have contributed massively to increased genomic data, demands parallel improvement in phenotyping technologies. Lack of high throughput phenotyping platforms and therefore lack of phenotypic data

to compliment genomic data is a major bottleneck in terms of progression in either plant breeding or understanding of the fundamental science itself (Le Marie *et al*., 2014; Kuijken *et al*., 2015).

Various root phenotyping platforms have been developed, with options ranging from field root phenotyping to controlled environment, lab and glasshouses phenotyping capabilities (Zhu *et al*., 2011; Judd *et al*., 2015). The advancement of root phenotyping platforms has seen a better throughput data in which more traits, individuals and treatments can be studied at the same time, through utilisation of various cultivation systems, enhancement of imaging platforms and data acquisition approaches (Kuijken *et al*., 2015).

The usefulness of downstream phenotyping methods depends on the method of cultivating the plants before any imaging and data acquisition (Paez-Garcia *et al*., 2015). There are many cultivation approaches for root phenotyping which can be broadly divided into soil-based medium and alternative non soil-based medium, which depends on the desired root evaluation outcomes. Cultivating plants in soil-based culture medium has always been a preferred method, as this mimics the environmental complexities encountered by plants when grown in the field. Observation and analysis of roots from plants grown in the fields involve excavation of the soil around the mature roots followed by manual measurements such as shovelomics (Trachsel *et al*., 2011; Colombi *et al*., 2015).

However, such studies are labour- and time-intensive with deteriorating RSA information due to loss of fine roots. The challenges may be solved by growing plants under a lab- or glasshouse-based environment such as soil-filled containers and rhizotrons (Kerbiriou *et al*., 2013; Yuan *et al*., 2016) and also flat cartridges (Nagel *et al*., 2012). However, soil heterogeneity in term of soil structure and composition serves as environmental noise, complicating the identification of actual responses of RSA to specific condition in soil due to strong genetic and environmental interactions (Kuijken *et al*., 2015; Paez-Garcia *et al*., 2015).

There are several alternatives that reduce the difficulties and issues surrounding the soil-based cultivation system. These involved the usage of non-soil, artificial medium providing a more controlled and standardised environment (Kuijken *et al*., 2015). These systems often allow higher throughput output and flexibility of imaging options such as flatbed scanners (Shi *et al*., 2012; Gruber *et al*., 2013; Adu *et al*., 2014) and digital single-lens reflex (DSLR) camera (Atkinson *et al*., 2015; Thomas *et al*., 2016). Most commonly used method is on agar-based petri dishes (Gruber *et al*., 2013; Shi *et al*., 2013). Agar-based petri dishes system is highly controllable lab-based approach, providing advantages for evaluation of root growth in real time, high-throughput data output from a single experiment, ability to create a repeatable condition, small space for large number of samples, easy handling and cleaner roots for imaging (Paez-Garcia *et al*., 2015). It also gives the ability to control precisely the

amount of nutrients being used for differential nutrient availability assessment (such as Shi *et al*., 2013 and Gruber *et al*., 2013) in contrast to soil-based method. However, high sucrose content and root illumination may create unnecessary artefact responses, which may alter true representation of root responses in comparison to natural environments (Karthikeyan *et al*., 2007 and Xu *et al*., 2013). Improved agar-plate system using dark petri dishes and in the absent of sucrose have been reported (Xu *et al*., 2013). However, these dark petri dishes are not easily available.

Germination paper is also widely used in root phenotyping and serves as an alternative to agar-based system. The usage of germination paper instead of agar petri dishes reduces the need of aseptic conditions in preparing the setup and for growth condition and more replicates can be sown on a limited space. Growth pouch system is one example of paper-based application, with setup involving the sandwich of seeds in between blotting paper and polythene film (Hund *et al*., 2009; Atkinson *et al*., 2015; Thomas *et al*., 2016). Utilising growth pouch system also allows root phenotyping with bigger root system for example wheat (Atkinson *et al*., 2015) and barley (Rattanapichai and Klem, 2016), which are difficult to grow in smaller vessel with artificial culture medium such as agar petri dishes. Seedling root traits may not always represent later stages of root phenotypes in a specific condition (Watt *et al*., 2013), however, study by Thomas *et al*. (2016) which utilises the paper-based growth pouch system has proven otherwise. The development of rhizoslides, an optimised form

of growth pouch system (i.e. growth-between-papers system), enables better observation of post-embryonic root system such as crown roots (Le Marie *et al.*, 2014). Rhizoslides were also used in split-nutrient root phenotyping, a similar concept being used in split pot method (Zandt *et al*., 2015).

Despite benefits and advantages of using root phenotyping approaches as previously mentioned, the root growth may be forced to unnatural 2-dimensional plane perspective as opposed to natural 3-dimensional root attributes found in the soil environment (Kuijken *et al*., 2015). Non-destructive, 3D root phenotyping of RSA within soil environment is a superior choice in this case, of which support growth for many developmental stages in soil with relevance to agronomic traits. Most reviewed platform would be the application of x-ray computed tomography (CT) technology in root phenotyping (Mooney *et al*., 2012; Kuijken *et al*., 2015). X-ray CT technology has been extensively used in medical field to view 3D cross-sectional internal anatomy model for diagnosis and monitoring, based on reconstruction of slices of 2D images, captured from different angles. The principle used in x-ray CT of root phenotyping is the same with the one used in medical field, in which images of the roots are composed as a result of attenuation differences of the rhizosphere. Lower resolution of the system may however impede the full *in silico* reconstruction of the roots 3D model due to the lost of information from the scanning session (Mairhofer *et al*., 2013), although

this may be improved through time with better procedures and equipments that support higher resolution (Mooney *et al*., 2012). This downside has lead to the needs of revalidation of the RSA through conventional 2D imaging method (Tracy *et al*., 2012), impeding the full potential of precise 3D phenotyping *in situ*, non-disturbed root growth of the plants. Additionally, the systems are immobile and expensive, often need long scanning time and also requiring specialist on site for operation and maintenance.

3D root growth in a container filled with non-soil medium such as phytagel and gellan gum, for instance, may provide advantages of studying 3D root traits under a more controllable condition (Fang *et al*., 2009; Clark *et al*., 2011; Topp *et al*., 2013). This however, also inherits certain practicality disadvantages of agar petri dishes method (i.e. the need of sterile condition and method, tedious preparation of gel, gel pouring, storage etc.). Alternative cultivation method would be hydroponics as this is more cost-effective and easier to setup. With the advent of 3D printing, recent study has reported the development of custom made mesh system which provides mechanical support and growth environment for the roots which absent in the hydroponics system, tested on several species which include rice, sorghum and maize (Piñeros *et al*., 2016). The mesh system is versatile as it is also compatible to solid medium such as Turface, widely commercialised solid-like, granular, unsaturated medium, which can be easily cleaned before imaging.

Many software packages for root imaging and quantitative data analysis have been developed in the past few years, offering enormous options of analysis in either 2D or 3D, and control of whether manual or more automated. However, manual correction is still needed (Kuijken *et al*., 2015), especially in a more automated analysis due to errors in detecting correct parts of the roots. Examples of softwares include SmartRoot (Lobet *et al*., 2011), RooTrak (Mairhofer *et al*., 2012b) and RootTrace (French *et al*., 2009), however, detailed discussion of each software is out of the review scope and readers are redirected to online sources (for example, www. plant-images-analysis.org; Lobet *et al*., 2013) for further information.

1.6 CONCLUDING REMARKS AND PERSPECTIVE

Over the course of history, there has been an enormous enhancement in crop productivity, which has been based on the successful selection of specific traits for crop breeding and improvement. The early domestication and breeding programmes have mostly been targeting the above-ground organs that have direct impact on economic value, consequently neglecting the improvement of roots due to the hidden nature of the roots and technical issues involved in studying RSA. The root research field has now appreciated the importance of RSA characterisation and selection, with much evidence linking to the improvement of overall crop productivity and yield. Nitrogen and

phosphorus, for example, are two most important minerals needed by the plants, however these are deficient in most soil. Diversification of allelic sources especially those from crop wild progenitors and also ones from underutilised plants is important in improving the crops, especially the RSA, as these allow for a wider exploitation of better nutrient and water adaptation strategies.

Climate change, increasing world population and needs for delivering food security demands for radical changes for sustainable agricultural production (especially with low input framework). This is also parallel with the second Green Revolution wave (i.e. producing high-yielding cultivars that can withstand challenging conditions such as drought and nutrient deficiencies) and the idea of developing a super-domesticate (Vaughan *et al*., 2007), while exploiting the plasticity nature of RSA in adapting to the environment it is exposed to (Paez-Garcia *et al*., 2015). Breeding for deeper root system, desirably together with enhanced nutrient and water uptake at reduced metabolic cost is more preferable and advantageous than shallow root system, as it can fit in many agricultural systems worldwide and increases the interception of both topsoil and subsoil edaphic resources. This also include the deployment of genotypic and phenotypic platforms, as valuable tools for selection in breeding and improvement programmes and to allow for the identification of root traits for optimal acquisition of resources.

1.7 RESEARCH MOTIVATION AND JUSTIFICATION

Roots are vital for the plants, as a sessile organism which cannot move around freely. It is important for nutrient and water uptake from the soil, acts as food storage especially in tubers and rhizobium crops, provide mechanical support and site of interaction between soil rhizosphere and plants. Root system architecture (RSA) improvement seems neglected especially by the breeders, possibly due to the hidden nature of it beneath the ground and invisible direct impact on economic value. Plus, the research on roots is tedious, time- and labour-intensive, impeding the effort of RSA improvement and over-concentration on above-ground phenotypes. However, it is believed that root study is important as a way to tackle climate change, improve plant adaptation to abiotic and biotic stresses, improve crop performance and increase food security.

The thesis focussed the study of root responses towards different phosphorus levels. Phosphorus is key component for adenosine triphosphate (ATP), which is vital for plant energy sources, and also key component for nucleic acids and phospholipids. However, it is immobile in soil, reducing the availability towards plants. Adapted RSA traits are vital for efficient P uptake in order to reach these immobile P ions, therefore, chosen to be studied in this thesis. Briefly, the plants will follow a collective adaptation root traits called topsoil foraging strategy, in which primary root is inhibited, increased in lateral root formation and in some

species such as white lupin (*Lupinus albus*), cluster or proteiod roots can be seen (Lynch and Brown, 2001; Williamson *et al*., 2001, Nibau *et al*., 2008; Péret *et al*., 2014; Rogers and Benfey, 2015). At higher P levels, reverse phenotypic responses were expected.

Furthermore, the thesis has chosen lettuce as the main plant material to be used throughout the project. Lettuce is a popular leafy vegetable crops, commonly consumed for its health benefits and contains a range of beneficial secondary plant metabolites, including, phenolics, ascorbate, α-tocopherol, lignans, as well as sesquiterpene lactones (García-Macías et al., 2007; Oh, Trick, & Rajashekar, 2009). Lettuce (*Lactuca sativa* L.) is an annual plant in the Compositae (Asteraceae) family under the section *Lactuca* (de Vries, 1997). It is a diploid with 2n=2x=18 chromosomes. Domesticated lettuce *L. sativa* was likely domesticated from its wild progenitor, *L. serriola* (Kesseli *et al*., 1991; de Vries, 1997), in which genomes consist of potential source of disease resistance genes (Maisonneuve *et al*., 1994) and also uncharacterised phenotypic QTLs (Johnson *et al*., 2000). These genotypes have no genetic barrier for gene flow and can be readily crossed (Lindqvist, 1960). Effect of domestication of lettuce is widely known for its variety of shape and colour of the leaves, distinct to its wild progenitor, together with decrease latex content and bitter taste, loss of prickles of stem and leaf, absence of early bolting, increase in seed size and head-formation and non-shattering seed properties (de Vries, 1997; Ryder and Whitaker, 1976).

Previous studies by Jackson (1995), have shown that wild lettuce and domesticated lettuce have very different root architecture. Wild lettuce has a relatively deeper root system with significantly longer primary roots than a domesticated lettuce of which inadvertently evolved to have a more shallower root system with more laterals at the topsoil horizon, with insignificant biomass differences between these two root systems under normal soil condition. These differences in soil exploration may incur issues in agricultural practices and multiple nutrients and water acquisition RSA traits tradeoff especially in low input agricultural systems. Less issues might be observed in crops grown under high input system as required nutrient and water can be easily captured by plants with shallow root system as in domesticated lettuce, because the nutrients are mostly applied and concentrated at the topsoil region.

The behaviour of lettuce roots in response to nutrient acquisition, especially P, has not been studied in great detail. Although the shallow rooting of domesticated lettuce RSA seems adapted to low P condition, this has not been properly characterised, especially in a wider spectrum of P levels. This also applies to wild lettuce RSA, which may show different strategies in response to different levels of P. Understanding the responses of the two lettuce species to different P levels would provide some insight on the impact of domestication on root plasticity towards phosphorus. In the event of higher P levels, as can be mimicked by topsoil environment in high input agriculture system, it is hypothesised

that topsoil foraging strategy is reduced, allocating biomass to roots for deeper soil exploration. This is important to capture deeper soil resources such as leached nitrogen and water for increased robustness in the plant. In the event of lower P level, it is unclear as to whether wild lettuce adopt topsoil foraging strategy, reducing the rooting depth, which would affect branching of the lateral roots. Lesson from the characterised RSA traits may be useful for improving modern lettuce or other plants, grown in challenging environment.

The root traits being investigated were divided into three main categories namely, global root parameters, primary root parameters and lateral root parameters. Global root parameters includes total root length (TRL), total root surface area (SA), average root diameter (AD) and total root volume (RV). These were calculated manually using primary and lateral data and further explained in **Section 2.6.** Primary root parameters includes primary root length (PRL), primary root surface area (PSA), primary root volume (PV), and primary root diameter (PD). Lateral root parameters on the other hand comprises of lateral root length, (LRL), lateral surface area (LSA), lateral root volume (LV), lateral root diameter (LD), lateral root number (LRN) and lateral root density (LRD). The image of lettuce roots with labels of root traits being investigated in Figure 1.2.

The species used in this research serve as model species for a wide range of crop species under Crops For the Future (CFF). Most of the

underutilised crops are still undergoing improvement, therefore, this research should guide researchers to target suitable root traits for sustainable agricultural systems. Improvement of underutilised crops is important to complement and to reduce over-reliance on major crops to ensure food security for ever increasing world population, and to provide and preserve cultural and dietary diversity especially to local indigenous people and poor farmers (Mayes *et al*., 2011).

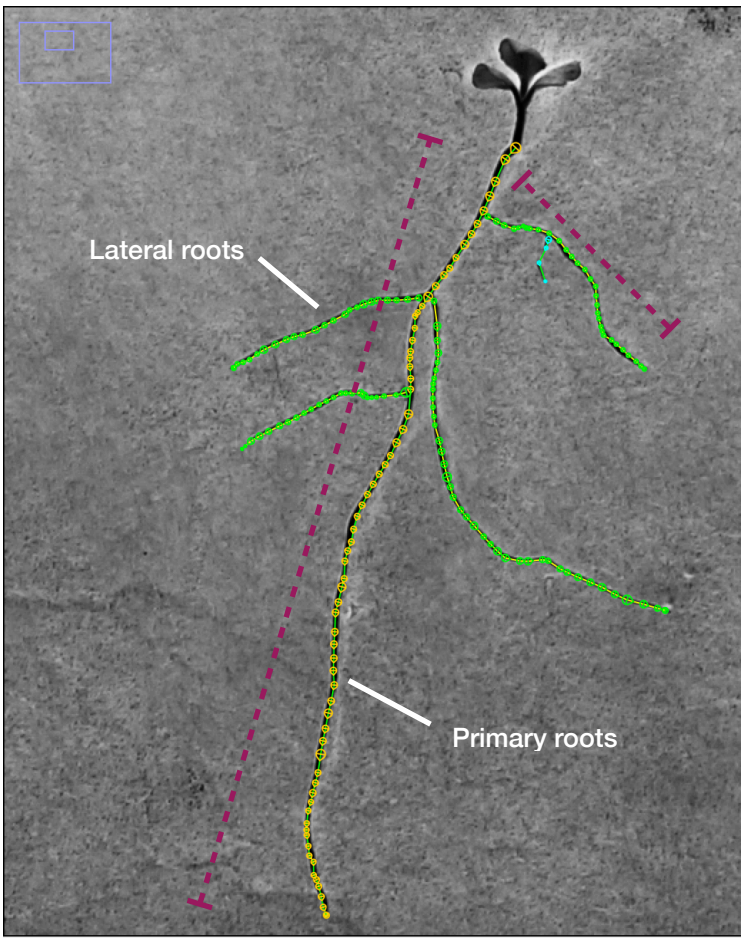


Figure 1.2 Main traits being examined in the project. Green and blue lines represent lateral roots and yellow line represents primary roots. The software automatically generated length, surface area and volume data of lateral and primary roots respectively. Purple line is estimated root length, with software will precisely measure the pixels being segmented. Surface area and volume were estimated based on algorithms related to the width of the segmented roots. Lettuce shown was at 14 days after subculture.

1.8 PROBLEM STATEMENT

This project identified and quantified traits in wild and domesticated lettuce that adapted towards different phosphorus levels using different root phenotyping platforms, and recommending traits that should be maintained or selected as crops (especially underutilised crops) are improved through breeding programme.

1.9 GENERAL AIMS

a. Understand the root physiology, root phenomics and phosphorus demand in wild and domesticated lettuce.
b. Explore root phenotyping platforms to study the root traits shown by lettuce following treatments of phosphorus levels.
c. Identify and quantify quantitative trait locus (QTL) that is related to the difference in lettuce root traits.
d. Trial on quantifying root structure using X-ray microcomputed tomography (μCT).

1.10 GENERAL OBJECTIVES

a. Quantifying and analysing root traits using agar- and paper-based phenotyping methods.

b. High-throughput data collection through scanner- and camera-based imaging systems and trait measurements *in silico*.
c. Performing QTL analysis on lettuce mapping population under paper-based pouches environment, with QTL comparison using dense and framework maps.

1.11 APPROACHES USED

This research attempted to explore different techniques, especially high throughput approaches to study RSA and to make comparison between the techniques and to identify the advantages and disadvantages of the techniques in studying root traits. Two approaches were used to screen for RSA. The agar-based media and paper-based growth pouch. Plants were then subjected to 2D imaging which utilise flatbed scanner and DSLR camera and *in silico* root traits segmentation and measurements using SmartRoot platform (Lobet *et al*., 2011). Preliminary studies utilised 3D images of lettuce roots generated by X-ray micro-computed tomography (uCT), performed in the state-of-the-art Hounsfield Facilities located in Sutton Bonington Campus, University of Nottingham, UK. Quantitative trait loci (QTL) analysis was done to access the region of genome that contribute to the responses of root RSA to different phosphorus levels following a mapping population screening using paper-based growth pouch system.

1.12 THESIS OVERVIEW

Chapter 1 presents a detailed review of literature on RSA and highlights progress made on molecular tools in crop improvements and root phenotyping platforms. In Chapter 2, General Materials and Methods are presented, which cover the common materials (i.e. the parental genotypes and also the mapping population) and general methods used throughout the study. This is followed by Chapter 3, in which lettuce root system architecture is explored in agar-based system under different phosphorus levels. The chapter presents the specific methods used in preparing the petri dishes with different P treatments, and also results and discussion of the studied traits. In Chapter 4, paper-based phenotyping approach is used to study lettuce root system architecture grown under different phosphorus levels. Chapter 5 presents the analysis of quantitative trait locus (QTL) lettuce root architecture using data generated from the mapping population, domesticated (*Lactuca sativa* cv. Salinas) × wild (*Lactuca serriola* acc. UC96US23) lettuce recombinant inbred lines (RIL). The thesis ends with some general discussions and also explores wider perspectives (Chapter 6) in assessing roots in 3-dimensions (3D).

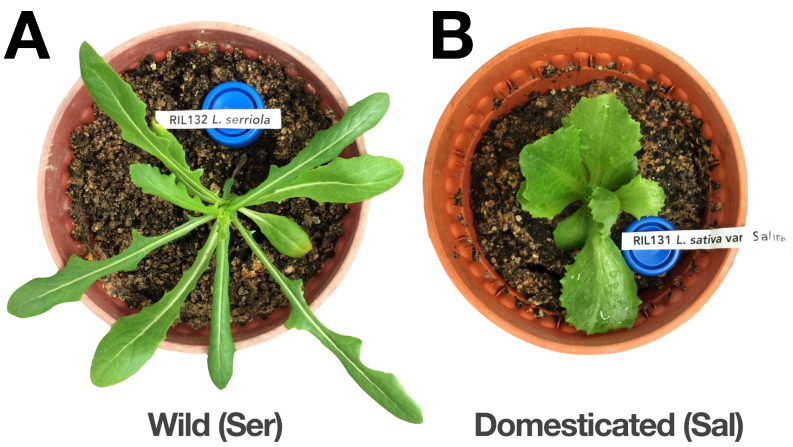
CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 PLANT MATERIALS

2.1.1 Lettuce parental genotypes

Lettuce parental genotypes used in this study are iceberg type domesticated lettuce, *Lactuca sativa* cv. Salinas, and its wild progenitor *Lactuca serriola* acc. UC96US23 (Zhang *et al*., 2007). Both genotypes differ greatly in their characteristics, both shoots and roots (Johnson *et al*., 2000) (examples showed in Figure 2.1A-D). In term of root architecture, *L. sativa* has been described to have a shallower root system than its wild relative, *L. serriola* which has a deeper root system (Jackson, 1995) (example as shown in Figure 2.1E-F).



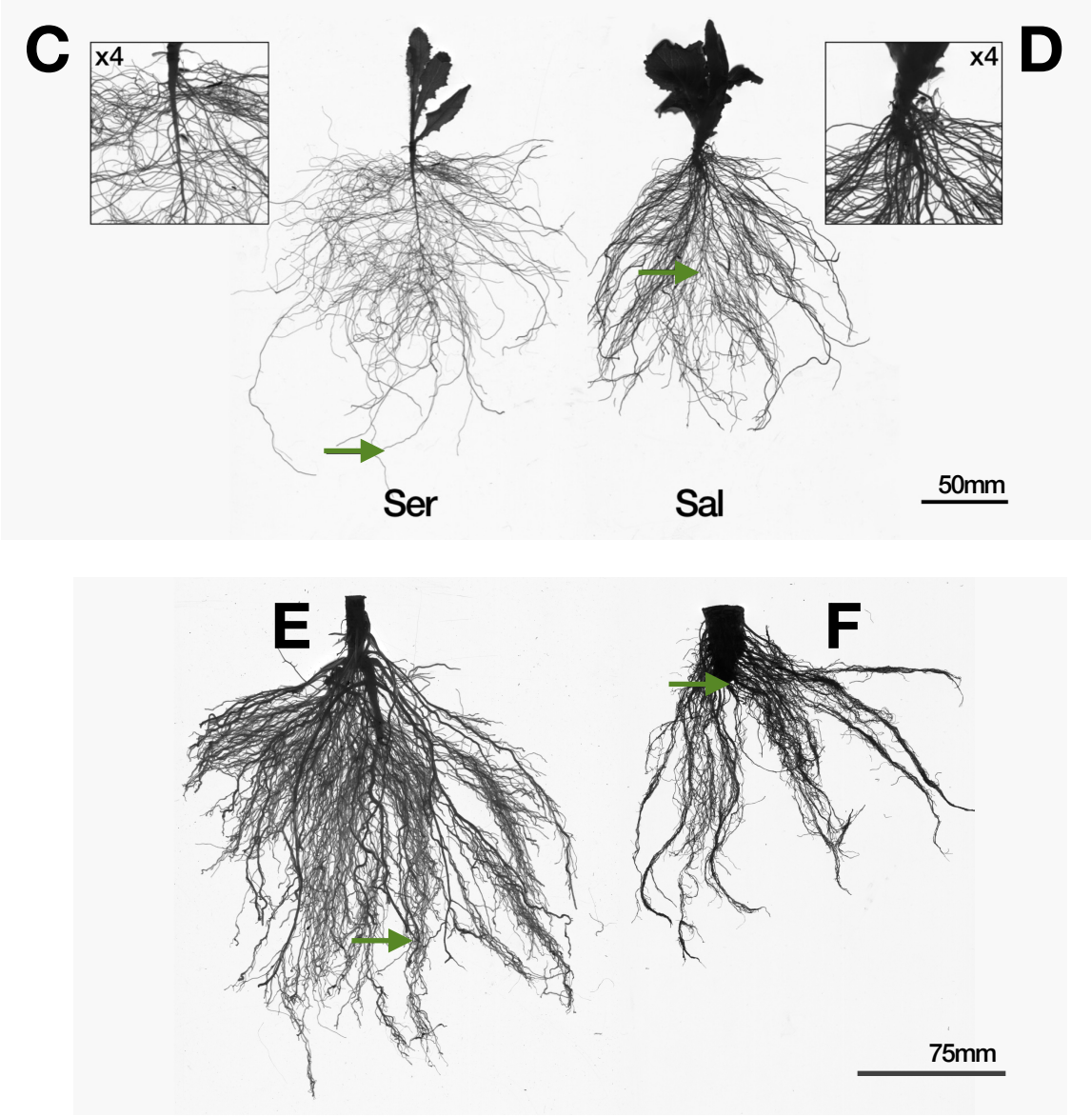


Figure 2.1 Comparison of lettuce parental phenotypes. A and B are the examples of above soil differences phenotypes. C and D are the scanned images of the root systems at 8 weeks. Arrows showed the estimated tip end of primary root. Boxes represents the lateral density near primary root base and the thickness of the base roots, enlarged four times. E and F are the root images of matured lettuce parental genotypes. Scale bar for C and D=50mm. Scale bar for E and F=75mm.

2.1.2 Lettuce mapping population

91 lines out of 130 F_8 recombinant inbred lines (RIL) mapping population which were generated from an interspecific cross between iceberg cultivar of *Lactuca sativa* cv. Salinas × *Lactuca serriola* acc. UC96US23 were used. Both parental and RIL seeds were propagated under controlled environment and obtained from A. L. Tozer Ltd., Surrey, United Kingdom.

2.2 GERMINATION ASSAY

A germination assay consisted of a 35mm diameter filter paper lined in a 35 mm petri dish. The filter paper was moistened with sterile deionised water (SDW) to facilitate the germination process.

2.3 SEED SURFACE STERILISATION

The seed surface sterilisation process used in the experiments was optimised from the method described by Webb (1992) and Di Salvatore *et al*. (2008) due to an observation of slow germination which affected the growth of germinated seedlings. The seeds were sterilised in 10% (v/v) fresh sodium hypochlorite (NaOCl) solution for 10 minutes with occasional shaking in between. The seeds were then rinsed thrice with sterile deionised water (SDW).

2.4 SEED STRATIFICATION

To improve seed germination rate and synchronisation, the sterilised seeds were cold treated at 8-12ºC for three days.

2.5 GENERAL PLANT GROWTH CONDITIONS

The seeds were grown in a controlled environment growth room with constant temperature of 24ºC (±2ºC), 16-h photoperiod and lighting provided by fluorescent tube lamps at an average light intensity of 135 µmol photons m^{-2} s^{-1}. All seed was grown for 14 days after being transferred to the treatment medium (i.e. agar-filled petri dishes and paper-based pouches) before being imaged.

2.6 ROOT SEGMENTATION AND TRAITS' MEASUREMENTS

Images of roots were loaded into ImageJ (Abramoff *et al*., 2004) software for root segmentation to reconstruct 2-dimensional root images and these were measured using SmartRoot (SR) plugin (Lobet *et al*., 2011). RSA segmentation of each individual started by tracing the primary root (PR) using 'Trace' function and then followed by automatic selection of lateral roots (LR) using 'Find laterals' function. Any missed or extra laterals on the traces were visually examined afterwards and were fixed manually using 'Append node' function (Figure 2.2). From these root tracings, SR

automatically measures PR and LR length, diameter, surface area and volume and also LR number. Measurements from SR plugin were exported, merged and arranged to a single spreadsheet.

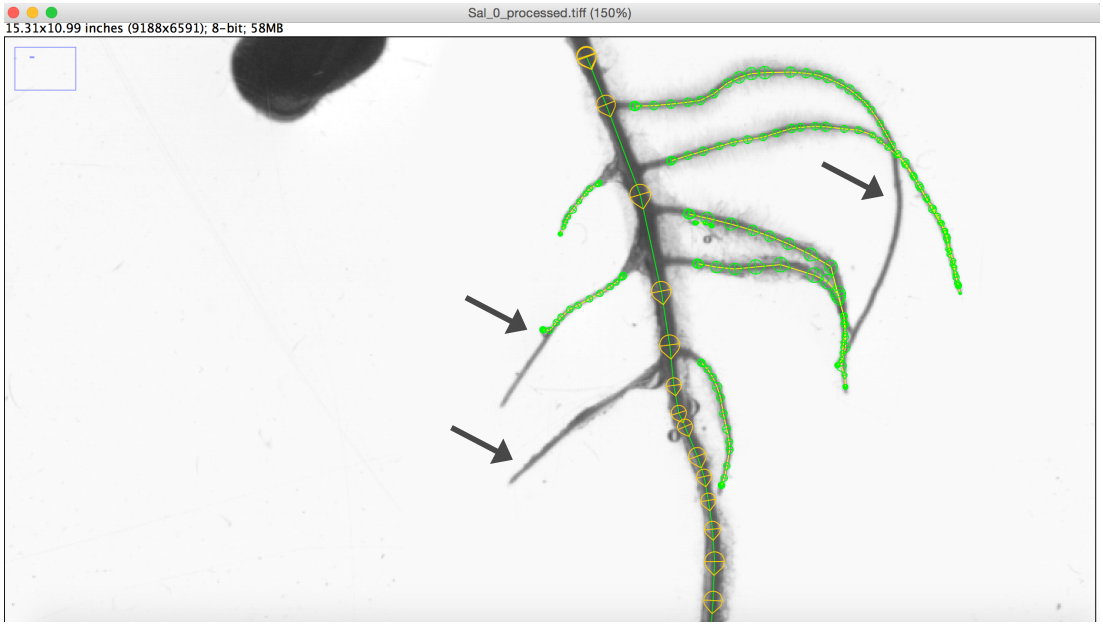


Figure 2.2 Example of traced roots in SmartRoot plugin in ImageJ. Line represented by yellow nodes was a primary root and lines with green nodes were lateral roots. Arrows showed missed laterals by automatic selection of laterals by using 'Find laterals' function and manually fixed using 'Append node' function.

Further traits including total root length (TRL), average root diameter (AD), total surface area (SA) and total root volume (RV) and lateral root density (LRD) were calculated using Microsoft Excel (version 2015) as follow:

$$TRL = \sum PRL + \sum LRL \quad (i)$$

where TRL is total root length, PRL is primary root length and LRL is lateral root length.

$$AD = x_{(\sum PD, \sum LD)} \quad (ii)$$

where AD is average root diameter, x is average, PD is primary root diameter and LD is lateral root diameter.

$$SA = \sum PSA + \sum LSA \quad (iii)$$

where SA is total surface area, PSA is primary root surface area and LSA is lateral root surface area.

$$RV = \sum PV + \sum LV \quad (iv)$$

where RV is total root volume, PV is primary root volume and LV is lateral root volume.

$$LRD = LRN/PRL \quad (v)$$

where LRD is lateral root density, LRN is lateral root number and TRL is primary root length (i).

2.7 SEED QUALITY DETERMINATION

In this experiment, two tests were done, namely germination and 2,3,5-triphenyl tetrazolium chloride (TZ; Sigma-Aldrich) assay of seed viability tests were done. Differences of percentage obtained from this two tests resulted in percentage dormancy in the seed genotype.

The germination test indicates the potential of seed lots to emerge. The test in this study involved germinating 10 seeds per line with missing data. The seeds were germinated on filter paper wetted with 1ml of sterile deionised water (SDW) in each 35mm round petri dish for 5 days in a controlled environment growth room at a continuous 24ºC, 16-hour photoperiod daylight and 135 μmol photons m^{-2} s^{-1}. The seeds were first stratified for three days by keeping at a temperature between 8-12ºC prior to transfer to a growth room. After 5 days in the growth room, the number of seeds that had germinated (i.e. seeds with visible emerged radicle from micropylar endosperm region) was recorded and converted to a percentage. This was recorded as the seed germination percentage (SG%).

Briefly, the TZ assay of seed viability involved staining of seeds with TZ solution which indicates the presence of viable seed activity. The staining is due to the reduction of colourless TZ into red coloured, non-diffusable formazan, in a reaction that occurs in all respiring living tissues (Patil and Dadlani, 2009). Prior to TZ staining, seed pre-conditioning was done to allow complete hydration of seed tissue; prevent damage to cotyledons and embryos axes while cutting seeds; initiate and activate the seeds' germination and to maximise the staining of the seeds (Patil and Dadlani, 2009). Based on the method described for lettuce by Patil and Dadlani (2009), the selected lettuce seeds were moistened for 18 hours on wet

(SDW) filter paper in a 35mm petri dish, at room temperature. Subsequently, the seeds were cut longitudinally through midsection of distal end (Figure 2.3).

Figure 2.3 Seeds being cut longitudinally through midsection of distal end before being covered with 1% (w/v) TZ buffer solution.

1.5ml of 1% (w/v) TZ in phosphate buffer solution at pH 7 was used to cover the seeds and they were left to stain for 24 hours at room temperature. The phosphate buffer solution consists of 40% (v/v) 66.7 mM monopotassium phosphate (KH_2PO_4) solution and 60% (v/v) 83.7 mM disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_20$) solution. The buffer solution is important to maintain the neutral pH of the solution as

proper staining occurs only at neutral pH, manifesting seed viability (Patil and Dadlani, 2009). The seeds were then observed under a stereoscopic zoom microscope (SMZ1500, Nikon Malaysia Sdn. Bhd., Malaysia) to look for bright red staining in the seeds tissues. The bright red stain means the seed is viable for germination, little or very faded red stain means the seeds is not viable for germination anymore. The number of red stained seeds were recorded and converted to a percentage (seed viability percentage, SV%).

CHAPTER 3

AGAR-BASED PHENOTYPING APPROACH FOR LETTUCE ROOT SYSTEM ARCHITECTURE UNDER DIFFERENT PHOSPHORUS LEVELS

3.1 INTRODUCTION

Sessile lifestyle of plants requires responsive interaction with the environment. One way to achieve this is by having a dynamic root system architecture (RSA) in order to capture the essential requirements from the soil, especially under limiting nutrients and water conditions. Phosphorus (P) especially in the form of inorganic phosphate (P_i) in soil, is among the essential macronutrient needed by plants, for growth and development and also as key component of nucleic acids and phospholipids (Péret *et al*., 2014). P has low mobility in the soil, therefore intrinsic adaptive responses is essential to capture as much P as possible, especially during P deficiency events.

The plants grown under limiting P conditions develop shallow roots as a strategy to maximise topsoil foraging as P is more concentrated in the topsoil. Shallow root system means more lateral root (LR) formation with reduction in primary root (PR) growth. Under low P condition LR formation is induced by auxin-related and P perception pathway genes. This include *TRANSPORT INHIBITOR RESPONSE1* (*TIR1*; Pérez-Torres *et al*., 2008), *PDR2* (Ticconi *et al*., 2004) and *PHT1;9* (Remy *et al*., 2012). The whole

mechanism of reduced PR growth under low P is not fully understood (Péret *et al*., 2014). Only a few candidate genes have been identified which include *LOW PHOSPHATE ROOT* (*LPR*; Svistoonoff *et al*., 2007) and *ALTERED PHOSPHATE STARVATION RESPONSE1* (*ASPR1*; González-Mendoza *et al*., 2013). Shallow root system is however a tradeoff for efficient nitrogen (N) and water acquisition in the soil, as these are more concentrated at depths which require a deeper root system, which may jeopardise overall crop yield.

Domesticated lettuce, *Lactuca sativa* cv. Salinas, has a shallower root system than its wild predecessor, *Lactuca serriola* (Jackson, 1995) which is a perfect trait for capturing nutrients present in the topsoil region. On the other hand, a deeper root system possessed by the wild lettuce has a better adaptation to acquire nutrient and water from the soil because the ability to forage different horizons of the soil. Heavy P fertilisation in domesticated lettuce fields has proven not to significantly increase yield production (Johnstone *et al*., 2005) suggesting limited uptake and utilisation of yield-related nutrients especially N and water.

In this chapter, analysis of RSA of wild and domesticated lettuce was done to understand the impact of wild-domesticated intrinsic genetic differences and the effect of differential P levels on the RSA. The knowledge may be beneficial for other plants especially underutilised crops, which may still be packed with useful innate genes conferring

adaptive root traits for efficient uptake by nutrients and water. As farming shifts towards a more sustainable approach with less dependence on the external inputs and optimising utilisation of variable resource availability, deeper root system of wild lettuce and other species is a more favourable trait for exploitation.

In this experiment, agar-based screening method is used to study RSA in the lettuce genotypes under different P levels in a typical laboratory RSA screening conditions. Conventionally, agar-based method is usually employed to screen RSA as it is a highly controlled laboratory method. This technique provides advantages for evaluation of root growth in real time, high-throughput data output from a single experiment, ability to create a repeatable condition, small space for large number of samples, easy handling and cleaner roots for imaging (Paez-Garcia *et al*., 2015). It also allows for a precise control of the amount of nutrients being used for differential nutrient assessment (such as Shi *et al*., 2013 and Gruber *et al*., 2013) in contrast to soil-based method.

3.2 MATERIALS AND METHODS

3.2.1 Plant materials

Lettuce parental genotypes of *Lactuca serriola* acc. UC96US23 (wild lettuce; Ser) and *Lactuca sativa* cv. Salinas (domesticated lettuce; Sal) were used in this experiment. More information is provided in **Section 2.1.1.**

3.2.2 Experimental design

A randomised complete block design (RCBD) was used in the experiment. In a block, both lettuce parental genotypes were subjected to five different phosphorus (P) treatments (i.e. 0, 6, 312, 625 and 1250 µM). There were four independent blocks, with four biological replicates of each parental line for every P concentration in a block. Each block was grown at a different time scale, with petri dishes distributed randomly in a controlled environment growth room, located at the Biotechnology Research Centre (BRC), University of Nottingham Malaysia Campus.

3.2.3 Plant growth condition

The seeds were grown under sterile conditions at all times. The seeds were first sterilised as described in **Section 2.3**. In this experiment, assessment of seeds was done in two steps, one at seed germination stage and another at the end of growth stage, in order to maximise the number of individuals available for analysis. Germination screening involved germinating twenty sterilised seeds on germination assay (preparation described in **Section 2.2)** and randomly choosing the best four seedlings to be subcultured in the treatment assay.

The chosen seedlings were subcultured onto treatment assay which contain half strength (0.5×) Murashige and Skoog (MS) nutrient media (1962) with modified monopotassium phosphate (KH_2PO_4) concentrations of either 0, 6, 312, 625 and 1250 µM (Shi *et al.*, 2013). For the lettuce seeds grown under lower KH_2PO_4 levels, KCl was used to balance the K^+ ion. The pH of the media was adjusted to pH 5.8 using 1 M sodium hydroxide (NaOH) before adding the Agargel™ (Sigma-Aldrich) powder. The media was autoclaved at 121°C and 15 psi for 20 minutes. 80ml of media was dispensed to its respective vented labelled square polystyrene tray (120×120×70mm; Greiner Bio-one GmBH, Frickenhausen, Germany) in a laminar flow cabinet. The media was allowed to solidify completely

and stored under tissue culture growth conditions. Components in the media are summarised in Table 3.1.

Seeds were sown 3 cm from the top edge of the tray. To ease the subculturing process, a laminated paper with traced position was put under the petri dishes. All plates were sealed with parafilm 'M' (Pechiney Plastic Packaging, Menasha, USA). The seedlings were transferred to the controlled environment growth room and grown according to conditions described in **Section 2.5.** All plates were positioned vertically to ensure downwards growth of the roots from the top of the agar.

Table 3.1 Type of stocks prepared and the components of media solutions used in the experiment. *This is recipe for a complete nutrition of half-strength (0.5x) MS nutrient media.

Components	0 µM P	6 µM P	312 µM P	625* µM P	1250 µM P
Volume (L)	1	1	1	1	1
Sucrose 3% (m/v; g)	30	30	30	30	30
Agargel™ (Sigma-Aldrich; g)	5	5	5	5	5
Macronutrients with P 10x (ml)	-	0.48	25	50	100
Macronutrients with KCl 10x (ml)	50	49.52	25	-	-
Micronutrients 100x (ml)	5	5	5	5	5
Iron stock 100x (ml)	5	5	5	5	5

3.2.4 Root imaging and image analysis

In this experiment, the RSA images were captured using a flatbed scanner (Epson Expression 11000XL, Epson, California, USA) at 800 dots per inch (dpi) resolution in grayscale. The roots were delicately positioned in a root-positioning tray (Regent Instrument, Québec, Canada) that is filled with water prior to image capturing. RSA were segmented and measured based on the description provided in **Section 2.6**. Before images were loaded into ImageJ (Abramoff *et al*., 2004) software, all raw photos were enhanced using Pixelmator (Version 3.2, Pixelmator, Lithuania) to reduce image artefacts for instance tray scratches and water bubbles as SmartRoot plugin may define these as lateral roots present in the images.

3.2.5 Data collection

Measured data from segmented images were collected and used in the analysis. The raw data included three main categories of root parameters tested in the present study, namely, primary root, lateral root and global root parameters. Primary root parameters includes primary root length (PRL), primary root surface area (PSA), primary root volume (PV), and primary root diameter (PD). Lateral root parameters on the other hand comprises of lateral root length, (LRL), lateral surface area (LSA), lateral root volume (LV), lateral root diameter (LD), lateral root number (LRN) and lateral root density (LRD). Global root parameters includes total root

length (TRL), total root surface area (SA), average root diameter (AD) and total root volume (RV). LRD and all global root parameters were calculated based on description in **Section 2.6.**

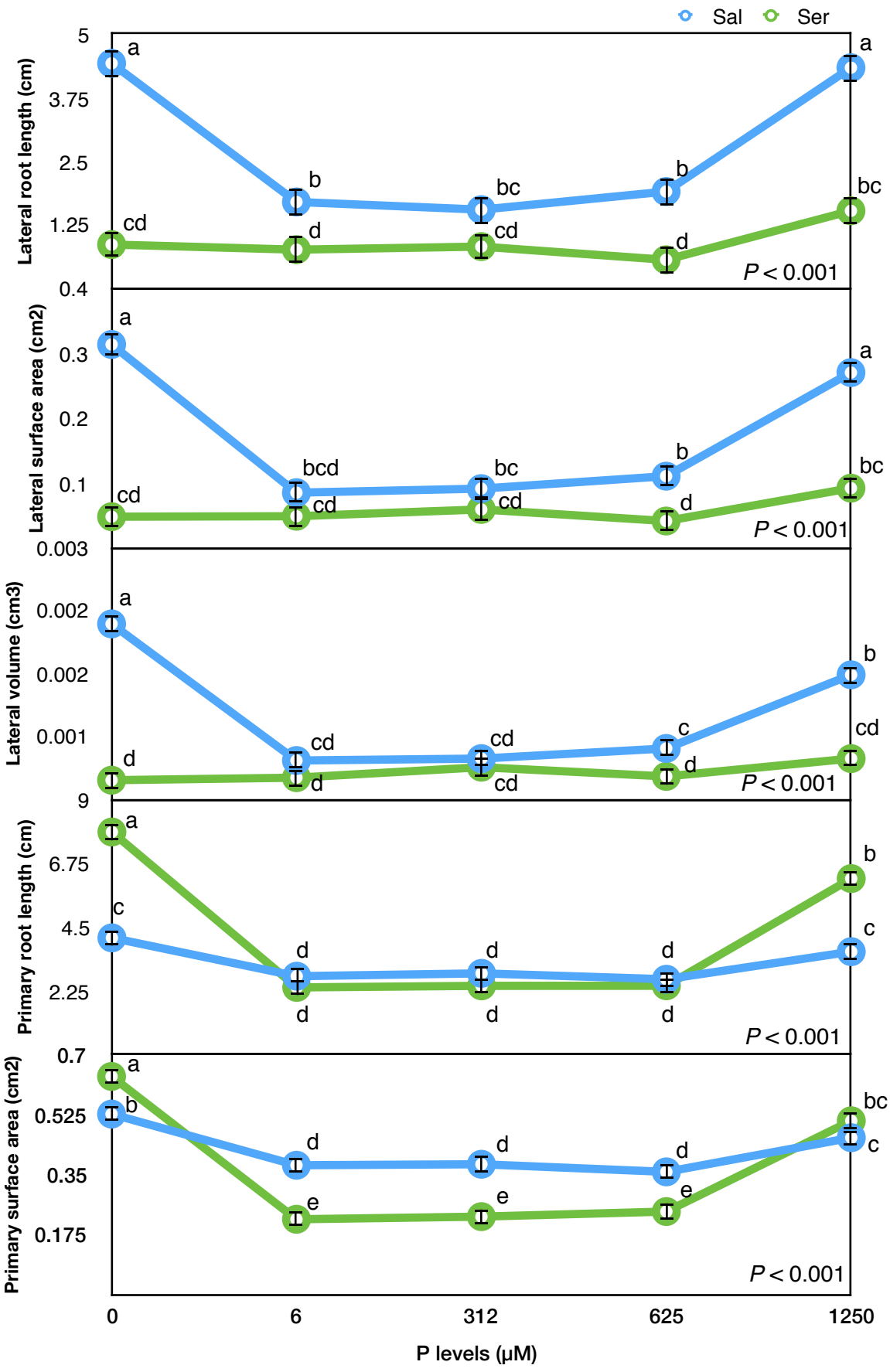
3.2.6 Statistical analysis

Raw data were entered into GenStat® (Release 17; VSN International, Oxford, UK). A two-way ANOVA tested for differences in the root parameters stated in **Section 3.2.5** in relation to lettuce parental genotypes grown under five different P levels. Fishers protected least significant difference (LSD) test was used as the multiple comparison test, set at 5%. Prior to the analysis, assumption of the normality of residuals and homogeneity of variances were tested using residual plot tools in GenStat®. All the analysis was presented according to minimal adequate model based on a top-down approach.

3.3 RESULTS

A total of 123 individuals were analysed in this experiment. Due to unavoidable circumstances, only 43 individuals of wild lettuce (*L. serriola* acc. UC96US23; Ser) were used for analysis as opposed to 80 individuals of domesticated lettuce (*L. sativa* cv. Salinas; Sal). There were issues related to Ser germination and therefore lack of Ser seedlings to be subcultured for treatment assay. An average of 8 Ser seedlings were used, with minimum of 6 and maximum 12 seeds per P treatment in the study.

Seven out of 14 root parameters showed significant interaction between lettuce parental genotypes × P levels. These include lateral root length (LRL) ($F_{(4,110)}$ = 11.42, P<0.001), lateral root surface area (LSA) ($F_{(4,110)}$ = 19.9, P<0.001), lateral root volume (LV) ($F_{(4,110)}$ = 27.53, P<0.001), lateral root diameter (LD) ($F_{(4,110)}$ = 2.82, P=0.029), primary root length (PRL) ($F_{(4,110)}$ = 28.01, P<0.001), primary root surface area (PSA) ($F_{(4,110)}$ = 15.91, P<0.001) and average root diameter (AD) ($F_{(4,110)}$ = 2.47, P=0.049) (Figure 3.1).


Sal
Ser
Lateral root length (cm)
5
3.75
2.5
1.25
0.4
a
b
bc
b
a
cd
d
cd
d
bc
P < 0.001
Lateral surface area (cm2)
0.3
0.2
0.1
0.003
a
bcd
bc
b
a
cd
cd
cd
d
bc
P < 0.001
Lateral volume (cm3)
0.002
0.002
0.001
9
a
cd
cd
c
b
d
d
cd
d
cd
P < 0.001
Primary root length (cm)
6.75
4.5
2.25
0.7
a
d
d
d
b
c
d
d
d
c
P < 0.001
Primary surface area (cm2)
0.525
0.35
0.175
a
b
d
d
d
bc
c
e
e
e
P < 0.001
0
6
312
625
1250
P levels (µM)

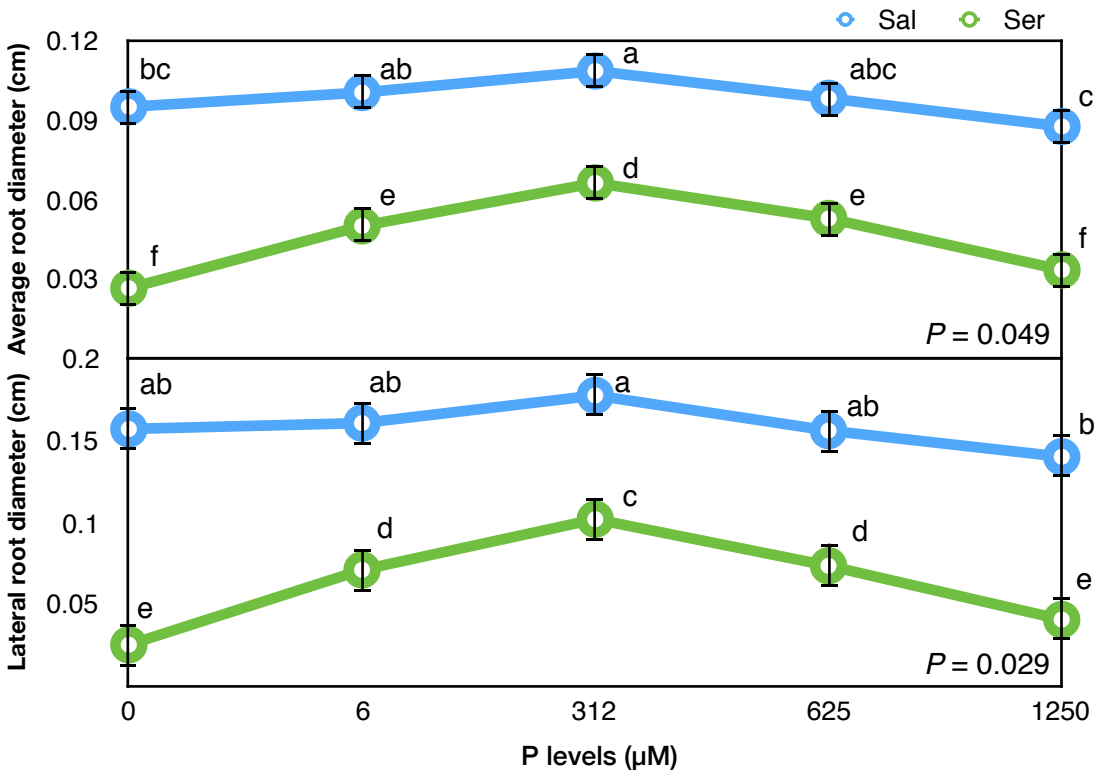


Figure 3.1 Means of phenotypes with significant interactions of lettuce parental genotypes × P levels. Different letters indicates significant different based on Fishers protected LSD at 5%. Sal is domesticated lettuce (while Ser is wild lettuce. Error bar = SED. n_{Sal}=80, n_{Ser}=43.

Multiple comparison tests revealed that these root parameters of both lettuce parental genotypes were significantly affected by the two extreme P treatments of 0 and 1250 µM P. In Sal, means of AD, LD, LRL, LSA and LV were significantly higher (P<0.001) than Ser at the two P extremes. In contrast, PRL and PSA were higher in Ser than that of Sal at the P extremes. A non-significant but consistent and similar growth pattern was observed between 6 and 625 µM P for these traits for the respective genotypes and phenotypes, however, significant differences (P<0.001) can be seen in term of mean values for AD, LD and PSA. Mean values for the range was significantly higher for Sal in AD and LD and vice versa for PSA.

Furthermore, the results that consider only P treatment (as the main factor) showed that at extreme P levels of 0 and 1250 µM, total root length (TRL), total surface area (SA), total root volume (RV) and primary root volume (PV) were also significantly affected (P<0.001). The two parental genotypes were significantly different for these traits (P<0.001), with mean Sal higher than Ser of between 13.7% to 47.8%. In contrast, primary root diameter (PD) was smaller in extremes 0 and 1250 µM P (P<0.001) with Ser showed a significant by lower mean than Sal (P<0.001). Treatment of 312 µM P significantly produced more lateral roots (LRN) (P<0.001) in Sal than Ser (P<0.001). Plus, lateral root density (LRD) showed a significant mean peak at 312 µM P and lowest at extremes 0 and 1250 µM P (P<0.001). Mean of Sal was significantly greater in LRD (P<0.001) by 59.4%. These results are summarised in Tables 3.2 and Table 3.3. Example of seedlings' images from each P treatments are also shown in Figure 3.2.

Table 3.2 Means of P treatments on measured root traits (with no significant interaction). *Different letters means significant different based on Fishers protected LSD at 5%. n, number of observation; F, F-value; *P*, level of significance; SEM, standard error of mean; SED, standard error of difference; LSD, least significance difference value. TRL = total root length; SA = total root surface area; RV = total root volume; LRN = lateral root number; LRD = lateral root density; PD = primary root diameter; PV = primary root volume.

P treatment	n	TRL (cm)	SA (cm^2)	RV (cm^3)	LRN	LRD (cm^{-1})	PD (cm)	PV (cm^3)
0	27	8.641a	0.7628a	0.00693a	4.619c	1.05d	0.0288b	0.00577a
6	22	3.81c	0.3652c	0.003676c	6.462ab	2.397b	0.03472a	0.003318c
312	22	3.843c	0.3788c	0.003724c	7.542a	2.783a	0.03497a	0.003292c
625	27	3.79c	0.376c	0.003746c	5.8b	2.288b	0.03635a	0.003307c
1250	25	7.863b	0.6639b	0.005605b	5.383bc	1.418c	0.03081b	0.004619b
	$F_{(4,110)}$	85.5	96.5	49.5	7.6	31.0	19.2	32.4
	P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	SEM	0.2650	0.0193	0.0002	0.4030	0.1299	0.0007	0.0002
	SED	0.3740	0.0273	0.0003	0.5700	0.1837	0.0010	0.0003
	LSD	0.7420	0.0541	0.0006	1.1300	0.3641	0.0020	0.0005

Table 3.3 Means of lettuce parental genotypes on measured root traits (with no significant interaction). Sal is domesticated lettuce while Ser is wild lettuce. n, number of observation; F, F-value; *P*, level of significance; SEM, standard error of mean; SED, standard error of difference. TRL = total root length; SA = total root surface area; RV = total root volume; LRN = lateral root number; LRD = lateral root density; PD = primary root diameter; PV = primary root volume.

Genotype	n	TRL (cm)	SA (cm^2)	RV (cm^3)	LRN	LRD (cm^{-1})	PD (cm)	PV (cm^3)
Sal	80	6.000	0.594	0.006	8.670	2.828	0.038	0.005
Ser	43	5.180	0.424	0.003	3.250	1.147	0.029	0.003
	$F_{(1,110)}$	12.1	97.1	251.9	226.4	209.4	183.7	169.5
	P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	SEM	0.1670	0.0122	0.0001	0.2550	0.0822	0.0005	0.0001
	SED	0.2370	0.0173	0.0002	0.3610	0.1162	0.0006	0.0002

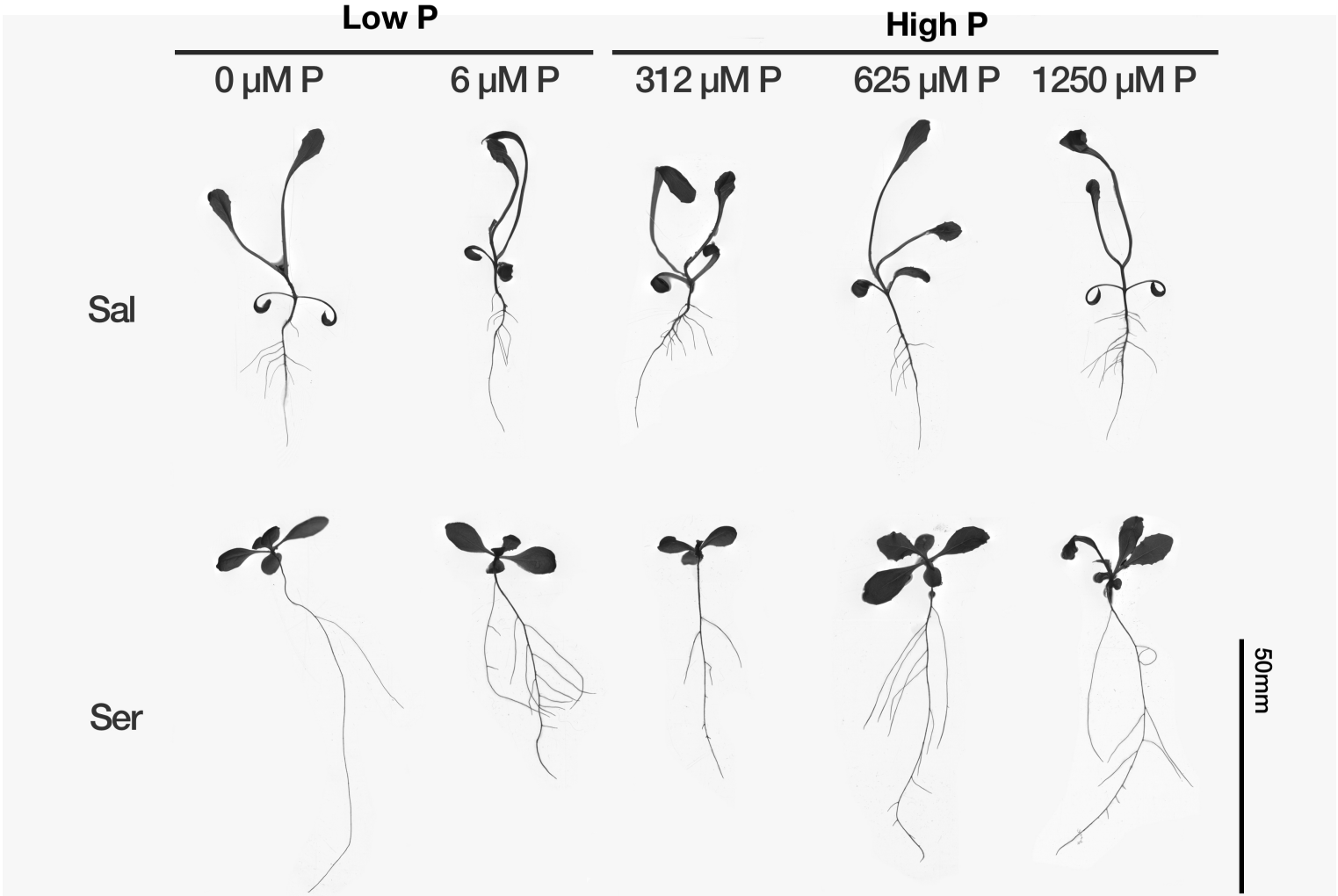


Figure 3.2 Example of parental lettuce seedlings from each P treatment. Sal is domesticated lettuce while Ser is wild lettuce. The plants were harvested at 14 days after being subcultured into treatment petri dishes. Scale bar = 50mm.

3.4 DISCUSSION

The present study showed some distinct but complex RSA between wild and domesticated lettuce genotypes showing possible differential adaptation to different P applications (Figure 3.2). Primary root length (PRL) decreased from higher to lower P levels in both domesticated lettuce (*L. sativa* cv. Salinas; Sal) and wild lettuce (*L. serriola* acc. UC96US23; Ser), especially from 1250 µM to 650 µM P. This is consistent with observation in other plants (for example Hammond *et al*., 2009; Yang *et al*., 2010; Shi *et al*., 2013), albeit a more relatively subtle PRL reduction observed in Sal.

However, in domesticated lettuce, PRL growth is more favourable at higher P levels, perhaps mimicking heavy P fertilisation in the field. Further, heavy P fertilisation has resulted in no significant increase in lettuce yield production (Johnstone *et al*., 2005). Nevertheless, responses of RSA to high P level with PRL elongation may increase the ability to exploit deeper soil resources. The longer PRL at extreme P level was more prominent in Ser in this case. This indicates genotypic difference between both parental lettuce in adapting higher P level which may derive from domestication processes.

Furthermore, previous study by MacBeath and co-workers (2011) on wheat has shown that wheat utilised 62% subsoil P as opposed to only

20% of topsoil when some P fertiliser were applied to the wheat. These results showed the importance of subsoil root exploration for P as well while at the same time acquire yield-related nutrient such as nitrogen for improved productivity.

On the other hand, P starvation condition reduced primary root growth and promotes formation of lateral roots (Wiliamson *et al*., 2001 and Péret *et al*., 2014). Interestingly, an increase in PR growth was seen at 0 µM for both wild and domesticated lettuce parental genotypes, which is atypical as P deficiency should dramatically inhibit PR growth (Wiliamson *et al*., 2001 and Péret *et al*., 2014). The reason behind this is not fully understood.

The presence of sucrose and illumination in reference to the Arabidopsis root study, have shown effects on the root growth in agar-based method as artefact responses (Karthikeyan *et al*., 2007 and Xu *et al*., 2013). There is increasing evidence to suggest sucrose regulates global plant responses to P starvation by inducing the expression of P starvation induced (PSI) genes of Arabidopsis (Lei *et al*., 2011) thus inhibit the PR growth. Growing roots in darkness without sucrose promotes PR growth too (Xu *et al*., 2013). Nevertheless, all of these relationships seems contradicting to the results.

However, looking at combined multiple stress signalling pathway of nitrate (NO_3^-) and phosphate (PO_4^{3-}), *HYPERSENSITIVE TO LOW Pi-ELICITED PR SHORTENING 1* (*HRS1*) gene may explain the increase of PRL mean at 0 µM P. This is because the presence of nitrate may promote the PR growth although the phosphate is absence (Medici *et al*., 2015). Other reason contributing to this issue may be due to the presence of P in the gel, contaminated from manufacturing line of agar supplier.

Furthermore, from visual observation of roots in Figure 3.2, represented root system of domesticated lettuce may suggest that seedlings were not entirely in P-stressed conditions as the RSA looks similar at low and high P levels, although analysed to be statistically different, especially at extreme P levels. The domesticated lettuce has larger seed size as compared to wild lettuce (data not shown), suggesting higher seed P content to be used before total depletion. Plus, shoot traits information need to be addressed in the future, as the difference in RSA of wild and domesticated lettuce towards different P levels might be allometric, especially between shoot and root traits. Typical P deficiency response includes reduction in shoot biomass with increase of root:shoot ratio, possibly due to preferential assimilate distribution to the roots (Freeden *et al*., 1989; Mollier and Pellerin, 1999; Vance *et al*., 2003).

Additionally, post-embryonic developmental changes in RSA under different P levels, which include lateral root (LR) formation, is important in maximising P uptake (Giehl *et al*., 2014). LR formation is a tightly co-ordinated event (Péret *et al*., 2009), of which can be modulated by P levels. Examples from Arabidopsis studies have shown LR formation stimulation under P limiting environment (Williamson *et al*., 2001; Lopez-Bucio *et al*., 2002 and Gruber *et al*., 2013).

Based on the P treatment factor, significant lower LRN and LRD can be seen in lettuce grown in very limiting or high P conditions ($P<0.001$). These root responses were complex and inconsistent compared to studies by López-Bucio and co-workers (2002), of which Arabidopsis seedlings grown under 10 µM produced abundant lateral roots as compared to those grown against P level of more than 100 µM. This however, is compensated by longer lateral root length (LRL) at both extreme P levels of 0 and 1250 µM P in Sal and only at 1250 µM in Ser, balancing the limitation of the plants to initiate more LR formation. Higher lateral surface area (LSA) and smaller lateral root diameter (LD) were also observed, suggesting a 'feedforward' mechanism to provide more efficient nutrient uptake. In a bigger picture, these adaptation strategies by lettuce seedlings were hardly match the topsoil foraging strategy seen in other plants. The results obtained may be species-specific, however, roots in more matured lettuce should be compared as well, as these results may not be representative.

Furthermore, the use of agar-based assessment method posed some challenges throughout the course of this experiment. Examples include the needs of sterilisation for all components of the experiments to avoid nutrient medium contamination, time consuming protocols of agar plating and seed plating and some inconsistency related to seed growth on agar-based medium. Time taken for seeds to germinate varied significantly within wild lettuce, Ser, and affected the experimental design, data collection and analysis processes. Missing data in Ser dataset reduced the number of samples for analysis. Microscopic observation of a random selection of Ser seed embryo (results not shown here) showed the seeds were viable therefore the inconsistency observed could be due to seed coat dormancy. Some seedlings showed callus-like growth and were not included in the analysis.

3.5 SUMMARY

The experiment aimed at phenotyping lettuce root traits under different P levels. The results showed clear differences between wild and domesticated lettuce in the way they adapt to different P levels, especially at the very limiting or high P concentrations. Wild lettuce showed enhanced PR growth while domesticated lettuce showed enhanced LR formation, especially at a very low and high P extremes. Furthermore, under a very low P concentrations, the results suggest that lettuce was hardly conformed to topsoil foraging strategy. Lack of LR formation under limiting P levels in domesticated lettuce was compensated through additional root structures that optimise nutrient and water uptake for example LSA and LD. The compensated different adaptation strategies seen in domesticated lettuce and wild lettuce may imply species-specific responses by lettuce. Additionally, the method used in this experiment has generated a useful high throughput data to the analysis. However, the method posed some logistic challenges in generating the data which slows down data generation. Issues with wild lettuce inconsistent germination and abnormal growth of some seeds reduce the number of seedlings available for analysis.

CHAPTER 4

PAPER-BASED PHENOTYPING APPROACH FOR LETTUCE ROOT SYSTEM ARCHITECTURE GROWN UNDER DIFFERENT PHOSPHORUS LEVELS

4.1 INTRODUCTION

Paper-based system is a reliable and robust method for 2-dimensional (2D) root system architecture (RSA) phenotyping. The method has garnered more attention recently and several studies have used this technique to screen RSA of many species including maize (Le Marié *et al*., 2014), wheat (Atkinson *et al*., 2015), barley (Rattanapichai and Klem, 2016) and *Brassica* species (Thomas *et al*., 2016).

Similar to agar-based system, paper-based system has several advantages including evaluation of root growth in real time, high-throughput data output from a single experiment, ability to create a repeatable condition, small space for large number of samples, easy handling and cleaner roots for imaging (Paez-Garcia *et al*., 2015). This method can be utilised in a non-aseptic environment for example glass- and shade houses. Plus, non-destructive roots images can also be obtained. For example, a combination of high-resolution scanner was utilised with paper-based method to screen root phenotype in real-time (Adu *et al*., 2014), unveiling more beneficial RSA that could be incorporated in breeding programme. Additionally, the roots were kept at

dark condition at all time, mimicking the natural soil environment as opposed to the agar-based method.

A recent study has also explored the potential use of the system for a split root system to study different root responses at different nutrient levels on the same plants (Zandt *et al*., 2015) . This is a modification of the previous method of split pot method (Grossman and Rice, 2012; Sartoni *et al*., 2015), in which roots were split in a pot to study root responses to patches of nutrient in soil. Each nutrient patch can be precisely controlled in comparison to soil-based split pot method. Combined with other advantages, the system may offer more reliable and robust results.

In this experiment, the main aim was to carry out RSA screening of wild and domesticated lettuce parental genotypes using paper-based assessment method grown under different P conditions. The experiment explored the paper-based screening method execution and its comparative advantages and disadvantages to the agar-based screening method.

4.2 MATERIALS AND METHODS

4.2.1 Plant materials

Lettuce parental genotypes, *Lactuca serriola* acc. UC96US23 (wild lettuce; Ser) and *Lactuca sativa* cv. Salinas (domesticated lettuce; Sal) were used in this experiment. Details are provided in **Section 2.1.1.**

4.2.2 Experimental design

A randomised complete block design (RCBD) was used. There were four blocks; a block consisted of three phosphorus (P) treatments of 0, 7.5 and 15 mg l^{-1} (after 15% dilution), and the two lettuce parental genotypes. A total of 16 replicates were used per genotype, with four seedlings per block per genotype. All block was grown at the same time, grown in a controlled environment growth room, located at the Plant Science Building, Sutton Bonington Campus, University of Nottingham, UK.

4.2.3 Plant growth condition

In this experiment, the seeds were grown in paper-based pouches. The seeds were first sterilised as reported in **Section 2.3** and then stratified (see **Section 2.4**) before they were germinated (as described in **Section 2.2**). The pouches and growth assembly were based on Atkinson *et al*.

(2015), and optimised for lettuce (J. Roberts, personal communication, 17 September 2015). Each pouch consists of a sheet of blue germination paper (24×30 cm; Anchor Paper Company, St Paul, Minnesota, USA), addition of a layer of kitchen tissue layer (23×22.5 cm, 2-ply; ALDI Stores, Warwickshire, UK) from the one described previously in Atkinson *et al*. (2015) and both overlaid with a black polythene film (75 µm thick; Cransford Polythene Ltd, Suffolk, UK) (Figure 4.1A). Kitchen tissue layer was added to increase and maintain high moisture level for lettuce seeds. Each pouch were fixed to each side-face of acrylic rod (316×15×5 mm; Acrylic Online, Hull, UK) by using 18 mm bulldog-type fold-back clips at each end of acrylic rod. A label sticker with genotype and treatment identification was affixed onto the black polythene film for easy identification reference.

The acrylic rods with growth pouches were fitted in custom-made aluminium (104×62×102 cm; KJN Ltd, Leicester, UK) frame with side black polypropylene panels (101×31×0.3 cm and 63×31×0.3 cm; Cut Plastic Sheeting, Devon, UK) assemblies in the controlled environment chamber. The black side panels maintain darkness condition for the roots. The assemblies supported the acrylic rods with its toothed acrylic holders which suspend each pouch in a set position. A total of 90 acrylic rods can be arranged in each frame across three different rows of toothed acrylic holders (Figure 4.1B). The base of the pouches consists of nine drip trays, positioned in three columns and three rows, which are then filled with

nutrient solution for the seeds. The bottom part of the pouches were submerged in the nutrient solution and nutrient solution were drawn up to the seeds located at the top of the pouches via capillary action. Prior to this, the pouches were first submerged in the nutrient solution until fully saturated. The nutrient solution levels were checked in every two days and maintained at least half of the drip tray.

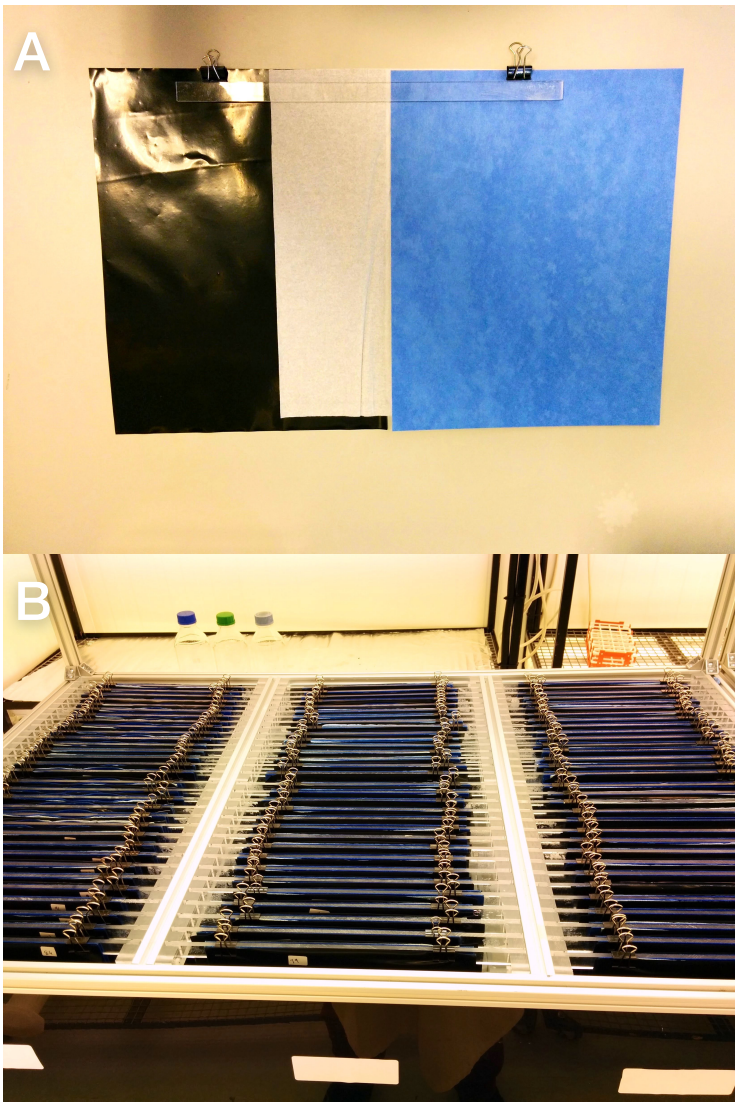


Figure 4.1 Growth assemblies of the lettuce seeds. A) Layers of growth pouch, clipped together on both side of acrylic rod. B) Growth frame assembly.

A modified recipe of Hoagland's solution at 15% dilution with recommended major elements concentration for lettuce (Schon, 1992) was used. Separate components of macronutrients stock solutions were mixed to get a full strength (1×) nutrient solution. These included; three monopotassium phosphate, KH_2PO_4 concentrations (0, 50 and 100 mg l^{-1}); magnesium sulphate heptahydrate, $MgSO_4.7H_2O$; calcium nitrate tetrahydrate, $Ca(NO_3)_2.4H_2O$; potassium chloride, KCl (to balance K^+ ion where lower KH_2PO_4 were used); iron chelate stock solution ferric sodium EDTA, FeNaEDTA and a single mixture of micronutrients stock solution which consist of boric acid, H_3BO_3; manganese sulphate hydrate, $MnSO_4.H_2O$; zinc sulphate heptahydrate, $ZnSO_4.7H_2O$; copper sulphate pentahydrate, $CuSO_4.5H_2O$; and sodium molybdate dihydrate, $Na_2MoO_4.2H_2O$. To prevent precipitation with phosphate salt, $Ca(NO_3)_2.4H_2O$ stock solution was added first, followed by KH_2PO_4 stock solution and then pH adjusted to ~6.0 using 0.5 M KOH solution, before adding other stock solutions. Components of stock solutions and their concentrations are listed in Table 4.1.

Each pouch consisted of two seeds placed ~5 cm from each other, located at ~5 mm deep from top of the pouch. The seeds were secured in place with moist tissue surrounded the seeds. The growth condition for the seeds are described in **Section 2.5.**

Table 4.1 Components of nutrient solution and their concentrations.

Salts	Molecular weight	Stock solution concentration (mg l^{-1})	1× solution concentration (mg l^{-1})	Volume added (ml l^{-1})	15% dilution concentration (mg l^{-1})
Macronutrients *(separate stock solutions)*					
KH_2PO_4	136.09	36290	100	2.756	15
			50	1.378	7.5
			0	0	0
KCl	74.5513	19880	200	2.683	30
			250	3.353	37.5
			300	4.024	45
$MgSO_4.7H_2O$	246.47	92430	65	0.703	9.75
$Ca(NO_3)_2.4H_2O$	236.15	236150	200	0.847	30
Iron chelate					
FeNaEDTA	367.05	18350	36.70	2	5.505
Micronutrients *(single stock solution)*					
H_3BO_3	61.83	1860	1.860	1	0.279
$MnSO_4.H_2O$	151	1510	1.510		0.1725
$ZnSO_4.7H_2O$	287.55	290	0.290		0.0435
$CuSO_4.5H_2O$	249.68	250	0.250		0.0375
$Na_2MoO_4.2H_2O$	241.95	120	0.120		0.018

4.2.4 Root imaging and image analysis

After fourteen days of growing, the roots were exposed by removing the black polythene layer and RSA images were taken using a digital single-lens reflex (DSLR) camera (Canon EOS 1100D, Canon Inc., Tokyo, Japan), remotely controlled using Canon software on a laptop. The DSLR camera was attached to a copy stand at a fixed height of 60cm (Figure 4.2). Marks were made on the copy stand to ensure consistent placement of the pouches for every photo snapshots. RSA were segmented and measured based as described in **Section 2.6**.

Figure 4.2 Imaging setup for paper-based method. These components are very portable and can be set up where it is convenient.

4.2.5 Data collection

Measured data from segmented images were collected and used in the analysis. The raw data included three main categories of root parameters tested in the present study, namely, primary root, lateral root and global root parameters. Primary root parameters includes primary root length (PRL), primary root surface area (PSA), primary root volume (PV), and primary root diameter (PD). Lateral root parameters on the other hand comprises of lateral root length, (LRL), lateral surface area (LSA), lateral root volume (LV), lateral root diameter (LD), lateral root number (LRN) and lateral root density (LRD). Global root parameters includes total root length (TRL), total root surface area (SA), average root diameter (AD) and total root volume (RV). LRD and all global root parameters were calculated based on description in **Section 2.6.**

4.2.6 Statistical analysis

Raw data were loaded into GenStat® (Release 17; VSN International, Oxford, UK). Before the analysis, PSA, PV, TRL and LSA were transformed with log function, LRL with reciprocal fourth root function and LV with square root function. LRN cannot be normalised by transformation, therefore subjected to non-parametric analysis. The normality of trait distribution was confirmed through Anderson-Darling test at 5% (Appendix 1).

A two-way ANOVA was used to test for differences in root parameters of the two lettuce parental genotypes grown under three different P levels. Fishers protected least significant difference (LSD) test was used as the multiple comparison test, set at 5%. Prior to the ANOVA analysis, assumption of the normality of residuals and homogeneity of variances were tested using residual plot tools in GenStat®. All analysis was presented according to minimal adequate model based on a top-down approach.

Spearman's rank correlations (r_s) were calculated for all traits measured. Pearson's product-moment correlations coefficient (r_p) were calculated for parametric root traits observed in this paper-based method. "rcorr()" function from "Hmisc" package was used in R Studio (Version 0.99.903, R Studio Team, 2015), which give out outputs of *r* values, *n* number of observations analysed in the data matrix and *P*-values of all pair-wise correlations. *r* values with $0.2 \leq |r| \leq 0.5$, $0.5 < |r| < 0.8$ and $|r| \geq 0.8$ were defined as weak, moderate and strong, respectively. A correlogram combined with its respective *r*-values was produced by using "corrplot ()" function of "corrplot" package. The correlogram was organised according to angular order of the eigenvectors (order = "AOE"; Friendly, 2002). Coding lines are shown in Appendix 1.

4.3 RESULTS

4.3.1 RSA variation between lettuce parental genotypes

A total of 90 viable individuals were analysed in this study. An average of 14 Ser seeds were used, with minimum of 13 and maximum 15 seeds per P treatment. A 48-replicate was obtained across all treatments for domesticated lettuce (*L. sativa* cv. Salinas; Sal). 12.5% of data was missing in Ser. A typical image of sample roots were shown in Figure 4.3.

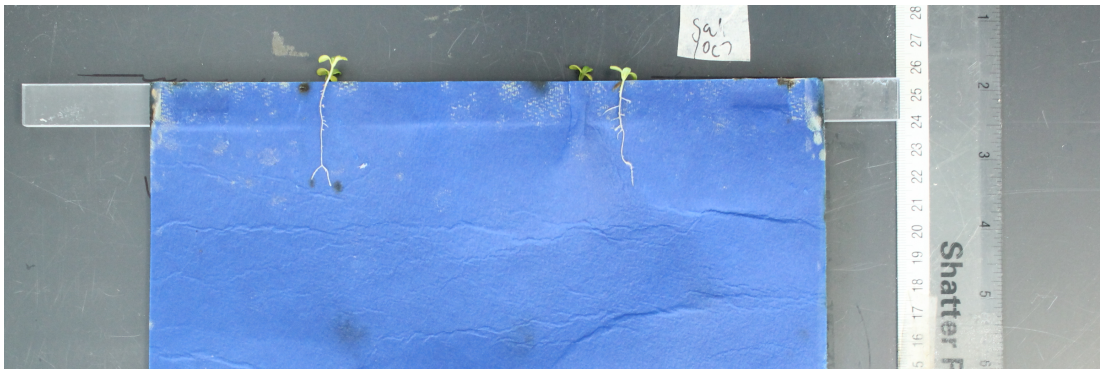


Figure 4.3 Typical image of sample roots from paper-based root phenotyping (cropped). The seedlings were at 14 days after being transferred to the pouches.

The two parental lines, *Lactuca serriola* acc. UC96US23 (wild lettuce; Ser) and *Lactuca sativa* cv. Salinas (domesticated lettuce; Sal) showed significant mean differences ($P \leq 0.006$) between all measured traits except for lateral root density (LRD). Means of all measured traits for Sal were higher in comparison to Ser. Mean total root length (TRL), total root surface area (SA), root average diameter (AD) and total root volume (RV) of Sal were 50.6%, 51.9%, 35.6% and 53.2% higher than Ser, respectively.

For primary traits, primary root length (PRL), primary root surface area (PSA) and primary root volume of Sal were all more than 50% higher than Ser. Mean of lateral root length (LRL), lateral root surface area (LSA), lateral root volume (LV), lateral root diameter (LD) and lateral root number (LRN) of Sal were 43.0%, 41.5%, 55.6%, 43.6% and 43.0% higher than Ser respectively. All details are summarised in Table 4.2-4.5.

In contrast, no any significant phosphorus (P) treatments on the lettuce seedlings were obtained from the analysis ($P \geq 0.08$). However, exception for trait primary root diameter (PD) which show a significant lettuce parental genotypes × treatments interaction ($F_{(2,81)} = 5.26$, $P=0.007$, Figure 4.4). Multiple comparison test for PD showed significant peak difference in mean PD at 0 mg ml^{-1} of P for both Sal and Ser ($P<0.05$). Mean PD showed declining trend in increasing P treatments, especially from 0 to 50 mg ml^{-1}, for both Sal and Ser. The declining trend in Sal was however more subtle in comparison to Ser.

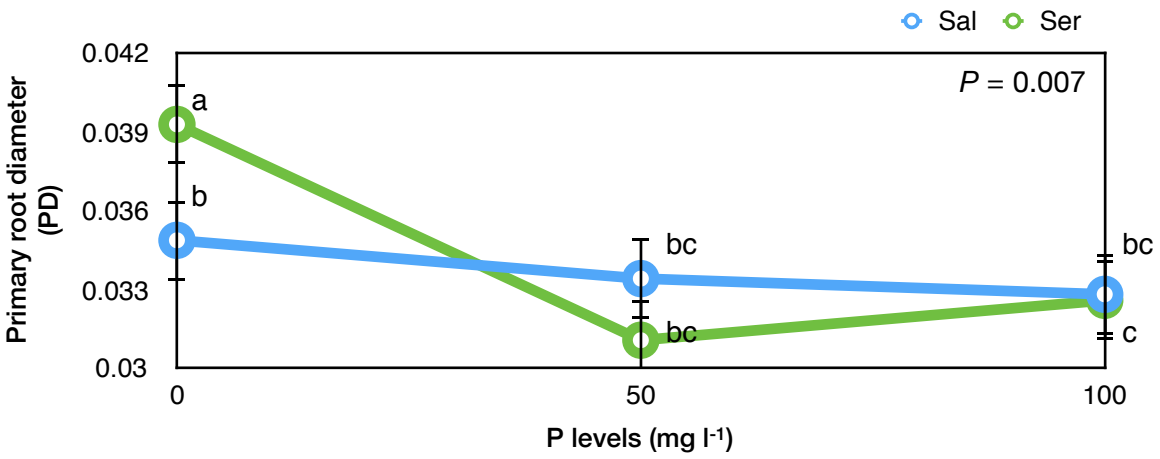


Figure 4.4 Significant genotype × treatments interaction of primary root diameter (PD).

Table 4.2 Means of P treatments on measured root traits (non-transformed data, with no significant interaction). n, number of observation; F, F-value; *P*, level of significance; ns, not significant; SEM, standard error of mean; SED, standard error of difference. SA=total root surface area; AD=average root diameter; RV=total root volume; PRL=primary root length; LD=lateral root diameter; LRN=lateral root number; LRD=lateral root density.

P treatment	n	SA (cm^2)	AD (cm)	RV (cm^3)	PRL (cm)	LD (cm)	LRN	LRD (cm^{-1})
0	31	0.360	0.080	0.003	2.131	0.123	4.260	2.070
50	29	0.335	0.084	0.003	2.264	0.135	4.850	2.350
100	30	0.321	0.071	0.003	2.302	0.110	3.990	1.710
	$F_{(2,81)}$	0.7	1.2	2.1	0.4	1.2	1.2	2.6
	P	0.527^{ns}	0.305^{ns}	0.127^{ns}	0.678^{ns}	0.314^{ns}	0.312^{ns}	0.08^{ns}
	SEM	0.024	0.006	0.000	0.144	0.012	0.407	0.201
	SED	0.035	0.008	0.000	0.204	0.017	0.575	0.285

Table 4.3 Means of P treatments on measured root traits (transformed data, with no significant interaction). n, number of observation; F, F-value; *P*, level of significance; ns, not significant. Sal is domesticated lettuce and Ser is wild lettuce. TRL=total root length; PSA=primary root surface area; PV=primary root volume; LRL=lateral root length; LSA=lateral root surface area; LV=lateral root volume.

P treatment	n	TRL (cm)	PSA (cm^2)	PV (cm^3)	LRL (cm)	LSA (cm^2)	LV (cm^3)
0	31	3.545	0.227	0.002	1.155	0.125	0.001
50	29	3.612	0.223	0.002	1.198	0.106	0.001
100	30	3.411	0.220	0.002	0.913	0.091	0.001
	$F_{(2,81)}$	0.1	0.0	1.1	0.4	0.7	1.2
	P	0.915^{ns}	1^{ns}	0.327^{ns}	0.644^{ns}	0.519^{ns}	0.313^{ns}

Table 4.4 Mean of lettuce parental genotypes on measured root traits (non-transformed data, with no significant interaction). n, number of observation; F, F-value; *P*, level of significance; ns, not significant; SEM, standard error of mean; SED, standard error of difference. Sal is domesticated lettuce and Ser is wild lettuce. SA=total root surface area; AD=average root diameter; RV=total root volume; PRL=primary root length; LD=lateral root diameter; LRN=lateral root number; LRD=lateral root density.

Genotype	n	SA (cm^2)	AD (cm)	RV (cm^3)	PRL (cm)	LD (cm)	LRN	LRD (cm^{-1})
Sal	48	0.457	0.0953	0.00404	3.046	0.1568	5.56	1.95
Ser	42	0.22	0.0614	0.00189	1.418	0.0884	3.17	2.13
	$F_{(2,81)}$	70.1	25.19	90.87	95.63	25.59	25.98	0.57
	P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.453^{ns}
	SEM	0.02	0.00478	0.00016	0.1177	0.00956	0.332	0.164
	SED	0.0282	0.00675	0.000226	0.1665	0.01352	0.47	0.233

Table 4.5 Mean of lettuce parental genotypes on measured root traits (transformed data, with no significant interaction). n, number of observation; F, F-value; *P*, level of significance. Sal is domesticated lettuce and Ser is wild lettuce. TRL=total root length; PSA=primary root surface area; PV=primary root volume; LRL=lateral root length; LSA=lateral root surface area; LV=lateral root volume.

Genotype	n	TRL (cm)	PSA (cm^2)	PV (cm^3)	LRL (cm)	LSA (cm^2)	LV (cm^3)
Sal	48	4.7170	0.3120	0.0029	1.4630	0.1350	0.0009
Ser	42	2.3280	0.1350	0.0012	0.7150	0.0790	0.0004
	$F_{(2,81)}$	57.7	147.8	166.4	8.0	12.1	10.9
	P	<0.001	<0.001	<0.001	0.006	<0.001	0.001

4.3.2 Correlations between root traits

There were overall general positive correlations between root traits measured in the paper based assessment system. In the Spearman's rank correlation (r_s) analysis (Figure 4.5), which involve all traits measured, the strongest positive correlations were between lateral-lateral, primary-primary and global-global root traits ($r_s \geq 0.82$, $P<0.001$). For example, there were strong correlations between lateral root volume (LV) and lateral root surface area (LSA) ($r_s = 0.99$); lateral root diameter (LD) and lateral root number (LRN) ($r_s = 0.97$); primary root length (PRL) and primary root volume (PV) ($r_s = 0.89$); PRL and primary root surface area (PSA) ($r_s = 0.97$); and total root length (TRL) and total root surface area (SA) ($r_s = 0.98$). Moderate or weak correlations were mostly seen between different root classes. These includes relationship between LV and PV ($r_s = 0.4$); and LRN and PRL ($r_s = 0.58$). Lateral root length (LRL) particularly showed negative correlations to all other traits at different r values. Strongest correlations were showed between trait LV ($r_s = -0.96$) and LSA ($r_s = -0.99$). Weak relationship observed between trait LRL and PRL ($r_s = -0.49$). Primary root diameter (PD) showed no significant correlations to most of the traits. Lateral root density (LRD) showed no significant correlations to other traits classes, the primary and global root traits.

The usage of Pearson's product-moment correlations coefficient (r_p) analysis for parametric dataset increases the power of *r* values for the correlations pairwise tests. In the analysis, an overall positive correlations was observed in Figure 4.6. Strongest correlations were detected for traits in the same classes, lateral-lateral, primary-primary and global-global root parameters. For example, there were strong correlations between LV and LSA (r_p = 0.99); TRL and SA (r_p = 0.98); and PSA and PV (r_p = 0.97). Similar to Spearman's rank correlation analysis, root traits of different classes were either moderately and weakly correlated. For instance, moderate correlation can be seen between traits LV and and RV (r_p = 0.74) and weak correlation can be observed between trait LSA and PSA (r_p = 0.42). In this analysis, LRL showed negative correlations to all other traits. The strong correlations were shown between LRL and mostly lateral traits and weaker correlations to primary root traits. PD showed no significant correlations to almost all other traits except for TRL (r_p = -0.27) and PRL (r_p = -0.32), albeit very weak. LRD did not show significant results to RV, PSA, PV and PRL.

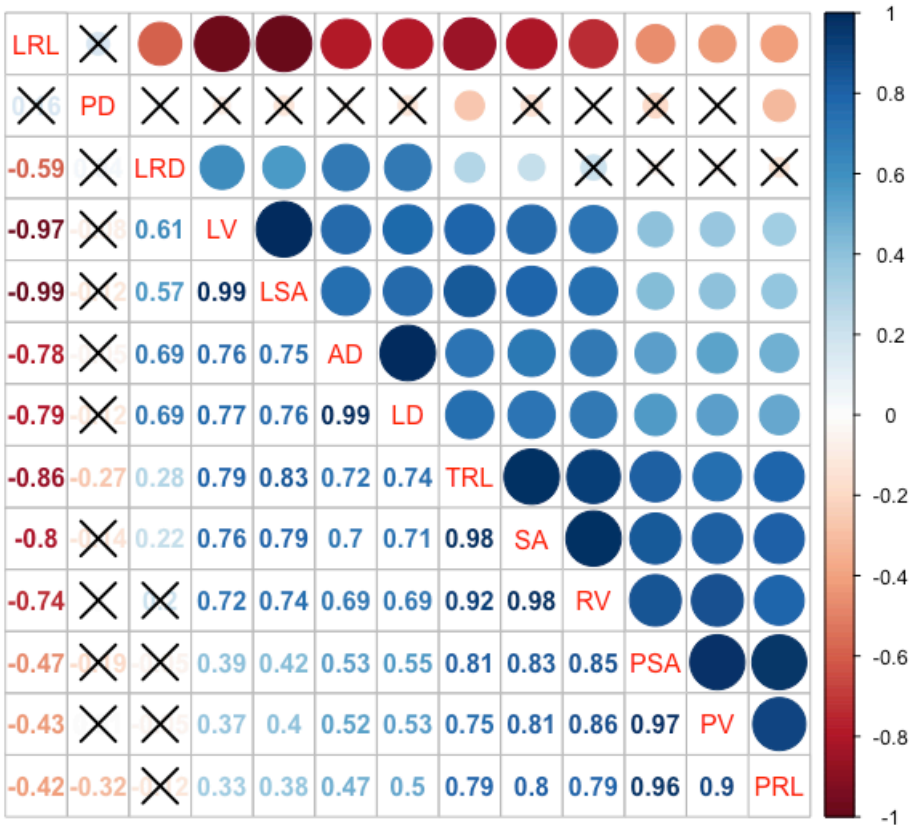


Figure 4.5 A correlogram of traits analysed in paper-based method based on Spearman's rank correlation (r_s) analysis. Blue circles are showing positive correlations whilst the red showing negative correlations. The circles are organised in angular order of the eigenvectors (Friendly, 2002). X means non-significant correlations ($P > 0.05$).
PD = primary root diameter; LRD = lateral root density; LV = lateral root volume; LSA = lateral root surface area; LRL = lateral root length; AD = average root diameter; LD = lateral root diameter; LRN = lateral root number; TRL = total root length; SA = total root surface area; V = total root volume; PRL = primary root length; PV = primary root volume; PSA = primary root surface area.

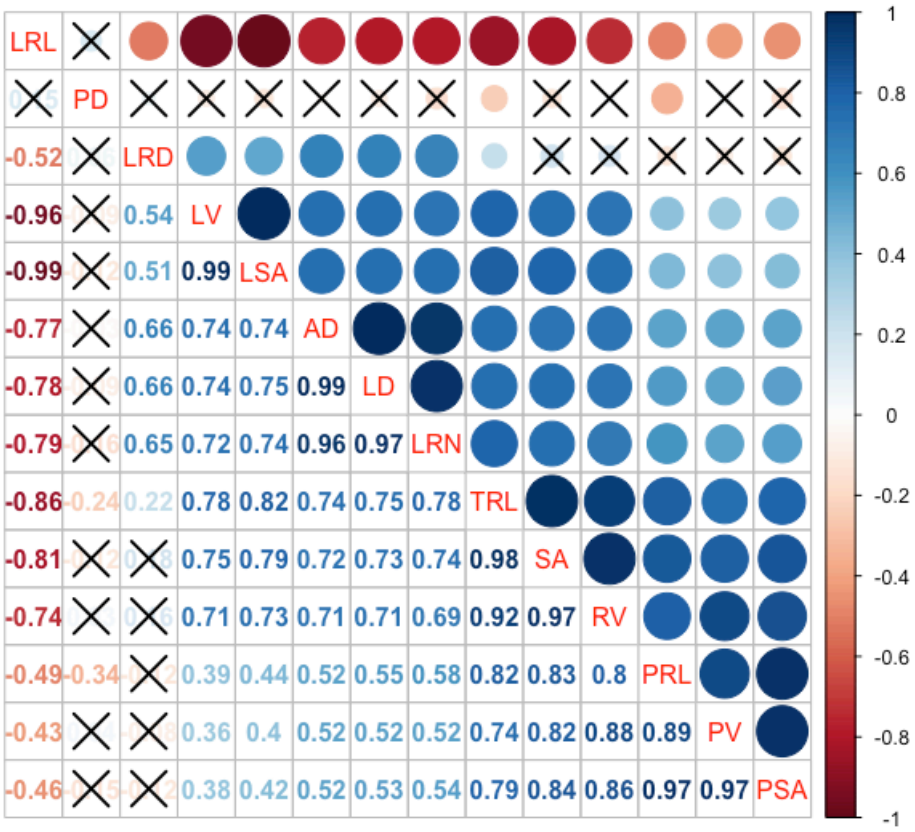


Figure 4.6 A correlogram of traits analysed in paper-based method based on Pearson's product-moment correlations (r_p) analysis. Blue circles are showing positive correlations whilst the red showing negative correlations. The circles are organised in angular order of the eigenvectors (Friendly, 2002). X means non-significant correlations ($P > 0.05$). PD = primary root diameter; LRD = lateral root density; LV = lateral root volume; LSA = lateral root surface area; LRL = lateral root length; AD = average root diameter; LD = lateral root diameter; LRN = lateral root number; TRL = total root length; SA = total root surface area; V = total root volume; PRL = primary root length; PV = primary root volume; PSA = primary root surface area.

4.4 DISCUSSION

4.4.1 Phenotypic variation of lettuce parental genotypes on RSA

In this experiment, a complex adaptation strategy has been observed in both wild (*Lactuca serriola* acc. UC96US23; Ser) and domesticated (*Lactuca sativa* cv. Salinas; Sal) lettuce under paper-based assessment method. The results showed significant differences between parental genotypes, the wild and domesticated lettuce. In this experiment, the roots of Sal were generally more established than Ser. Ser roots have significant shorter primary roots (PR) and lateral roots (LR), and reduced number of LR. Traits such as surface area, volume and diameter were also mostly dominated by Sal. This may indicate seed vigour, domestication trait that aid in better seed establishment on field. Other reason contributing to this is perhaps the larger size of domesticated lettuce seeds in comparison to wild lettuce (data not shown), with virtually more nutrient available before depletion. Seed P content should be addressed in the future to validate this inconsistent dataset.

Typically, higher P environments promotes PR growth and lower P environments inhibits PR growth and encourage lateral root (LR) formation (Wiliamson *et al*., 2001 and Péret *et al*., 2014). However, a definite conclusion cannot be drawn from the observations as most of the differences were not significant for P treatments in paper-based screening

method. Although P treatments applied did not affect the RSA significantly, the RSA at these P levels showed some hints for the growth trends. From the results, although not significant, all mean traits of Sal showed declining trend when more P was applied. The trend for Ser was rather complex, however, showed a generally increasing trend with increasing P level. The results may suggest different adaptation strategy by both Sal and Ser in adapting to different P levels. However, the observations did not conform to typical responses to P, suggesting a species-specific adaptation. Furthermore, having shoot traits data might give better insights of P dynamics in these lettuce, as allometric growth of source-sink allocation might rule these differences. In this experiment, relatively smaller size of shoots was observe in all seedling even though the seedlings were already at 14 days after being subcultured to paper-based pouches (data not shown).

Correlations data have shown a cluster pattern of strong correlations between lateral-lateral traits and primary-primary traits, which may indicate separate intrinsic root control and responses of the parental lettuce lines towards its environment. Weaker correlations, on the other hand, were seen between lateral-primary traits. Hypothesis of different QTL region controlled by lateral and primary traits can be tested. However, interestingly, PD have shown very weak or non-correlations to most of other traits observed. Negative correlations between PD and both TRL and PRL were not surprising as both traits are growth-related which

needs efficient carbon utilisation and compartmentalisation. This negative associations however a trade-off of one of the steep, cheap and deep ideotype phene sets for deeper water and nitrogen (N) acquisition, which demand a larger PD with few but long laterals along with cold tolerant roots (Lynch, 2013).

4.4.2 Paper-based method execution

The cost of the consumables of both agar- and paper-based methods were estimated less than £1 per individual plants. Nevertheless, this can be compared to advantages of growing plants in a relatively minimum aseptic environment in paper-based method. The pouches and nutrient solution did not need to be sterilised prior to sowing in contrast to agar-based method which require a sterilised petri dishes and media, prepared under thorough aseptic conditions. However, the seeds still needs to be sterilised, in order to reduce the chances of mortality due to fungus contamination from high moisture environment coming from the tissue and germination paper. This has been one of the most time-consuming step in this experiment. Furthermore, number of replicates for wild lettuce, Ser for analysis has improved in this experiment with only 12.5% missing data as opposed to 46.25% missing data in agar-based method. Missing data was due to mortality or germination arrest after being transferred to blue germination paper.

From previous study, the estimated time for obtaining root data (sowing, imaging and data analysis) can come down to less than 2 minutes per individual plant (Thomas *et al*., 2016), however, in this experiment the time taken to obtain the images and to analyse the data took longer than that mainly because in most cases, the roots were intertwined with kitchen tissue layer fibre. To capture images of these, the roots needed to be deliberately untangled from the kitchen tissue fibre and this has become increasingly tedious. This has been a set back in order to maintain high moisture level for the lettuce seeds, as the blue germination alone was too dry for the lettuce seeds to grow properly (results not shown). This has also hindered other analysis related to undisturbed RSA for example root angle and convex hull. Nevertheless, the use of digital single-lens reflex (DSLR) camera has simplified the lettuce imaging processes as compared to using the water-filled tray on a scanner used in agar-based assessment method whilst at the same time maintaining the high-resolution of the captured images. Moreover, the whole processes can be easily adopted in simple laboratories with no sophisticated equipments, therefore giving advantages to root researchers in developing countries.

Higher dilution factor and higher P treatments as compared to agar-based system cannot be achieved, as preliminary tests have shown stunted seedling growth and reduced root system size, if grown with higher dilution factor nutrient solutions (results not shown). 15% dilution factor has been the most effective to grow the lettuce seedlings from seeds in

this method (J. Roberts, personal communication, 17 September 2015). As a consequence, no significant treatment differences were seen in this experiment except for primary root diameter (PD) trait. However, trend of growth can be seen from the results, giving some insights of responses to P levels.

Tissue P level investigation may perhaps overcome the issues, giving an insight of plant responses related to the P treatments according to the level of tissue P. This can be done through inductively coupled plasma mass spectrometry (ICP-MS). Nevertheless, significant lettuce parental genotype effects can still be observed, albeit relatively low number of individual plants being used in the experiment, suggesting very strong genetic variances between both of the lettuce parental genotypes. This also qualify for the identification of quantitative trait locus (QTL) which may be represented by either of the lettuce parental genotypes related to the RSA.

4.5 SUMMARY

In this experiment, parental genotypes of lettuce root system architecture (RSA) were assessed, with alternative method, paper-based root assessment method. No significant P treatments was seen in this experiment which might be due to low levels of P in the nutrient solution. Nevertheless, most of the traits have shown a higher significant mean values for domesticated lettuce in comparison to wild lettuce, suggesting strong intrinsic differences between the parental genotypes. Domesticated lettuce has consistently shown preference of LR formation for adaptation to its environment. Additionally, the strong differences are suitable for QTL identification in the mapping population for RSA. Furthermore, minimum requirement of aseptic condition has been a huge advantage over agar-based method. The whole setup was easily replicated, especially by those in a less equipped laboratories. In this experiment, a tissue layer was added to the pouch as the lettuce seeds demands higher moisture content for growth. However, the addition of tissue layer slowed down the data collection. As a result, some of information from undisturbed RSA cannot be recorded.

CHAPTER 5

QUANTITATIVE TRAIT LOCUS (QTL) ANALYSIS OF LETTUCE ROOT ARCHITECTURE USING A PAPER-BASED PHENOTYPING APPROACH

5.1 INTRODUCTION

Quantitative or complex traits often account for the majority of agronomically important crop traits, such as; yield, disease resistance, abiotic stress resistance and the efficiency of water and nutrient use of many crops. In term of root system architecture (RSA), the quantitative trait changes are likely to have been driven by domestication and breeding, which may lead to different spatial arrangement of the roots (de Dorlodot *et al*., 2007). These quantitative traits are often polygenic or controlled by multiple gene loci. In most cases, root morphology is regulated by a suite of small-effect loci, although a few individual loci may have major effects (de Dorlodot *et al.*, 2007), such as the maximum root length QTL detected on chromosome 11 in F_2 population rice crosses of 'Bala' and 'Azucena' varieties, which explain nearly 30% of the variation (Price and Tomos, 1997).

Identification of QTL is important to investigate the genetic control of root traits and to understand phenotypic responses to different environmental conditions (de Dorlodot *et al.*, 2007). This identification can be, at least partly, achieved through QTL analysis, identifying which regions of genes in the genome are associated with significant genetic control through

mapping and QTL analysis, before different approaches such as positional cloning and candidate gene approaches (Salvi and Tuberosa, 2005) are applied to identify the actual genes. A genetic linkage map consists of polymorphic genetic markers and their analysis against accurate phenotypic data in a segregating population is an important tool to successfully locate the associated QTL to the linkage map regions.

Moreover, the QTL analysis opens up the possibility of utilising underutilised crop wild relatives (CRW) traits, which could enhance domesticated cultivars. These potentially beneficial traits may have been eliminated under domestication or breeding selection processes, particularly where the main selection criterion is yield, potentially causing a modern cultivar genetic bottleneck (Tanksley and McCouch, 1997; Consonni *et al.*, 2005; Doebly *et al.*, 2006; Wang and Chee, 2010). CRW can be a rich source of genetic diversity, which has continued to evolve under natural selection, providing us with a dynamic, larger gene pool to be exploited (Brozynska *et al.*, 2015). For instance, loci for tap root length and the ability to extract deeper water sources, has been shown to be driven by wild lettuce allele (Johnson *et al.* 2000). Another example is from the introgression of wild barley into a domesticated barley background, which has improved root and shoot traits, with 15 chromosomal regions identified with co-localised QTLs differing between the wild and cultivated barley (Naz *et al.*, 2014).

In previous chapters, significant RSA variation between lettuce parental genotypes was observed, indicating strong intrinsic genetic variation between wild and cultivated species. In this chapter, the aim was to identify and quantify the QTLs related to RSA in a mapping population of the cross between wild × domesticated lettuce, focusing on the intrinsic genetic variation observed with the paper-based assessment method. The population genetics were analysed in term of the population phenotypic variation, segregation, and correlation between traits, followed by a QTL analysis of the traits. Here, the hypothesis of lateral and primary root traits being controlled by different QTL regions was also tested. The QTL regions were analysed by using a dense map and also a framework map for QTL region validation. It is believed that this analysis is the first report describing the lettuce RSA QTL using this mapping population, specifically grown using the paper-based culture environment.

5.2 MATERIALS AND METHODS

5.2.1 Plant materials

91 F_8 recombinant inbred line (RIL) derived from wild lettuce, *Lactuca serriola* acc. UC96US23 (wild lettuce; Ser) crossed with *Lactuca sativa* cv. Salinas (domesticated lettuce; Sal) (Zhang *et al*., 2007) were used in this experiment with more information provided in **Section 2.1.2.** Parental lines as described in **Section 2.1.1** were also used to perform comparisons with the population dataset.

5.2.2 Plant growth conditions and data collection

The lettuce RIL population were grown as described in **Section 4.2.3.** RSA was segmented and measured based on the trait description in **Section 2.6.**

5.2.3 Experimental design

A 15% dilution of nutrient solution was used in this experiment to study the intrinsic genetic variation of the RIL mapping population using the paper-based method and identify any QTL involved in RSA. A total of 91 RIL plus two parental lines were sown in a randomised complete block design (RCBD) which involved a total of two blocks, with 4 replicates of

each line per block, in controlled environment growth room at Plant Science Building, Sutton Bonington Campus, University of Nottingham, UK.

5.2.4 Statistical analysis

The raw data were entered into GenStat® (Release 17; VSN International, Oxford, UK). Mean, median and range of the data were obtained to visualise the divergence of the traits in the mapping population. To analyse segregation of the traits, mean data (and its standard error of mean) of each trait was used and plotted against the genotypes. Correlation tests were calculated and the results were plotted as described in **Section 4.2.5** to study the correlations of RSA traits within mapping population. Principal component analyses (PCA) was performed using varimax rotation in R Studio (Version 0.99.903, R Studio Team, 2015) using "princomp()" function and plotted using "ggfortify" package. Based on Kaiser-Guttman Criterion's (Yeomans and Golder, 1982), only the first two principal components (PC) were retained and characterised. Codes were shown in Appendix II.

Predicted means of the phenotypic traits were generated by using Reduced Maximum Likelihood (REML) analysis and were used to conduct the QTL analysis. Prior to QTL analysis, the dataset of primary root length (PRL), primary root surface area (PSA), primary root volume (PV), total root

length (TRL), total root surface area (SA) and total root volume (RV) were transformed for normalisation. Average root diameter (AD), primary root diameter (PD), lateral root length (LRL), lateral root surface area (LSA), lateral root diameter (LD), lateral root volume (LV), lateral root number (LRN) and lateral root density (LRD) were all subjected to non-parametric analysis as the distribution could not be normalised by transformation. The normal distribution of all traits and transformed traits were tested using an Anderson-Darling normality test at 5% (Appendix II).

The sources of variation in the analysis considered as random factors were variation between blocks and number of replicates per block. Each block was sown at different times and each block had variation in number of replicates due to germination and growth issues. A random term which include all terms, [(Block/Replicate) + (Genotype)] and no defined fixed factors was used to allocate sources of variation for individual traits. Replicate component variation was nested in the Block component. Subsequently, the means of the traits were estimated by using a fixed term [Genotype] and random term of [(Block/Replicate)].

5.2.5 QTL analysis

5.2.5.1 *QTL file preparation*

In order to be able to perform QTL analysis in MapQTL® (Version 6, Kyazma B. V., Netherland), three important files were needed to be prepared which are 1) quantitative data or 'the phenotypic' file; 2) marker scores or 'the locus' genotypic data file; and 3) population map file.

To produce a quantitative data or phenotypic file, mean values generated by REML analysis in Genstat® for the traits were obtained and exported to a spreadsheet. It was formatted with genotypes or line information in rows and traits in columns. Missing data and missing lines were marked with an asterisk (*). This spreadsheet was then saved as text delimited (.txt) file. To make this file usable with the MapQTL® software, the extension of this file was changed to ".qua". Header information for this file were inserted which defined the number of traits, number of individuals, and the symbol representing the missing data (*) as shown in Figure 5.1A.

The marker scores file contains the genotypic data alleles of the markers. This is also called a locus genotypic data file. The locus genotypic data file extension was changed to ".loc" in order for it to be recognised by MapQTL® software. A header has been inserted to this file as well, which defines the name of the population, type of population (in this case RI8

i.e. RIL population of F_8), number of individuals involved, and number of loci evaluated, as shown in Figure 5.1B. The phenotypic and genotypic data file name needs to be exactly the same in order to function properly in MapQTL® software.

The population map file contains information on the name and position of the markers. The extension of this file is ".map". This file does not need any header instructions. Two maps were used in this study, the framework and dense maps. The construction of the framework map is described in the next section.

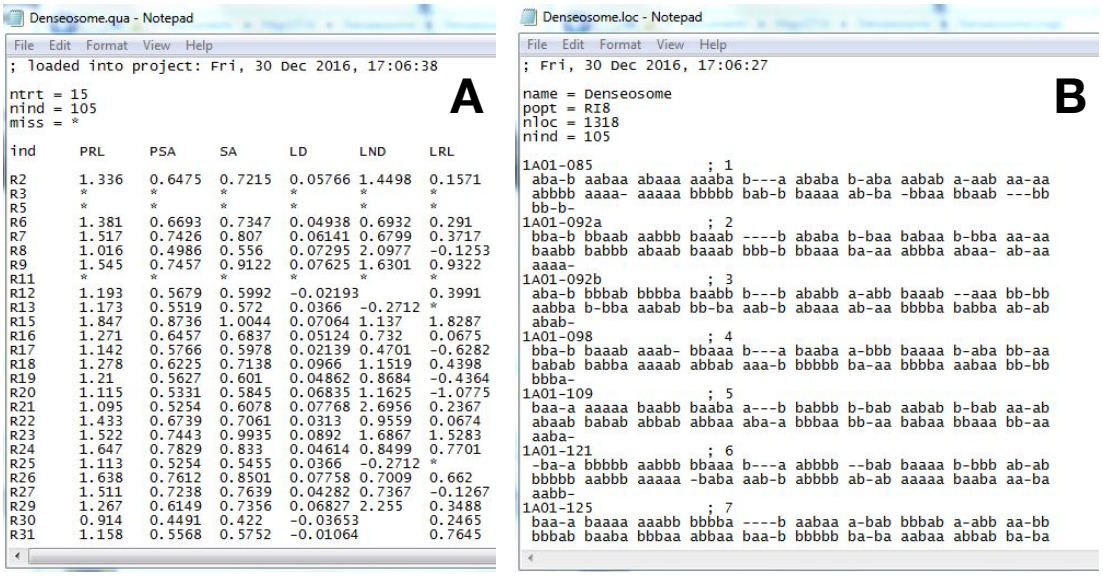


Figure 5.1 Examples of A) qua file and B) loc file with information headings on each file.

5.2.5.2 *Construction of a framework linkage map*

A framework linkage map was used in the study through creation of a spaced marker map, with markers eliminated from a pre-existing denser

map. By using JoinMap® (Version 4.1, Kyazma B. V., Netherland), a list of markers in a pre-existing dense map used in Zhang *et al.* (2007) were first screened to identify those which show double recombination events within short genetic distances and also markers with more than ~30% missing score data of the whole population. Markers were also tested for segregation distortion based on chi squared (χ^2) test and these were eliminated if the markers showed any significant distortion. A total of 242 remaining markers (from a total of 1335 markers) were grouped into nine linkage groups (LG) based on the highest LOD grouping trees, presumably representing nine chromosomes.

These groups were then further thinned out to around 5-10 centiMorgan (cM) marker location interval (where possible). The final framework map was finalised using the regression mapping algorithm and Haldane's mapping function, with other mapping settings set to default. Framework maps loose little in term of IM-based QTL detection power (given even genome coverage) but can help to resolve problems with incorrect local order. KW analysis uses only marker ranking without explicit use of locational information.

5.2.5.3 *QTL analysis approach*

Into the MapQTL® (Version 6, Kyazma B. V., Netherland) software, phenotypic file, locus genotypic data file and population map file were

loaded. In this study, the default settings of the software were used, which include regression model algorithm, LOD test statistic, F2 fit dominance, 1.0 cM mapping step size, maximum five neighbouring markers, maximum 200 iterations, $1.0x10^8$ functional tolerance value, *P*-value for automatic cofactor selection of 0.020 and 1000 permutations. All traits and the entire map were selected at this point to start the QTL analysis.

Primary root length (PRL), primary root surface area (PSA), primary root volume (PV), total root length (TRL), total root surface area (SA) and total root volume (RV) traits were first analysed by using the interval mapping (IM) analysis model. A permutation test (PT) with 1,000 iterations was done prior to IM analysis, and logarithm of odds (LOD) of the Genome-Wide (GW) score at $P \leq 0.05$ for each trait was recorded. A LOD score of a QTL which is equal to or above the respective GW score for each trait in IM analysis were considered significant QTLs. QTL with LOD scores up to 1 LOD drop below the threshold were considered to be 'putative QTL'.

Subsequently, the normally distributed or transformed-normal traits were analysed by using multiple QTL mapping (MQM) analysis to evaluate whether it was possible to narrow down the closest marker linked to the QTLs, through the use of cofactors. The forward method (van Ooijein, 2009) was used in this study, with cofactors to be tested in MQM analysis firstly identified and chosen based on automatic cofactor selection (ACS) analysis. MQM analysis was considered final when the cofactors ceased

to change. The positions of these cofactors were visually verified through LOD profiles and tables produced by MapQTL®.

For average root diameter (AD), primary root diameter (PD), lateral root diameter (LD), lateral root length (LRL), lateral root number (LRN), lateral root surface area (LSA) and lateral root volume (LV), these were analysed by using Kruskal-Wallis (KW) non-parametric, single marker-based QTL analysis. The significant QTLs for each trait were declared if KW statistics passed $P \leq 0.005$ or four asterisks (*) or more (van Ooijein, 2009). The QTLs obtained were not as specific as MQM analysis, rather comparable to that of IM analysis. The results lack the locational information as the KW analysis was based on the sum ranks of all markers.

Benjamini-Hochberg (BH) false discovery rates (FDR) multiple comparison tests for non-parametric dataset were used to confirm any false positive significant markers. *P* values of the K* values from the KW analysis were used to determine the adjusted *P* values. The FDR values were obtained through “p.adjust()” function from “FSA” package (Ogle, 2016) in R Studio (Version 0.99.903, R Studio Team, 2015). The maximum acceptable FDR was set at 5%. Code lines are shown in Appendix II. Highest K* in a region were considered as the most probable genuine QTL peak for the traits (highlighted in the results).

To map the QTLs, the population map file was first loaded into MapChart (Version 2.30, Wageningen UR, Netherland) software. To map non-parametric QTLs, location of significant QTL were determined based on the nearest markers. These locations were marked with the significant level and the QTL trait represented. To map other traits analysed by MQM analysis, the highest value of the QTL LOD score profile was identified in the IM or MQM analysis results. Confidence intervals of 1- and 2-LOD drops of the QTL peak were identified and represented in the MapChart software. The method was applied to both QTL analysis using the framework and also the dense map reported by Zhang *et al.* (2007).

5.2.6 Seed quality determination

In this study, 211 seeds (29%) from a total of targeted 728 seed replicates from 91 lines have been considered missing due to failure of seeds to either germinate or grow properly. In order to include these genotypes in the dataset, at least partly not due to obvious viability and dormancy issues, a germination and 2,3,5-triphenyl tetrazolium chloride (TZ; Sigma-Aldrich) assay of seed viability tests was done. These test results can be used to determine the dormancy percentage (SD%) of the lines based on percentage differences between both tests. The details of the test were described in **Section 2.7.**

5.3 RESULTS

5.3.1 Seed viability, germination rate and dormancy levels

In this experiment, 29% of the whole targeted replicates were considered missing in the dataset, in which the RIL seeds sown did not produce seedlings that can be measured and included in the analysis. Based on Table 5.1, it is shown that 88.7% (i.e. seed viability % [SV%]) of the seeds from missing data lot is fully viable for the QTL studies and is not inviable due to seed damage. Seeds with viability less than 90% were discarded from analysis. 36.5% showed some degree of dormancy, nevertheless, these genotypes were accepted in the analysis.

Table 5.1 Seed viability %, seed germination % and seed dormancy % of seeds from missing data lot. *SD% = SV%-SD%.

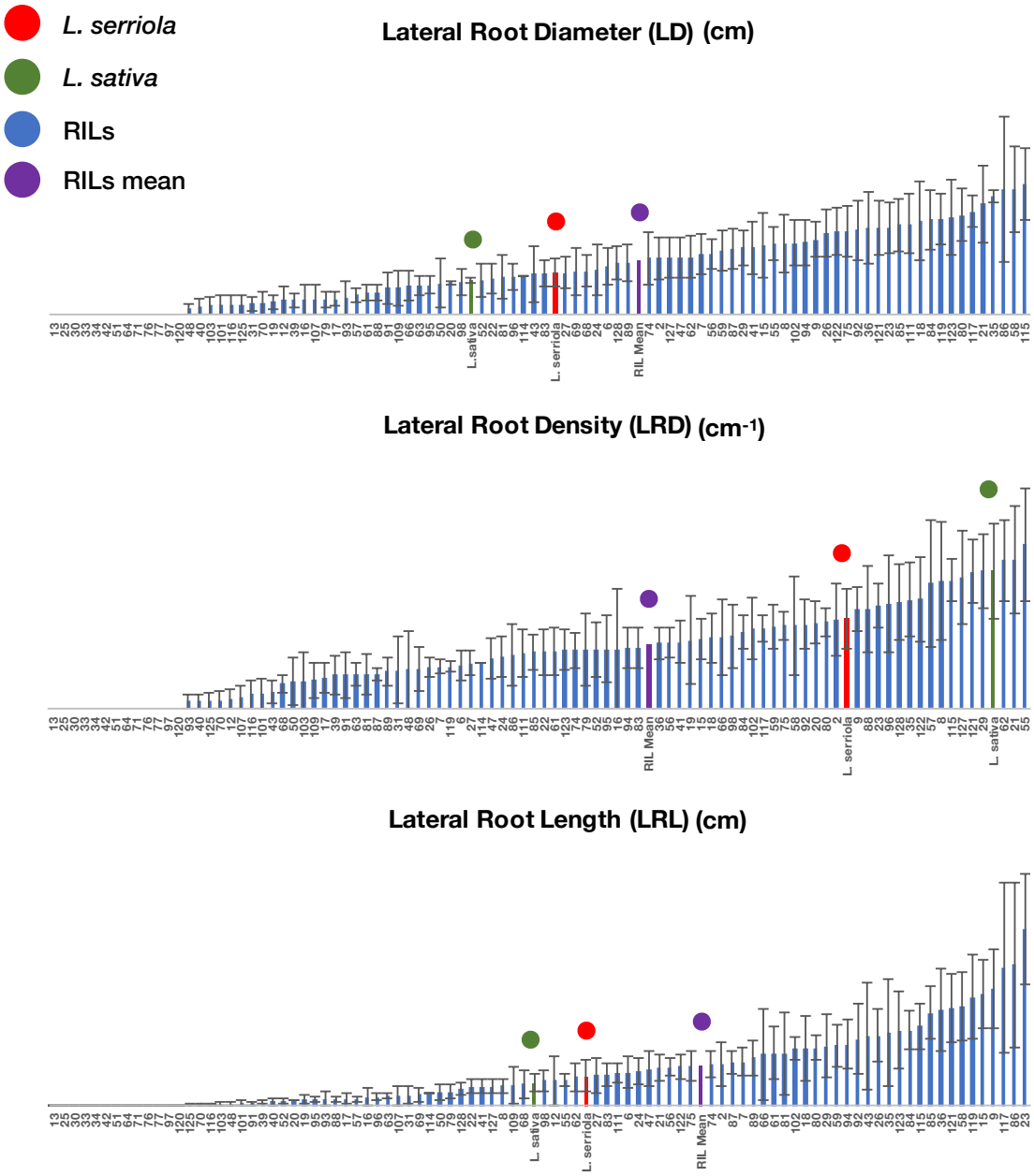
Genotype	Seed Viability (SV) %	Seed Germination (SG) %	Seed Dormancy* (SD) %
17	100.0	100.0	0.0
19	100.0	100.0	0.0
20	100.0	100.0	0.0
21	100.0	100.0	0.0
22	100.0	100.0	0.0
26	100.0	100.0	0.0
30	100.0	33.3	66.7
31	100.0	10.0	90.0
34	100.0	0.0	100.0
35	100.0	90.0	10.0
36	100.0	50.0	50.0
40	100.0	100.0	0.0
42	0.0	0.0	0.0
43	100.0	100.0	0.0
47	100.0	80.0	20.0
48	100.0	90.0	10.0
50	100.0	100.0	0.0
51	100.0	100.0	0.0
52	100.0	100.0	0.0
57	100.0	100.0	0.0
58	100.0	100.0	0.0
59	100.0	100.0	0.0
61	100.0	100.0	0.0
63	100.0	100.0	0.0
64	100.0	0.0	100.0
66	100.0	100.0	0.0
71	100.0	10.0	90.0
76	100.0	60.0	40.0
79	100.0	100.0	0.0

Genotype	Seed Viability (SV) %	Seed Germination (SG) %	Seed Dormancy* (SD) %
81	100.0	30.0	70.0
83	100.0	100.0	0.0
85	100.0	100.0	0.0
86	100.0	100.0	0.0
91	100.0	100.0	0.0
93	100.0	100.0	0.0
95	90.0	70.0	20.0
97	100.0	20.0	80.0
101	90.0	0.0	90.0
103	100.0	10.0	90.0
107	40.0	33.3	6.7
109	100.0	100.0	0.0
114	75.0	0.0	75.0
115	100.0	100.0	0.0
116	100.0	0.0	100.0
117	100.0	100.0	0.0
118	100.0	100.0	0.0
119	100.0	100.0	0.0
120	100.0	100.0	0.0
122	100.0	100.0	0.0
123	100.0	100.0	0.0
125	100.0	0.0	100.0
127	100.0	90.0	10.0
L. sativa	100.0	100.0	0.0
L. serriola	90.0	20.0	70.0

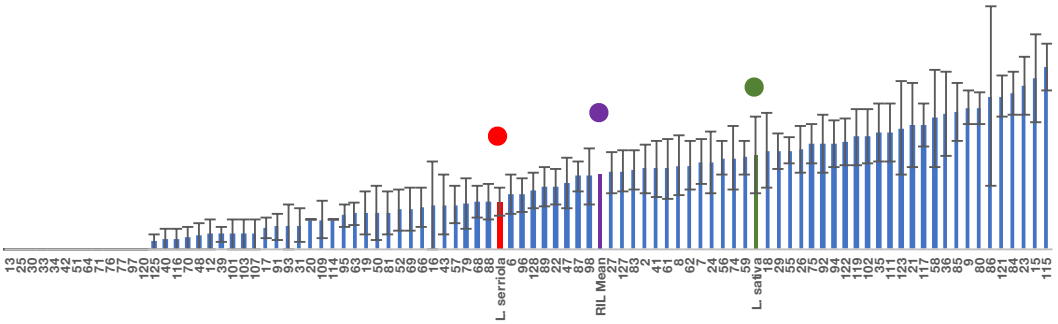
5.3.2 Phenotypic variation within the mapping population

A total of 517 viable seedlings were analysed in this experiment. Generally, broad differences can be seen from the segregation plots of mean of pre-normalised data for each of the traits within the mapping population, except for primary root diameter (PD). The mean of domesticated lettuce (Sal) were higher than wild lettuce (Ser) in most of the traits except for lateral root surface area (LSA), lateral root density (LRD), lateral root volume (LV) and average root diameter (AD). Most of the trait means of the RILs were higher than the parental lines, except for PD, primary root volume (PV), lateral root number (LRN), and LRD. The mean of RILs of trait PD, LRN and LRD were in between the parental line means, while mean RILs of PV was equal to Sal. Additionally, transgressive segregations were evident in most of the traits i.e. some RILs showed trait means of both higher and lower extremes than the two parental lines. This was observed generally in all traits (Figure 5.2, Table 5.2).

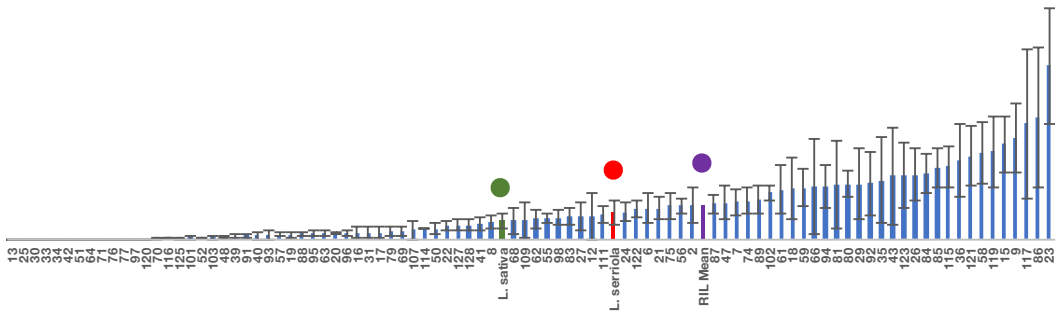
All the primary and global root traits normalised. The distributions are shown in Appendix II. On the other hand, the absence of lateral roots which can be observed in some lines (Figure 5.2: LRN), has a subsequent effect on other related lateral root phenotypes. This large number of 'zero' scores affected the distribution of the population data and hindered further data normalisation for these traits.


L. serriola
L. sativa
RILs
RILs mean
Lateral Root Diameter (LD) (cm)
Lateral Root Density (LRD) (cm-1)
Lateral Root Length (LRL) (cm)

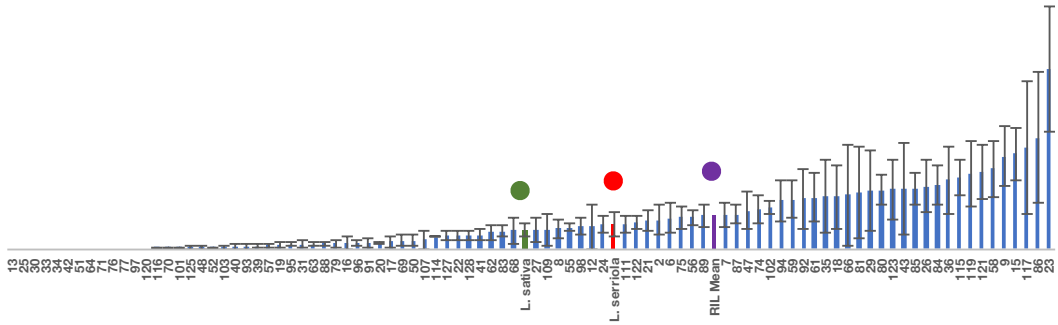
Lateral Root Number (LRN)



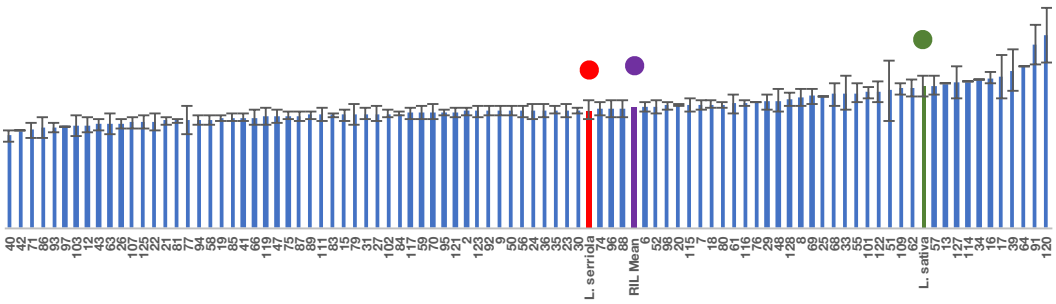
Lateral Root Surface Area (LSA) (cm^2)



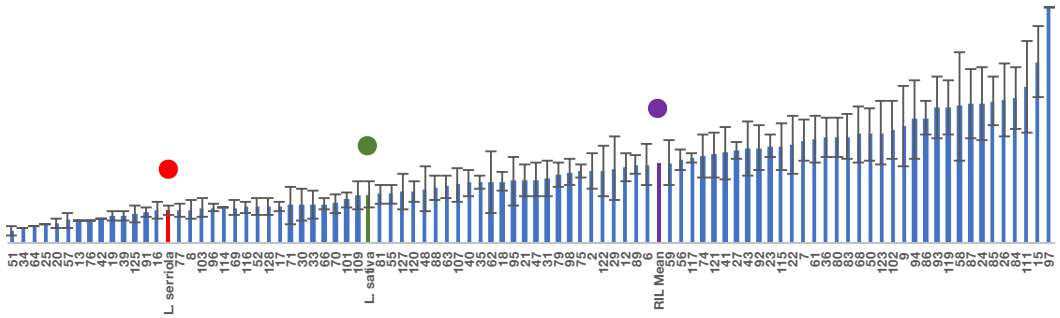
Lateral Root Volume (LV) (cm^3)



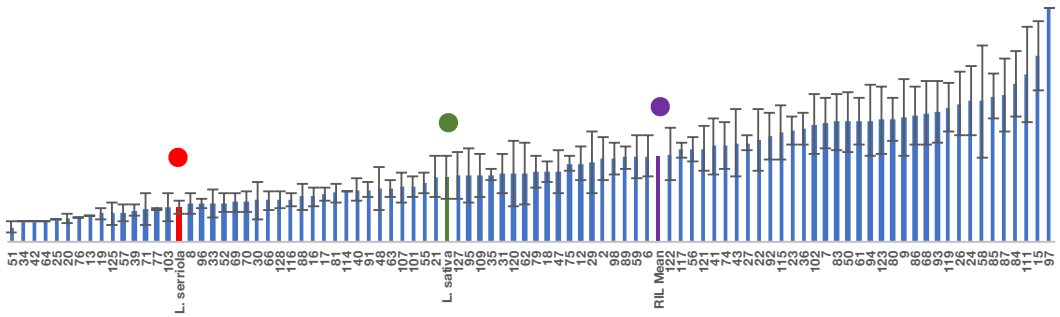
Primary Root Diameter (PD) (cm)



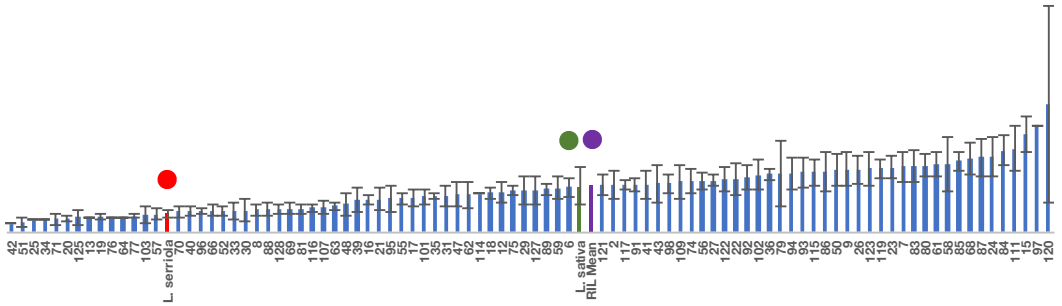
Primary Root Length (PRL) (cm)



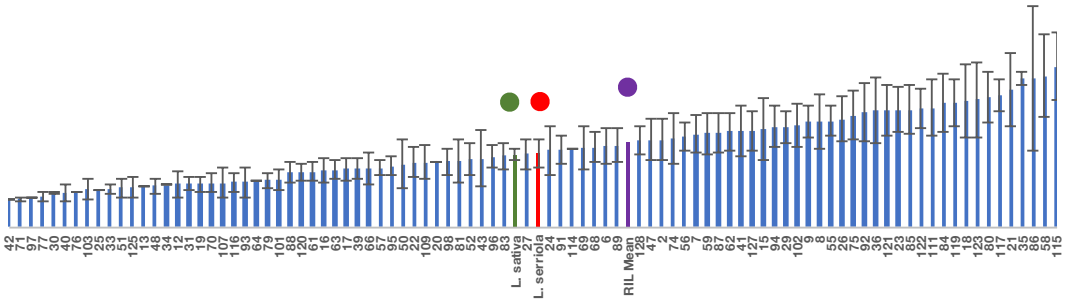
Primary Root Surface Area (PSA) (cm^2)



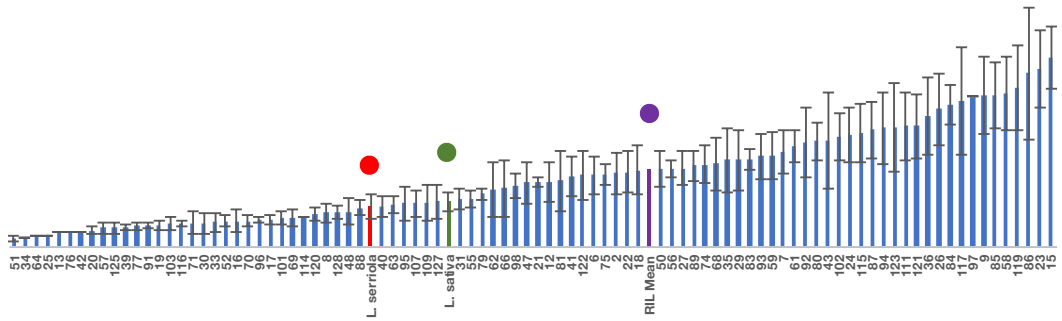
Primary Root Volume (PV) (cm^3)



Total Average Root Diameter (AD) (cm)



Total Root Length (TRL) (cm)



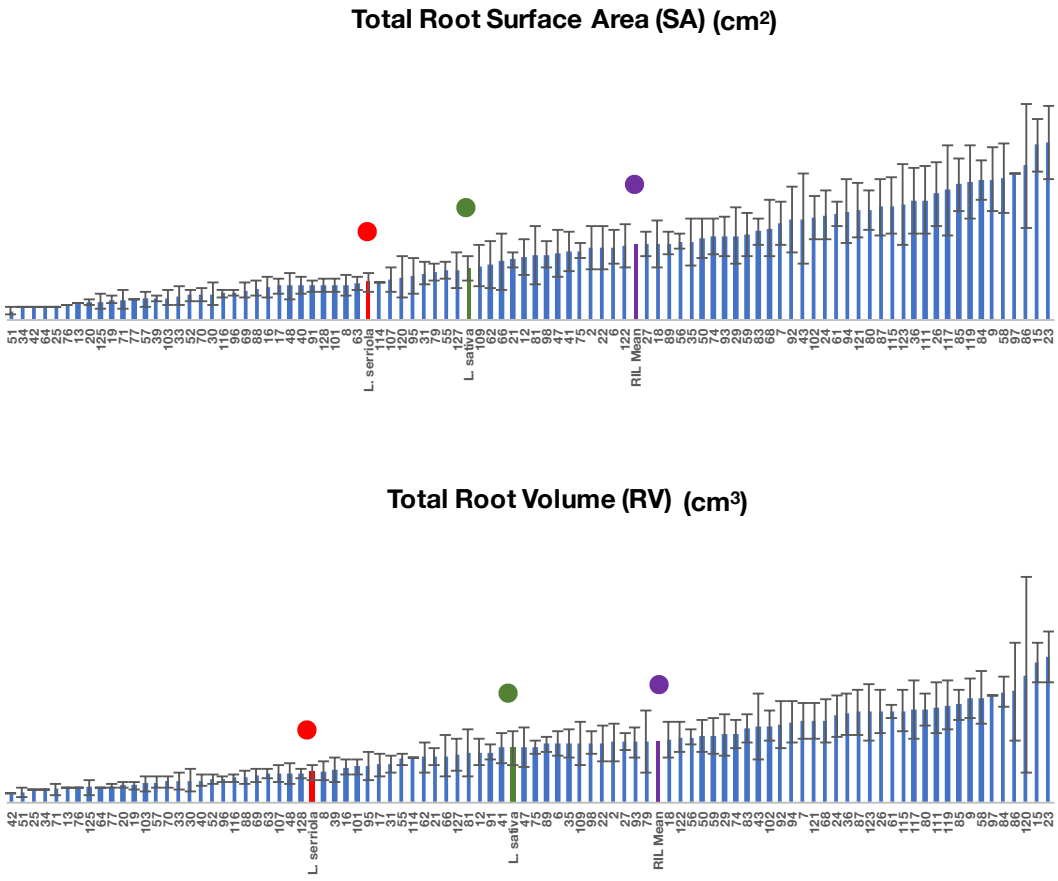


Figure 5.2 Segregation across the population of root traits grown using the paper-based method. Each bar graph is a ranking of 91 RILs plus two parental genotypes, *Lactuca sativa* (domesticated lettuce), *L. serriola* (wild lettuce) and RIL mean. Title on top of each graph represents its respective root traits. Each bar representing mean of eight replicates across two blocks of experimental run. Green, red and purple circles each representing *L. sativa, L. serriola* and RIL mean respectively. PD = primary root diameter; LRD = lateral root density; LV = lateral root volume; LSA = lateral root surface area; LRL = lateral root length; AD = average root diameter; LD = lateral root diameter; LRN = lateral root number; TRL = total root length; SA = total root surface area; V = total root volume; PRL = primary root length; PV = primary root volume; PSA = primary root surface area.

Table 5.2 Pre-normalised parental genotype mean, median and RILs range values for measured root traits within the mapping population. PD = primary root diameter; LRD = lateral root density; LV = lateral root volume; LSA = lateral root surface area; LRL = lateral root length; AD = average root diameter; LD = lateral root diameter; LRN = lateral root number; TRL = total root length; SA = total root surface area; RV = total root volume; PRL = primary root length; PV = primary root volume; PSA = primary root surface area. *Lactuca serriola* is the wild lettuce and *L. sativa* is the domesticated lettuce. RILs = recombinant inbred lines. Values followed by ± are standard error of means (SEM).

Trait	Parents				RILs		
	L. serriola		*L. sativa*		Min	Max	Mean
	Mean	Median	Mean	Median			
PRL (cm)	1.231±0.191	1.0970	1.854±0.510	1.8640	0.1879	15.1817	3.0590
PSA (cm^2)	0.1314±0.026	0.1142	0.250±0.083	0.2189	0.0010	1.4907	0.3300
PD (cm)	0.036±0.003	0.0374	0.043±0.003	0.0417	0.0173	0.0799	0.0367
PV (cm^3)	0.001±0.0003	0.0012	0.003±0.001	0.0023	0.0000	0.0291	0.0032
LRL (cm)	1.211±0.620	0.6443	0.900±0.356	0.8708	0.0000	16.9838	1.6380
LSA (cm^2)	0.099±0.048	0.0634	0.069±0.027	0.0670	0.0000	1.5448	0.1330
LD (cm)	0.043±0.014	0.0348	0.034±0.003	0.0353	0.0000	0.2858	0.0539
LV (cm^3)	0.0008±0.0004	0.0006	0.0006±0.00002	0.0005	0.0000	0.0131	0.0010
LRN	1.625±0.461	2.0000	3.250±1.315	2.5000	0.0000	12.0000	2.5780
LRD (cm^{-1})	1.474±0.493	0.9868	2.255±0.790	2.4845	0.0000	8.2055	1.0450
TRL (cm)	2.442±0.696	1.7230	2.754±0.576	3.2020	0.1879	21.8568	4.6970
SA (cm^2)	0.230±0.059	0.1841	0.320±0.077	0.3340	0.0010	2.0714	0.4630
RV (cm^3)	0.002±0.0005	0.0018	0.004±0.001	0.0032	0.0000	0.0291	0.0043
AD (cm)	0.039±0.008	0.0358	0.039±0.003	0.0387	0.0086	0.1600	0.0453

Principal component analyses (PCA) in Figure 5.3 showed some similar classes of trait clustering together, especially between the lateral root traits and primary root traits. The first two components of variation explained 88.1% of the variations. Component 3 and greater have variation percentage (%) below the Kaiser-Guttman Criterion's threshold. In PCA plots, clustering of vectors is an indication of correlation among traits, while vector length and direction indicate association with a particular component (Burton *et al*., 2014). Interestingly, LRL, LRN, LRD and LSA loadings were positively directed in y-axis, while the PRL, PSA, TRL and SA loadings were all separated and directed in an opposite direction in the biplot. LRL, LRN and LRD were closely clustered together. Since the LRL and PRL were calculated together to obtain the TRL value, it is clear that the vector of TRL is the vector sum of LRL and PRL, showing the association of LRL, PRL and TRL. Same case applied to association between LSA, PSA and SA components. The results showed overall explained variation by primary and global root traits for PC1, whilst PC2 by lateral root traits.

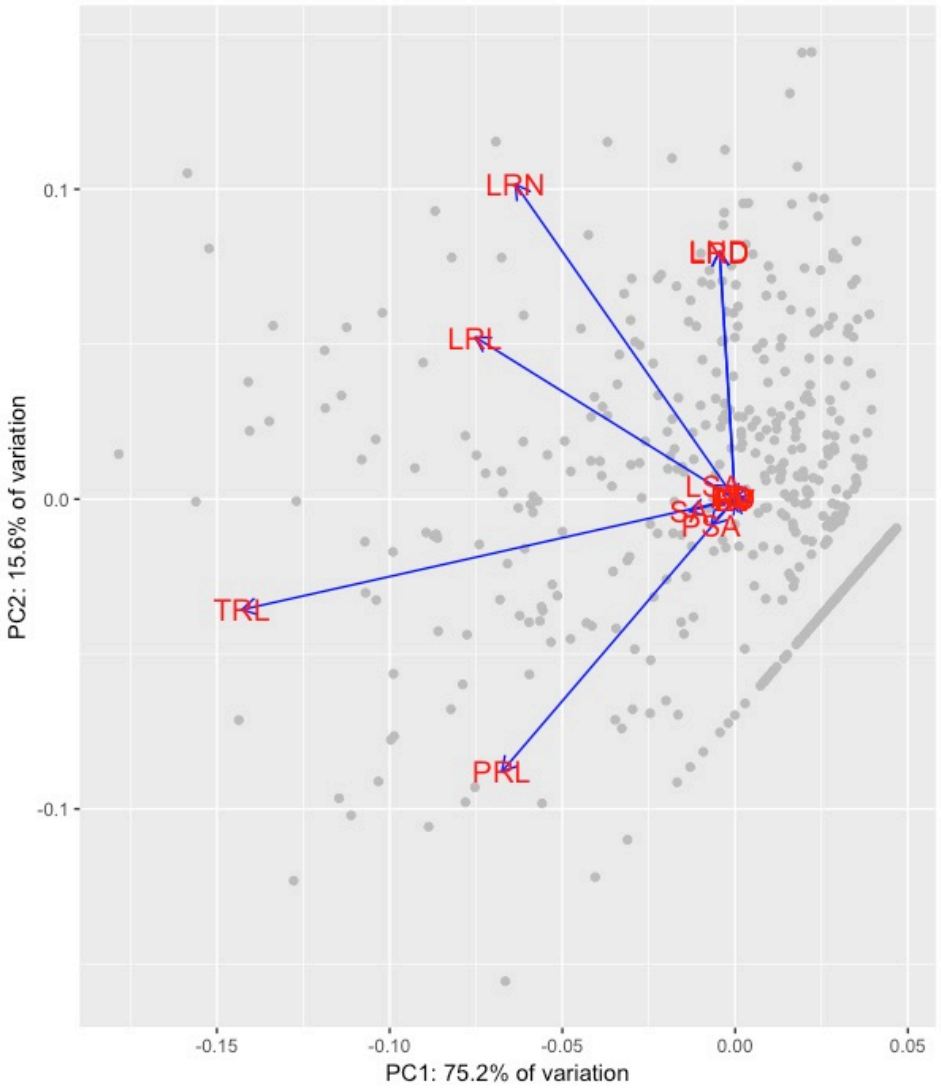


Figure 5.3 Principal component analysis (PCA) of root architectural traits in the recombinant inbred lines (RIL) mapping population of L*actuca serriola* acc. UC96US23 × *Lactuca sativa* cv. Salinas (wild × domesticated lettuce). The x and y axes are components 1 and 2, respectively. Axis labels include explained variation percentage (%) by each of these components. PD = primary root diameter; LRD = lateral root density; LV = lateral root volume; LSA = lateral root surface area; LRL = lateral root length; AD = average root diameter; LD = lateral root diameter; LRN = lateral root number; TRL = total root length; SA = total root surface area; RV = total root volume; PRL = primary root length; PV = primary root volume; PSA = primary root surface area.

5.3.3 Correlation between traits

Correlation results showed a general positive correlations between root traits within the population. Based on the Spearman's rank correlation (r_s) analysis (Figure 5.4), strongest correlations ($| r | \geq 0.8$) can be seen between same classes of lateral-lateral, primary-primary and global-global root traits. Some strong primary-global root traits correlation can be seen as well. These are consistent with trend showed by principal component analysis (PCA). For example, associations between lateral root length (LRL) and lateral surface area (LSA), lateral root diameter (LD) and lateral root volume (LV), primary root volume (PV) and total root surface area (SA), and total root length (TRL) and total root volume (RV) have shown r_s values of 0.99, 0.85, 0.89 and 0.92 respectively, values near to perfect relationship of 1. Additionally, groups of either moderately ($0.5 < | r | < 0.8$) or weakly ($0.2 \leq | r | \leq 0.5$) correlated were visibly prominent between lateral-primary root traits such as PV and LV ($r_s = 0.47$) and TRL and lateral root density (LRD) ($r_s = 0.27$). Most of primary root length (PRL) and primary root surface area (PSA) showed negative correlations to other traits, for instance, PSA and LSA ($r_s = -0.51$) and PRL and lateral root number (LRN) ($r_s = -0.46$). However, these two itself were strongly positive correlated to each other ($r_s = 0.97$). Some inconclusive correlation trend were seen in PRL, PSA and PD such as PRL-LRD, PSA-LRD, and PD-PV.

Similar pattern of results can be seen when parametric traits being analysed using Pearson's product-moment correlation (r_p) analysis (Figure 5.5). Most of the pairwise showed positive correlations except for PSA and PRL. Most of relationship of PSA and PRL were negatively correlated except for correlation between these two traits itself, which show strong positive correlation (r_p = 0.96). The results with Pearson's correlation analysis also showed that most of the pairwise has strong correlations which include relationship between SA-TRL (r_p = 0.97), RV- PV (r_p = 0.93) and PSA-TRL (r_p = -0.89).

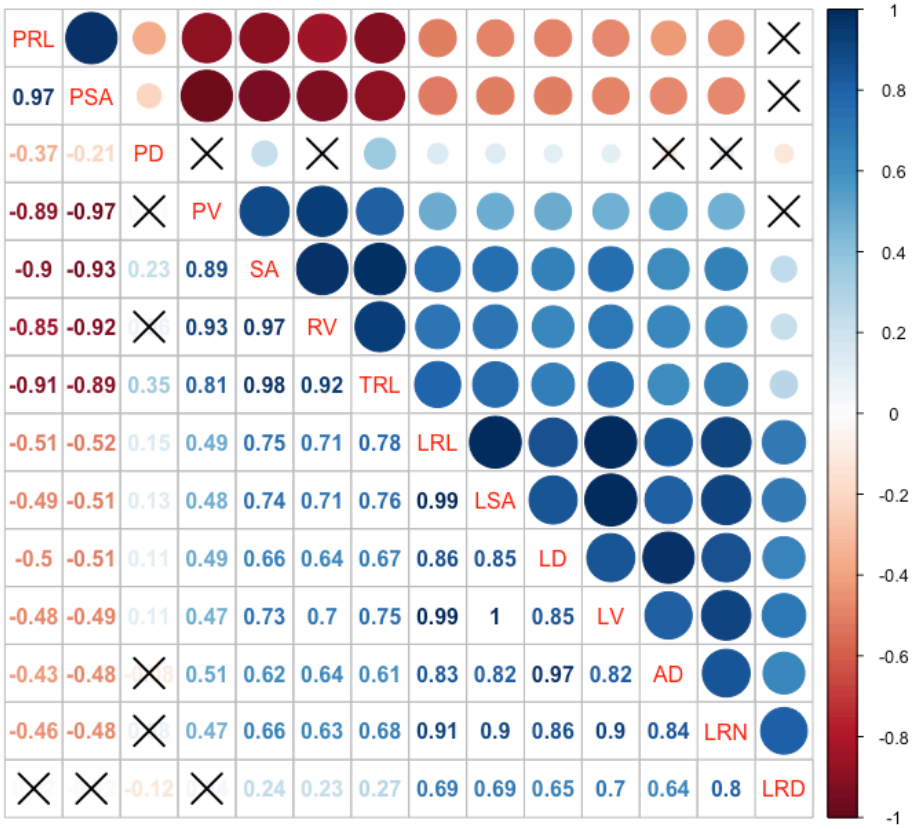


Figure 5.4 A correlogram of traits analysed using the paper-based method based on Spearman's rank correlation (r_s) analysis. Blue circles are showing positive correlations whilst the red showing negative correlations. The circles are organised in angular order of the eigenvectors (Friendly, 2002). X means non-significant correlations ($P > 0.05$). PD = primary root diameter; LRD = lateral root density; LV = lateral root volume; LSA = lateral root surface area; LRL = lateral root length; AD = average root diameter; LD = lateral root diameter; LRN = lateral root number; TRL = total root length; SA = total root surface area; V = total root volume; PRL = primary root length; PV = primary root volume; PSA = primary root surface area.

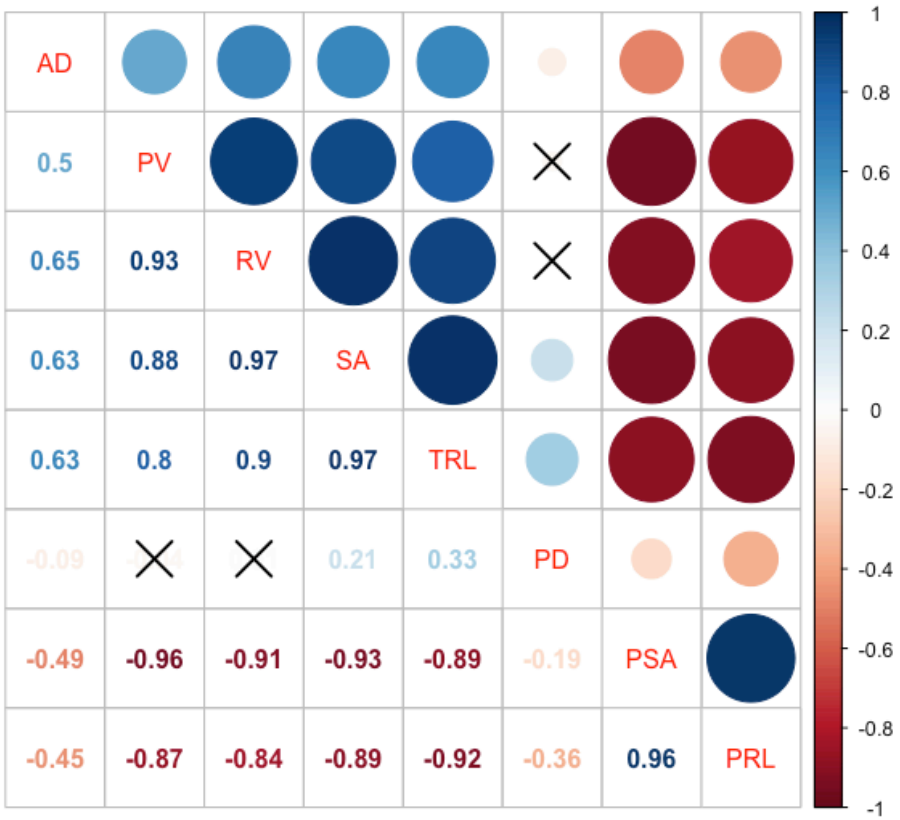


Figure 5.5 A correlogram of traits analysed in paper-based method based on Pearson's product-moment correlations (r_p) analysis. Blue circles are showing positive correlations whilst the red showing negative correlations. The circles are organised in angular order of the eigenvectors (Friendly, 2002). X means non-significant correlations ($P > 0.05$). PD = primary root diameter; AD = average root diameter; TRL = total root length; SA = total root surface area; RV = total root volume; PRL = primary root length; PV = primary root volume; PSA = primary root surface area.

5.3.4 Percentage variation contribution

Analysis of data through REML procedures generated allocation of variations contributed based on random term of [(Blocks/Replicates) + Genotypes] and no defined fixed factors. These variations sources were converted into percentages and summarised in Table 5.3. However, some of the original variance values in the Blocks x Replicates column generated by GenStat® have resulted in negative values, therefore, truncation has been done between sources of variation in order to convert these values into percentages.

Table 5.3 Percentage contributions for variance components generated from REML analysis of measured root traits in the mapping population. *0.00% values shown were truncated from negative variance allocation into zero values to convert variances into %. θA Wald test statistic was calculated to identify the significant sources of variation by using F-based statistic or χ^2 test on values marked with '^' (d.f. = 90; *** = $P < 0.001$).

Traits	Blocks	Blocks x Replicates*	Genotypes$^{\theta,***}$	Residual
TRL	39.86	0.00	32.97	27.17
SA	33.58	0.00	32.62	33.80
RV	28.41	0.00	28.58	43.01
LRN	7.86	0.00	27.47^	63.96
PSA	36.12	0.00	27.59	35.59
PRL	45.29	0.00	27.24	26.85
PV	26.00	0.00	22.94	51.06
AD	36.57	0.00	16.12	47.31
PD	18.63	0.25	18.77	62.35
LRL	17.34	0.69	19.08	62.89
LSA	15.17	0.52	18.33	65.97
LV	13.18	0.38	18.22	68.22
LD	40.64	0.00	16.49	42.86
LRD	8.49	0.00	9.81^	81.70

5.3.5 Root trait QTL mapping

A total of 13 traits related to the lettuce seedlings root system architecture (RSA) were recorded when the lines were grown using the paper-based method and analysed for quantitative trait loci (QTLs). Based on the distribution profile of the obtained phenotypic data, the results were classed into parametric Interval/multiple QTL mapping (IM/MQM) analysis and non-parametric Kruskal-Wallis (KW) QTL analysis.

5.3.5.1 *IM and MQM analysis for normally distributed or transformed-normal phenotypic data*

Multiple QTL mapping (MQM) analysis of both framework and dense map results' have shown six significant QTLs, represented by primary root length (PRL), primary root surface area (PSA), primary root volume (PV), total root length (TRL), total root surface area (SA) and total root volume (RV) traits. These QTLs were all clustered at a similar locus region nearest to marker LE1360 or LE0261, all situated on linkage group (LG) 1. No significant QTL were detected for average root diameter (AD) as all markers in the interval mapping (IM) analysis were below their respective genome-wide (GW) LOD threshold generated from the permutation test at $P \leq 0.05$, which was therefore not eligible for MQM analysis. However, a single putative QTL was detected when tested using dense map.

The effects of these locus on each trait using the framework map were as follows:

PRL: The significant QTL was identified at 23.1 cM on LG1 with a LOD score of 7.59 and phenotypic variation explain (PVE) of 31.7%. The nearest locus was represented by LE1360 located at 24.6 cM. This QTL has the highest PVE% compared to other traits.

PSA: A QTL was mapped at location 22.1 cM on LG1 with LOD score 6.79 and explained 29.9% of the total phenotypic variation. The nearest marker for the QTL location would be LE1360 at 24.6 cM.

PV: A QTL was mapped at location 22.1 cM on LG1 with LOD score of 5.29. The QTL location explained 24.2% of the PVE, with nearest marker reported as LE0261 located at 20.1 cM.

TRL: Location of QTL with highest LOD score of 6.46 was reported at 23.1 cM, with nearest marker LE1360 at 24.6 cM. The QTL explained 28.7% of the PVE.

SA: Nearest marker LE1360 representing the QTL with highest LOD of 6.12, explaining 27.4% of the PVE. It is located at 23.1 cM on LG1.

RV: Highest LOD of 4.93 was reported at location 22.1 cM on LG1 with PVE of 22.7%. The nearest marker is represented by LE1360 at 24.6 cM.

By using the dense map, the location of the QTL obtained was generally represented by a specific marker. The effects of these locus tested by using dense map are described as below:

PRL: The trait QTL was located at 42.8 cM of LG1 with LOD 8.18, represented by marker LE1360. It explained 34.8% of the PVE. This QTL has the highest PVE as compared to other traits.

PSA: Marker LE1360 represented the QTL at location 42.8 cM on LG1 with LOD 6.41, accounted for 28.5% of the PVE.

PV: Highest LOD of 5.59 of the trait was located at 42.8 cM, represented by marker LE1360, with total PVE of 25.7%.

TRL: A total of 28.7% explained phenotypic variation was seen in QTL at 42.8 cM on LG1. The location was represented by marker LE1360 with LOD score of 6.47.

SA: The trait was mapped with one significant QTL located at 42.8 cM on LG1. The significant QTL was linked with marker LE1360, with LOD score of 6.18 and accounted for 27.6% PVE.

RV: A significant QTL was identified that linked to marker LE1360 at 42.8 cM on LG1 with LOD 4.93. It explained 22.7% of total PVE.

AD: A putative QTL was identified at location 7.7 cM in LG7 that linked to marker LE3082. The LOD is 2.36, passing the putative QTL threshold of 2.3 for AD, which explained 11.6% of the PVE.

A summary of the comparison between MQM analyses utilising the framework and dense map with information on LOD score, position of QTLs, location of nearest markers, PVE and additive effect is presented in Table 5.4 and Table 5.5 respectively. Figure 5.6 shows the graphical representation of the confidence interval of the location of the QTL for each trait with significant QTL.

Table 5.4 Significant QTL associated with root traits measured within the mapping population using the framework map in MQM analysis. The QTL were determined using REML generated estimated means. *permutation test threshold by using 1000 reiterations at $P \leq 0.05$. Positive additive effect indicates QTL driven by *L. sativa* 'Sal' (domesticated lettuce) allele to trait value. PRL = primary root length, PSA = primary root surface area, PV = primary root volume, TRL = total root length, SA = total root surface area, RV = total root volume, LG = linkage group, PVE% = percentage of phenotypic variation explained.

Traits	LG	Position (cM)	Nearest marker	LOD	PT*	PVE%	Additive effect
PRL	1	23.1	LE1360 (24.6 cM)	7.59	2.9	31.7	-0.05
PSA	1	22.1	LE1360 (24.6 cM)	6.79	3.1	29.9	-0.11
PV	1	22.1	LE0261 (20.1 cM)	5.29	3.0	24.2	0.02
TRL	1	23.1	LE1360 (24.6 cM)	6.37	2.9	28.3	0.43
SA	1	23.1	LE1360 (24.6 cM)	5.91	3.1	26.6	0.39
RV	1	22.1	LE1360 (24.6 cM)	4.62	3.1	21.5	0.02

Table 5.5 Significant QTL associated with root traits measured within mapping population using dense map in MQM analysis. The QTL were determined using REML generated estimated means. *permutation test threshold by using 1000 reiterations at $P \leq 0.05$. Positive additive effect indicates QTL driven by *L. sativa* 'Sal' (domesticated lettuce) allele to trait value. # indicates putative QTLs. PRL = primary root length, PSA = primary root surface area, PV = primary root volume, TRL = total root length, SA = total root surface area, RV = total root volume, LG = linkage group, PVE% = percentage of phenotypic variation explained.

Traits	LG	Position (cM)	Nearest marker	LOD	PT*	PVE%	Additive effect
PRL	1	42.8	LE1360 (42.8 cM)	8.18	3.4	34.8	-0.05
PSA	1	42.8	LE1360 (42.8 cM)	6.41	3.4	28.5	-0.11
PV	1	42.8	LE1360 (42.8 cM)	5.67	3.3	25.7	0.02
TRL	1	42.8	LE1360 (42.8 cM)	6.47	3.3	28.7	0.41
SA	1	42.8	LE1360 (42.8 cM)	6.18	3.5	27.6	0.38
RV	1	42.8	LE1360 (42.8 cM)	4.93	3.4	22.7	0.02
AD#	7	7.7	LE3082 (7.7 cM)	2.36	3.3	11.6	-0.49

5.3.5.2 *KW analysis for non-parametric phenotypic data distribution*

The non-parametric, single marker-based Kruskal-Wallis (KW) QTL analysis on lateral roots parameters namely average root diameter (AD), primary root diameter (PD), lateral root length (LRL), lateral root surface area (LSA), lateral root diameter (LD), lateral root volume (LV) and lateral root number (LRN) gave several significant peaks across LG1, LG4, LG5 and LG8. These peaks were shown to be different to those of the QTL locations of primary root parameters suggesting a different mechanism of control.

Based on results, QTL were detected at $P \leq 0.005$ in PD, LRL, LSA, LV and LRN across three linkage groups of LG1, LG5 and LG8 when tested with framework map. LRL, LSA and LV have showed similar QTL on LG5. No QTL was detected for AD and LD. The effect of the locus were shown below:

PD: A total of four significant QTLs were detected above *P*-value of 0.005 across the linkage groups of LG1 and LG8. K* values were between 9.241-11.136.

LRL: A significant QTL represented LRL at location 120.8 cM in LG5 with K* value of 9.377 with $P=0.005$. The marker representing the QTL is 1A06-109.

LSA: A significant peak was detected at location 120.8 cM in LG5 with K* value of 9.906 with *P*=0.005. The marker representing the QTL is 1A06-109.

LV: A similar peak was representing LV as well at location 120.8 cM in LG5 with K* value of 9.134 with *P*=0.005. The marker representing the QTL is 1A06-109.

LRN: In LG8, the marker LE0138 at location 99.0 cM surpassed the threshold value of *P*=0.005, with K* value of 7.933.

On the other hand, QTLs were detected in PD, LRL, LSA, LV, LD and LRN after phenotypic data was tested using the dense map. No QTL was detected from AD trait. The effects of these locus on each trait are described as follows:

PD: A total of 15 significant QTLs were detected which pass the *P*=0.005 threshold, scattered across three LGs, LG1, LG5 and LG8. K* values of these markers were between 8.056-11.136.

LRL: A total of 11 significant QTLs were identified, all located at clustered position in LG1 and LG5. K* values of these markers were between 8.162-11.155.

LSA: There were 13 significant QTLs were discovered, mapped across two LGs of LG1 and LG5. K* values of the mapped QTLs were between 7.974-11.411.

LV: A total of 12 significant QTLs were identified across three LGs, LG1 LG4 and LG5. K* values of these location were reported at between 8.006-12.193.

LD: A single QTL was identified at LG4 at position 30.3 cM which is represented by marker LE1313. K* value was reported at 9.451 with *P*=0.005.

LRN: Five QTLs passed the threshold level at *P*=0.005, scattered at LG1, LG4 and LG8, with K* value ranging from 7.933-9.736.

A summary of KW analysis of QTLs identified using the dense map with information of location of QTLs, representative markers, K* value, *P* value, adjusted *P* value and allelic means are shown in Table 5.6 and Table 5.7. These location of the QTLs are also shown in Figure 5.7.

Table 5.6 Significant QTL associated with root traits measured within mapping population using framework map using KW analysis. The QTL were determined using REML generated estimated means. Location with significant ($P \leq 0.005$) K* values qualify for QTL selection. Mean-a indicates mean allele originated from *L. sativa* 'Sal' (domesticated lettuce) while mean-b from *L. serriola* (wild lettuce). Highlighted marker imply the most probable genuine QTL peak in the region. LRL = lateral root length, LSA = lateral root surface area, LV = lateral root volume, LD = lateral root diameter, LRN = lateral root number, LG = linkage group.

Traits	LG	Position (cM)	Nearest marker	K*	*P*	Mean-a	Mean-b
PD	**1**	**15.9**	**M1730**	**11.136**	**0.001**	**3.062**	**2.972**
	1	20.1	LE0261	9.534	0.005	3.064	2.977
	1	24.6	LE1360	9.241	0.005	3.074	2.968
	8	**52.8**	**LE1089**	**9.887**	**0.005**	**2.942**	**3.067**
LRL	**5**	**120.8**	**1A06-109**	**9.377**	**0.005**	**1.839**	**0.086**
LSA	**5**	**120.8**	**1A06-109**	**9.900**	**0.005**	**0.149**	**0.072**
LV	**5**	**120.8**	**1A06-109**	**9.134**	**0.005**	**0.001**	**0.001**
LRN	**8**	**99.0**	**LE0138**	**7.933**	**0.005**	**2.717**	**1.529**

Table 5.7 Significant QTL associated with root traits measured within the mapping population using dense map using KW analysis. Mean-a indicates mean allele originated from *L. sativa* 'Sal' (domesticated lettuce) while mean-b from *L. serriola* (wild lettuce). Highlighted marker imply the most probable genuine QTL peak in the region. PD=primary root diameter, LRL = lateral root length, LSA = lateral root surface area, LV = lateral root volume, LG = linkage group.

Traits	LG	Position (cM)	Marker	K*	*P*	Mean-a	Mean-b
PD	1	33.5	L2222	8.103	0.005	3.043	2.976
	1	**35.6**	**M1730**	**11.136**	**0.001**	**3.062**	**2.972**
	1	37.8	LE3223	8.946	0.005	3.062	2.974
	1	39.0	LE0261	9.534	0.005	3.064	2.977
	1	39.7	E44/M48-F-114	11.105	0.001	3.055	2.973
	1	42.1	1A02-246	10.865	0.001	3.061	2.965
	1	42.8	LE1360	9.241	0.005	3.074	2.968
	1	43.1	LR0023	8.546	0.005	3.073	2.975
	1	43.6	1A01-139	9.721	0.005	3.064	2.981
	1	45.7	1A38-073 <N>	8.120	0.005	3.063	2.981
	1	46.5	LE0093	9.366	0.005	3.062	2.968
	5	95.8	1A12-126 <N>	8.056	0.005	3.059	2.988
	5	**99.5**	**M1908**	**8.789**	**0.005**	**3.072**	**2.962**
	8	48.8	1A12-107 <N>	9.310	0.005	3.005	3.068
	8	**57.7**	**LE1089**	**9.887**	**0.005**	**2.942**	**3.067**

LRL	1	87.3	1A10-195 b<N>	10.377	0.005	2.131	0.718
	1	88.7	1A10-195 a<N>	9.154	0.005	2.051	0.764
	1	**89.7**	**1A15-451 <N>**	**11.155**	**0.001**	**2.110**	**0.726**
	5	95.0	E35/M48-F-289<N>	8.162	0.005	1.861	0.987
	5	95.2	E35/M49-F-463	8.238	0.005	1.653	0.847
	5	95.5	E35/M48-F-487<N>	9.251	0.005	1.861	0.944
	5	96.1	E33/M59-F-176	8.549	0.005	1.806	0.971
	5	96.2	E45/M49-F-146<N>	8.515	0.005	1.679	0.821
	5	**99.5**	**M1908**	**10.425**	**0.005**	**2.459**	**0.838**
	5	99.8	1A06-109	9.377	0.005	1.839	0.863
	5	99.8	1A04-252	8.879	0.005	1.818	0.904
LSA	1	87.3	1A10-195 b<N>	10.631	0.005	0.177	0.057
	1	88.7	1A10-195 a<N>	9.379	0.005	0.171	0.062
	1	**89.7**	**1A15-451 <N>**	**11.411**	**0.001**	**0.176**	**0.058**
	1	90.0	1A10-554 <N>	8.911	0.005	0.181	0.060
	5	95.0	E35/M48-F-289<N>	8.276	0.005	0.150	0.083
	5	95.2	E35/M49-F-463	8.415	0.005	0.134	0.071
	5	95.5	E35/M48-F-487<N>	9.366	0.005	0.150	0.080
	5	96.1	E33/M59-F-176	8.749	0.005	0.146	0.081
	5	96.2	E45/M49-F-146<N>	8.856	0.005	0.136	0.069
	5	96.5	E33/M59-F-345	7.974	0.005	0.143	0.079
	5	99.5	M1908	9.472	0.005	0.197	0.075
	5	**99.8**	**1A06-109**	**9.906**	**0.005**	**0.149**	**0.072**
	5	99.8	1A04-252	9.171	0.005	0.147	0.075

LV	1	87.3	1A10-195 b<N>	11.199	0.001	0.0014	0.0004
	1	88.7	1A10-195 a<N>	9.977	0.005	0.0013	0.0005
	1	**89.7**	**1A15-451 <N>**	**12.193**	**0.001**	**0.0014**	**0.0005**
	1	90.0	1A10-554 <N>	9.412	0.005	0.0014	0.0005
	4	**63.4**	**1A12-445 <N>**	**9.105**	**0.005**	**0.0004**	**0.0013**
	5	95.2	E35/M49-F-463	8.006	0.005	0.0010	0.0006
	5	95.5	E35/M48-F-487<N>	8.577	0.005	0.0011	0.0006
	5	96.1	E33/M59-F-176	8.094	0.005	0.0011	0.0007
	5	96.2	E45/M49-F-146<N>	8.248	0.005	0.0010	0.0006
	5	**99.5**	**M1908**	**9.241**	**0.005**	**0.0014**	**0.0006**
	5	99.8	1A06-109	9.134	0.005	0.0011	0.0006
	5	99.8	1A04-252	8.433	0.005	0.0011	0.0006

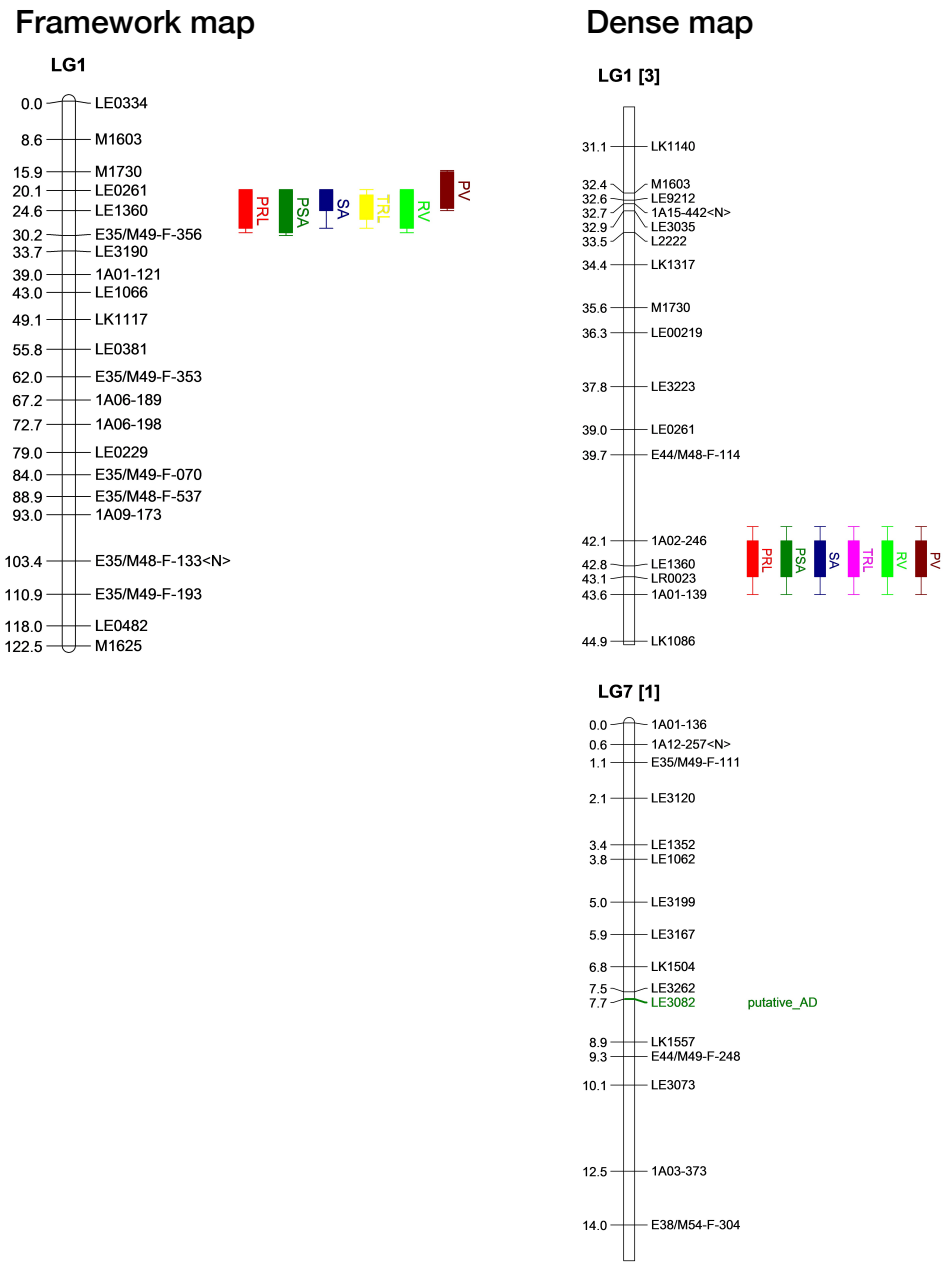
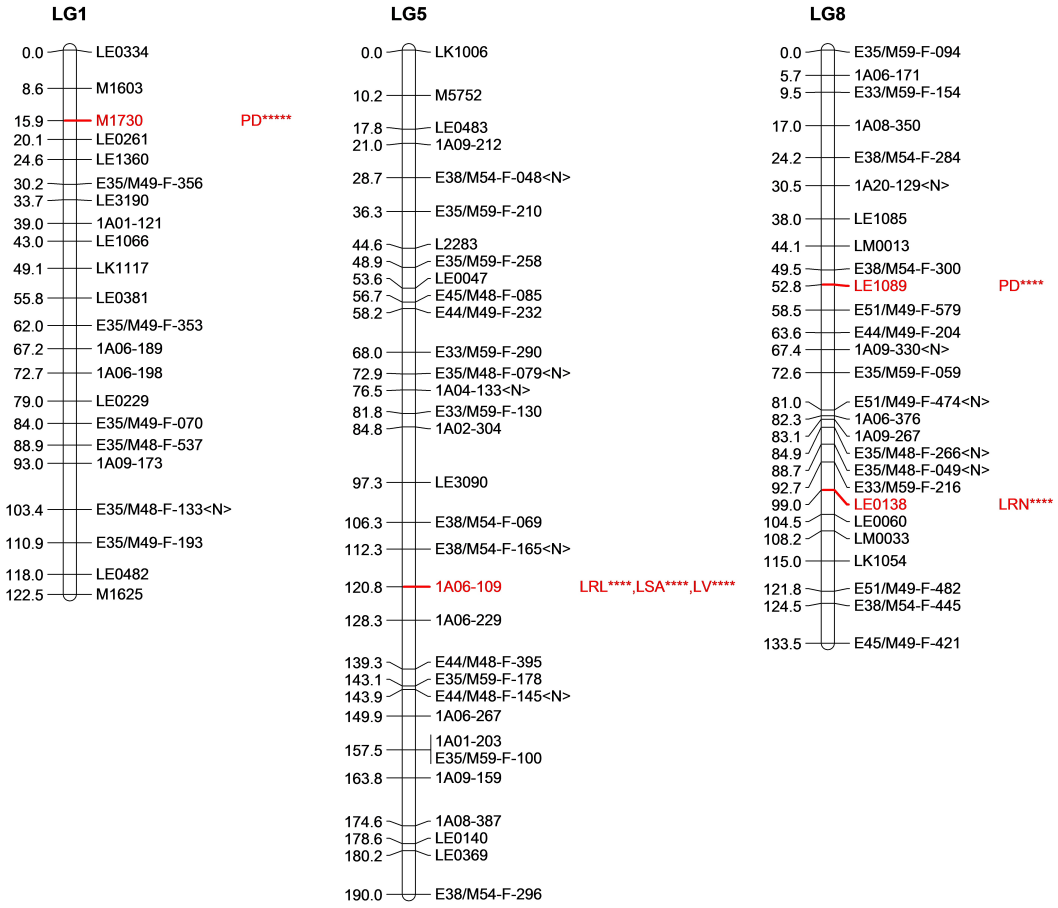


Figure 5.6 Position of QTLs analysed using the multiple QTL mapping (MQM) analysis. The figure compares the relative position of QTLs analysed using both maps. Rectangular boxes with confidence intervals besides LG1 map indicate significant QTL regions of root traits analysed with MQM procedures. The marker highlighted in green indicates the putative QTL. PRL = primary root length, PSA = primary root surface area, PD = primary root diameter, PV = primary root volume, TRL = total root length, SA = total root surface area, RV = total root volume.

Framework map



Dense map

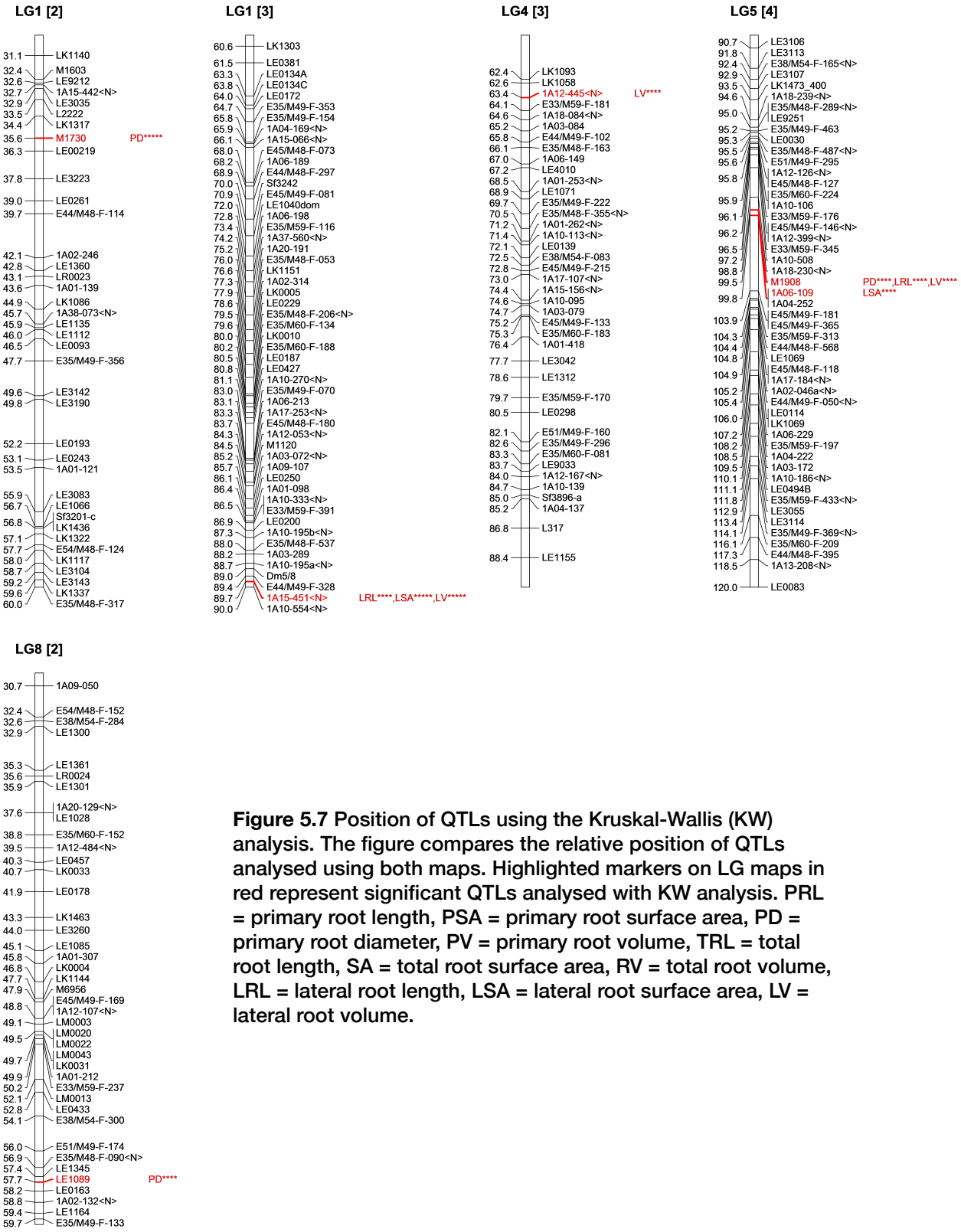


Figure 5.7 Position of QTLs using the Kruskal-Wallis (KW) analysis. The figure compares the relative position of QTLs analysed using both maps. Highlighted markers on LG maps in red represent significant QTLs analysed with KW analysis. PRL = primary root length, PSA = primary root surface area, PD = primary root diameter, PV = primary root volume, TRL = total root length, SA = total root surface area, RV = total root volume, LRL = lateral root length, LSA = lateral root surface area, LV = lateral root volume.

5.4 DISCUSSION

5.4.1 Intrinsic (without treatment) differences within mapping population genotypes

Crop domestication has been recognised since the beginning of recorded history, and helped to drive the transition from hunter-gatherer to settled agriculture (Childe, 1949; cited in Meyer *et al.*, 2012). Domestication of plants involved genetic modification of a wild species (through spontaneous mutation and farmer selection) to create a new form of plant altered to meet human needs (Doebley *et al.*, 2006). Domesticated lettuce, *Lactuca sativa* is one of hundreds of species which have undergone the domestication process, believed to be successfully cultivated from the weedy *L. serriola* (Kesseli *et al.*, 1991). These domesticated versions can be identified through the presence of 'domestication syndromes'.

In term of lettuce, the most obvious domestication syndrome factor would be the shape and yield of the leaves, as these hold very important economic value among consumers around the globe. However, the domestication processes has had significant effects particularly on root system architecture (RSA), as both matured wild and domesticated lettuce possess divergent root systems (Jackson, 1995). Wild lettuce has a relatively deeper root system than domesticated lettuce, which may have advantages in acquiring deeper soil resources than that of domesticated lettuce.

The evidence is apparent in the mapping population as the cross of *L. serriola* acc. UC96US23 × *L. sativa* cv. Salinas grown using the paper-based culture, reveals a very wide phenotypic in almost all measured root traits in the mapping population. This suggests high levels of genetic diversity between lines of the mapping population for these traits. The introduction of wild parent alleles may have beneficial effects as domesticated plants may contain a relatively narrower and more uniform genetic background as compared to their wild progenitors. This introduction of wild alleles has proven to improve domesticated lettuce performance in many previous studies reported previously, for example the primary root depth (Johnson *et al*., 2000), improving P uptake by better arbuscular mycorrhizal fungi colonisation (Jackson *et al*., 2002) and also fitness-related traits (Hartman *et al*., 2012).

Moreover, the cross has also led to the appearance of transgressive segregation in the mapping population as some RILs have shown higher or lower phenotypic values than the parental lines. This may open up accessions with more extreme traits' than the parental lines themselves for future crop improvement. These data have revealed the intrinsic genetic differences between parental lines, consistent with the results obtained in **Chapter 4**, and indicates the potential importance of wild allele introgression as one of factors for improving RSA traits.

Nevertheless, it can be seen too that most of the root traits' mean (and median) values of domesticated lettuce were relatively higher than that of wild lettuce, which may indicate better or faster seedling establishment, at least in the paper-based culture environment. Although seedlings were grown on blotting paper, this method is technically a hydroponic technique as the seedlings feed from nutrient solution reservoir, absorbed through capillary action. This may be also beneficial for lettuce RSA trait selection, when specifically grown under water-based culture such as hydroponics and aquaculture, as water-based cultures are important in growing leafy vegetables sustainably. An Arizona-based quantitative comparison between hydroponics and conventional soil-based agriculture has shown 11±1.7 times more yield with 13±2.7 less water demand in the hydroponic system (Barbosa *et al*., 2015). Selection of traits in individuals with better root volume, surface area and lateral root density, for example, may directly gain benefits from being grown under a water-based agriculture, in order to absorb more efficiently from the water-based nutrient mix reservoir.

Even so, overall population phenotypic variation may not be representative of larger seedlings or more matured lettuce, as the analysed seedlings were only grown for 14-days and have relatively small RSA, in comparison to other species that have utilised this method, such as wheat (Atkinson *et al.,* 2015) and Brassica species (Thomas *et al.*, 2016). Analysis using more matured plants may validate these differences

for the lettuce mapping population that are specifically grown under paper-based culture environment. Improved nutrient solution mix may also help in understanding differential nutrient deficiencies studies, as unfortunately, no significant phosphorus treatment effect could be reported in **Chapter 4**.

5.4.2 RSA traits QTL mapping

5.4.2.1 *Utilisation of framework map*

In this study, two variation of the genetic maps have been used to locate the position of significant QTLs of lettuce RSA traits, namely; framework and dense map. The framework map was constructed from a pre-existing dense map (Zhang *et al*., 2007), carefully spaced out to reduce the number of markers in linkage map. It is likely that having more markers in a linkage map i.e. having average marker intervals say less than 5 cM, may not generate more information about the targeted region as the chances of getting similar LOD with fewer marker intervals are relatively high (van Ooijen, 2009). More markers means more computations and memory requirement too (van Ooijen, 2009) and when missing data is present and relatively small population sizes, large numbers of markers may lead to mapping errors which can propagate through regions of the map.

This can be clearly seen in Tables 5.4 and 5.5 as the obtained QTLs using MQM analysis from a framework map have shown a very similar QTL region detection to the one that utilise the dense map. The similarity facilitates validation of the QTL locations obtained through identification of nearest markers to the QTL regions. Moreover, both QTL analysis using framework map and dense map have shown relatively high LOD and also, high explained variance percentage too. Sample size and quality of the trait data are far more important than number of markers on a map in getting more precise QTL locations and higher LOD scores.

On the other hand, more markers can be declared in KW analysis using dense map as opposed to framework map, as KW test successfully picked up the markers in the spaced region of the framework map. These have passed the threshold set at $P \leq 0.005$ for being declared as QTL for a trait, however, this has impede the identification the genuine peak of the traits of a QTL region (Figure 5.8). These markers were not present in the framework map as these might be taken out from the dense map, or too weak to be identified statistically. As the KW analysis did not explicitly involve any locational information of the map, the marker with the highest K* value may be called as the cofactor marker (in term of KW analysis), probably representing the most genuine QTL for the trait in a particular QTL region. These have been highlighted in Tables 5.6 and 5.7. The highlighted markers have also shown some similarities of markers representing a trait in comparison between framework and dense maps.

Comparison between maps serve as a method for validation for any QTLs identified.

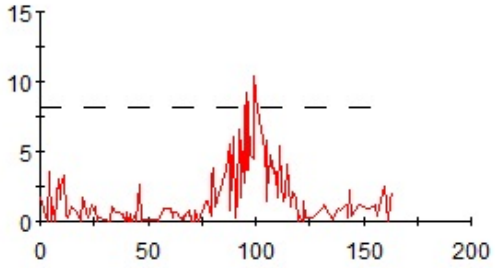


Figure 5.8 K* values distribution of lateral root length (LRL) in LG5. The markers above the dashed line passed the threshold KW statistics at $P \leq 0.005$.

5.4.2.2 *Pattern of QTL locations and its implications*

A number of QTLs were identified relating to lettuce RSA through parametric multiple QTL mapping (MQM) and non-parametric Kruskal-Wallis (KW) analysis. Through MQM analysis, of particular interest was the hotspot in the region of 15.9-30.2 and 41.7-45.0 cM of analysis using framework and dense map respectively (Figure 5.5). The region was co-localised with multiple QTLs for six root traits namely primary root length (PRL), primary root surface area (PSA), primary root volume (PV), total root length (TRL), total root surface area (SA) and total root volume (RV). This cluster is also known as consensus QTLs, and many species have shown this phenomenon as well (Zhang *et al*., 2004; Li and He, 2014). This clustering may indicate pleiotropic gene effect in which many traits were controlled by the same gene underlying this particular QTL region. These traits have the same characteristic of which involving the cell growth

particularly for primary root traits during seedling establishment. If the mechanism were as fundamental as this, it would also be expected that there could be an effect on the above ground parts of the plant.

These relationships of primary-primary and primary-global root traits were also consistent with correlation tests as these root traits were shown to have relatively high significant ($P \leq 0.05$) positive correlations (Figure 5.4 and Figure 5.5). This was also supported by principal component analysis (PCA) too (Figure 5.3). Interestingly, PRL and PSA, of which closely associated through PCA, were found to be increased by alleles from wild lettuce, *L. serriola* instead of domesticated lettuce, *L. sativa.* This is consistent with previous studies by Johnson *et al.* (2000) which identify root traits particularly PRL-related QTLs which have been driven by *L. serriola* allele. These traits were related to position near to 22.1-23.1 cM on framework map and on point 41.7-43.6 cM on LG1, which may suggest occurrence of domestication gene(s) that led to shallower rooting in the domesticated version.

On the other hand, KW analysis was used to locate the QTLs of trait average root diameter (AD), primary root diameter (PD), lateral root length (LRL), lateral root surface area (LSA), lateral root diameter (LD), lateral root volume (LV) and lateral root number (LRN). The KW analysis on these traits the using the framework map has identified eight QTLs, with at least one QTL per trait. Utilisation of dense map has unmasked 48 QTLs for

primary root diameter (PD), lateral root length (LRL), lateral root surface area (LSA) and lateral root volume (LV).

However, these massive number of QTLs can be analogised to the detection of QTLs in an interval mapping (IM) analysis. More specific range of QTL detection cannot be achieved, through MQM analysis, for example, as the distributions of these traits violate the assumptions of trait normal distribution. As a consequence of the deviation, the permutation test (PT) on these traits cannot be justified, therefore, IM or MQM analysis are technically failed to locate the region that reached genome-wide significance (Li and He, 2014). KW analysis did not take any considerations of the probability distribution of a trait, therefore, suitable for QTL analysis on these traits.

Furthermore, QTL clustering hotspots were also seen, particularly in LG1 and LG5. These traits control are in contrast to the ones obtained in MQM analysis, as these data suggests polygenic effects, in which many QTL regions control single traits. These traits are mostly lateral root traits, which are postembryonic roots, and depend on many hormonal and environmental signals and may be more influenced by the environment than the primary root traits (Nibau *et al.*, 2008). These QTL interactions were also consistent with correlation data which show a significant ($P \leq 0.05$) *r*-value correlations between lateral-lateral root traits (Figure 5.4 and Figure 5.5). This was supported by principal component analysis

(PCA) too (Figure 5.3). Moreover, some of PD's QTLs coincided with the cluster found using MQM analysis, suggesting similar control mechanism to the traits in the region, especially primary traits. Plus, differences in lateral-primary QTL regions answer the experimental hypothesis that these traits especially are mostly controlled by separate genomic regions. The contrasting primary-lateral root traits QTL regions were also consistent with studies by Johnson *et al.* (2000).

Although none of the markers co-locate to markers in the previous lettuce studies such as Johnson *et al*. (2000) and Zhang *et al*., (2007), possibly due to the different environment that the plants being exposed to (i.e. soil-based versus artificial medium), it was agreed that wild alleles improved the overall performance of domesticated lettuce. Based on the allelic effect of the QTLs and their clustered locations on the genetic map, it is suggested that any transgene integration is avoided to prevent loss of useful functions, especially in wild allele-driven root traits. Plus, the regions are non-annotated by any related genes, perhaps due to the nature of the markers itself, therefore, interested locus presented in the present study should be selected or spliced and maintained into the domesticated lettuce, perhaps through marker-assisted selection (MAS) breeding method for example introgression of wild alleles through near-isogenic lines (NIL) mapping population.

5.5 SUMMARY

In this chapter, QTL analysis of lettuce RSA using a paper-based method was carried out. A total of six QTLs and one putative QTL were obtained through MQM analysis, which mostly represent primary and global root traits. These QTLs were clustered in a hotspot located in LG1 which probably represents a domestication syndrome factor. A total of 48 QTLs was identified through KW analysis. Some clustering of trait QTLs were also seen, particularly dominated by lateral root traits. These clusters of trait loci may imply similar mechanism in controlling similar growth-related traits. The main difference between wild and domesticated lettuce is the rooting depth system which is influenced by primary root length (PRL). The results suggest a domestication QTL in LG1 for PRL, which is driven by wild lettuce. These clusters may benefit breeders in selecting many root traits of interest, particularly of primary and lateral root traits at a specific QTL region. Furthermore, QTLs obtained in this experiment can potentially be selected for soilless medium culture too, particularly for hydroponics and aquaculture. It is suggested that interested locus presented in the present study should be selected or spliced and maintained into the domesticated lettuce, perhaps through breeding methods.

CHAPTER 6

GENERAL DISCUSSIONS

6.1 PHENOTYPIC DIFFERENCES BETWEEN DOMESTICATED AND WILD LETTUCE PARENTAL GENOTYPES

Root serves as the epicentre of water and nutrient uptake for a plant, and thus it is the first organ to encounter avoid any drought and nutrient deficiencies. Root functions are achieved through the unique spatial structure of the root system commonly known as the root system architecture (RSA). Understanding the adaptation of RSA is an utmost priority as the soil resources are mainly heterogenous (Robinson, 1994). Ability of the roots to intercept essential resources is important for plant's survival, productivity and performance. The RSA adaptation is mainly influenced by genetic factors and the interaction with its growth medium, the environment (Malamy, 2005). Genotypic background determines the intrinsic root morphologies for a plant, whereas environmental components modify these root morphologies based on requirement at particular condition for its adaptation, reflecting high level of root plasticity (Gruber *et al*., 2013).

The study aimed to examine this interaction between parental genotypes of lettuces, namely *Lactuca sativa* cv. 'Salinas' and *Lactuca serriola* acc. UC96US23, at the seedling stage, and different phosphorus (P) concentrations. *L. sativa* cv. 'Salinas' is the domesticated version of its

wild progenitor *L. serriola* acc. UC96US23 (Kesseli *et al*., 1991), in which both possess different RSA at the matured stage (Jackson, 1995). Domesticated lettuce has a shallower RSA whilst wild lettuce has a deeper RSA, an intrinsic morphological shift which may be due to the domestication effect. A deeper RSA is advantageous as it can exploit the deeper resources of the soil while at the same time exploit the topsoil resources for the plants.

The lettuce RSA adaptation under wide array of P levels is poorly understood, despite the importance of the nutrient. This may be down to the fact that the domesticated lettuce has a shallower root system, which may be advantageous in acquiring P in topsoil. P is more abundant in the topsoil. P deficient environment exists and a topsoil foraging strategy has been reported for many species such as common bean (Lynch and Brown, 2001), rice (Panigrahy *et al*., 2009) and Brassica species (Shi *et al*., 2013). A topsoil foraging strategy in a reduced P availability environment promotes formation of lateral roots with primary root growth inhibition. Increasing P availability shows the opposite effect.

Based on the results reported in Chapter 3 and 4 through both agar- and paper-based screening methods, both parental genotypes seedlings have shown consistent significant difference in most of the traits measured. This indicates the intrinsic variation between the parental genotypes at genotypic level. In the agar-based assessment method, it was shown that

the domesticated lettuce has more prominent preference for lateral root traits as compared to wild lettuce which has bias towards primary root traits adaptation especially at the extreme ends of P concentrations. However, both parental lettuces did not follow the general topsoil foraging strategy in adapting to a low P level. Decreased formation of lateral root number (LRN) and its density (LRD) were observed in seedlings grown against decreasing P concentration. Primary root length (PRL) also increased at the lowest P level, which was unexpected given the general strategy of P foraging at lower P levels. Although the seedlings did not follow the topsoil foraging strategy, means for other traits especially lateral root length (LRL), surface area (LSA) and volume (LV) were increased, which may improve the P interception and uptake by the plants.

A higher P levels, these seedlings followed general adaptation strategies as reported in other species (Shi *et al*., 2013). PRL was promoted in both seedlings, suggesting a deeper nutrient acquisition mode, however, might be limiting in domesticated lettuce due to its intrinsic shallow root system. This may not be representative of mature lettuce, and further testing needs to be done in the future to confirm these results. These results suggest a unique strategy of the lettuce seedlings established under a wide array of P availability.

Further testing using paper-based method as reported in Chapter 4, showed insignificant differences at different P treatments, therefore comparison cannot be made between the two methods. Paper-based growth method has garnered more attention in the past few years due to its simplicity and versatility to be manipulated in many ways. For instance, Le Marie *et al*. (2015) modified the system to replicate split-pot method that can usually be done through a soil-based growth medium only. The convenience in preparing the whole setup in comparison to agar-based method was the main driver for the adoption of this screening method in the present study.

Interestingly, the QTL analyses have identified a number of QTLs, with some of the QTLs co-localised at the same locus. Of particular interest would be primary root related traits especially PRL. PRL is the main difference that differentiates the intrinsic root morphology between the two parental genotypes. The related traits namely primary root length (PRL), surface (PSA), and volume (PV) were all clustered at the same locus. Other traits such as total root length (TRL), surface area (SA) and volume (RV) were also clustered within the same locus region. This may imply the possibility of domestication gene(s) identification, which control these traits. These QTLs were also driven by domesticated lettuce allele to the trait value, which may suggest mutation on the original allele in wild lettuce, explaining the phenotypic variation measured in the population. The locus was also identified separately from the QTLs that explained the

lateral root phenotypes, addressing the hypothesis that primary and lateral root traits were mostly controlled by separate loci on the genetic map. Characterisation of the locus may provide the blueprint of primary root control in lettuce and perhaps compared to other crops especially underutilised crops.

6.2 3-DIMENSIONAL ROOT SCREENING PROSPECT

The two screening methods used in this study did not involve soil. The phenotypes identified may not entirely represent the traits that are well adapted or how plant would respond to the real soil environment. The images were collected in 2-dimensional (2D) plane, in which some of the crucial 3-dimensional-related information such as root angle and convex hull from the RSA is lost.

A preliminary study was undertaken in the present study using X-ray microcomputed tomography (µCT; Mooney *et al*., 2012) to study the 3-dimensional (3D) lettuce roots. The study involved studying RIL128 genotype, grown in a soil column filled with field topsoil collected at the University of Nottingham, Sutton Bonington Dairy Farm. The experiment last for 14 days under glasshouse conditions. The roots were scanned using an industrial GE® Phoenix v|tome|x m X-ray CT scanner and reconstructed *in silico* to obtained 3D root images. A sample image is displayed in Figure 6.1. The experiment showed the potential of utilising

such technology for better root traits screening, potentially to be selected for, in which more adapted in a field condition. The sample figure shows a clear 'umbrella-shaped' root architecture, indicating a good topsoil foraging traits. This is different when compared to the results described earlier, in which the seedlings did not shows clear topsoil foraging strategy. The roots-soil environment interaction may have contributed to this difference.

This method however proved to be time-consuming (especially the meticulous root segmentation process through thousands of stacked x-ray images) and require further validation through 2D imaging as smaller roots may have not been captured by the µCT scanner due to the spatial resolution. Full mapping population screening may encounter more challenges, especially post-scanning processes, which involve time-consuming *in silico* root reconstruction and large data handling. An alternative option would utilise 3D agar-based method, with prospective high-throughput outcomes (Fang *et al*., 2009; Topp *et al*., 2013; Piñeros *et al*., 2016). Fang and co-workers (2009) described the utilisation of Phytagel of differential P levels with crops growing in cylinder, imaged with laser scanner to obtain the 3D root traits, with potentials to be used with lettuce in the future. This however may inherit disadvantages related to handling agar-based medium such as requirement for aseptic condition at all time.

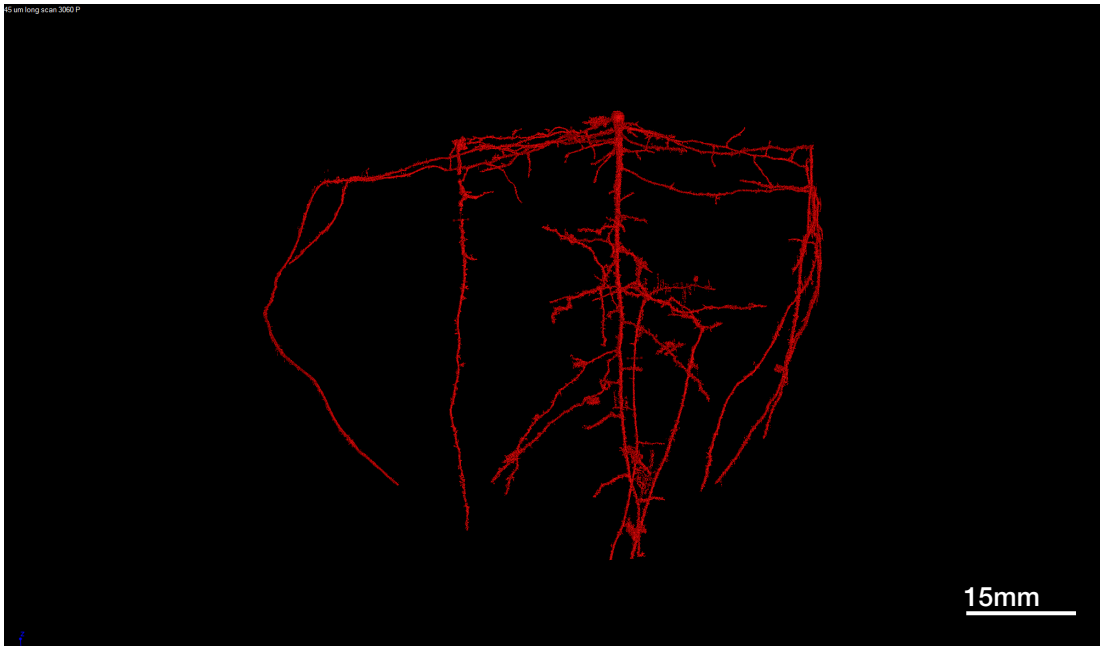


Figure 6.1 3D image of RIL128 lettuce, scanned by using GE® Phoenix v|tome|x m X-ray CT scanner. This image were scanned at 180kV and 200µA to give a resolution at 45µm. This image took 1 hour to finish scanning 3600 images before being reconstructed *in silico* into 3D volume. Cu filter was used to decrease image artefacts. Scale bar=15mm.

CONCLUSION

The present study utilised two main approaches in phenotyping the lettuce root system architecture (RSA). The first approach is agar-based phenotyping method which utilises the vertical agar-filled petri dishes with differential phosphorus (P) content. The key results obtained from the experiment includes the different adaptation strategies by both wild and domesticated lettuce. Wild lettuce showed enhanced primary root (PR) growth while domesticated lettuce showed enhanced lateral root (LR) formation, especially at a very low and high P levels.

An alternative phenotyping approach was tested in the present study, the paper-based root assessment method, utilised the vertical paper pouches for the seed's root to grow in predetermined P levels. The experiment consistently showed significant genotypic difference between the wild and domesticated lettuce. Additionally, the QTL analysis suggests clusters of trait loci which may control similar growth-related traits such as the difference between PR and LR loci clusters. The data also suggests domestication locus which relates to the difference in rooting depth between wild and domesticated lettuce in linkage group (LG) 1. The insignificant of P treatments, however, hindered further exploration of QTL which correlates different P levels and its RSA traits.

Nevertheless, the overall differences have shown effects of domestications on RSA traits between wild and domesticated lettuce. These genotypes were basically different to each other and its behaviour to the levels of P treatments. The main difference in rooting depth between wild and domesticated lettuce, for example, is very important to efficiently harness the deep water and nutrient resources, therefore reducing the dependency on the external water and fertiliser input.

The information obtained here is beneficial to other crops, especially wild genotypes or alleles for introgression in modern varieties and the underutilised crops. These wild genotypes and underutilised crops may contain unique or lost traits for a breeder to take advantages of in crop improvements. The present study serves as a reminder to breeders to consider root trait as an important component in breeding programmes especially for crops grown in rain-fed and low input systems. The hotspot especially in LG1 should be further studied, for specific gene identification, QTL splicing into another plant and syntenic comparison to major crops. Technologies such as x-ray microcomputed tomography (μCT) root visualisation should also be considered to understand more 3D-related root traits and its relationship to RSA.

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APPENDICES

APPENDIX 1

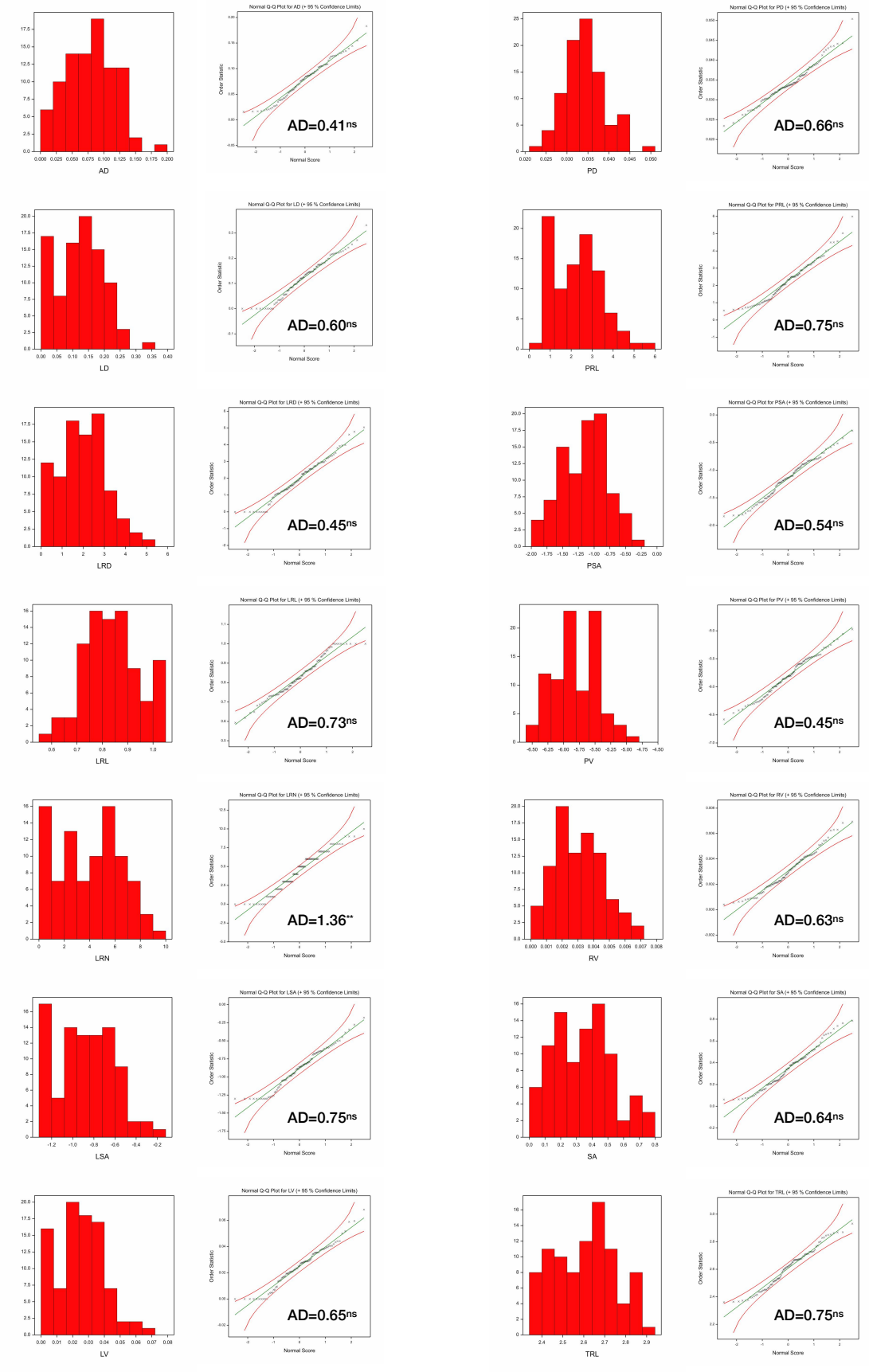


Figure AI.1 Distribution of root traits screened with paper-based assessment method with Anderson-Darling normality test results. Anderson-Darling test threshold was set at 5%. AD, Anderson-Darling value; ns, not significant; **, *P*=0.001.

Code AI.1 Coding lines to test Spearman's rank correlations (r_s) between root traits and correlogram visualisation in R language.

```
# loading files into the R environment, specifying the header is present,
files located on desktop file
Spearman_Dataset <- read.csv(
                                    file.choose(),
                                    header=T
                                    )

# attach the data
attach(Spearman_Dataset)

# review Spearman_Dataset
Pearson_Dataset

# review variable names
names(Spearman_Dataset)

# loading packages
library(Hmisc)
library(corrplot)

# correlations using rcorr() with p-values
# X being selected columns in dataset
Mcor <- rcorr(as.matrix(
                                Pearson_Dataset[,X]),
                                type="spearman"
                                )

# flattenCorrMatrix - obtained from sthda.com website
# cormat : matrix of the correlation coefficients
# pmat : matrix of the correlation p-values
flattenCorrMatrix <- function(cormat, pmat) {
  ut <- upper.tri(cormat)
  data.frame(
    row = rownames(cormat)[row(cormat)[ut]],
    column = rownames(cormat)[col(cormat)[ut]],
    cor  =(cormat)[ut],
    p = pmat[ut]
  )
}

#table of R and p values
flattenCorrMatrix(Mcor$r, Mcor$P)

#printing the correlation matrix
Mcor1 <- signif(Mcor$r, 2)

#printing the p-values of the correlations
Mcor2 <- signif(Mcor$P, 2)
```

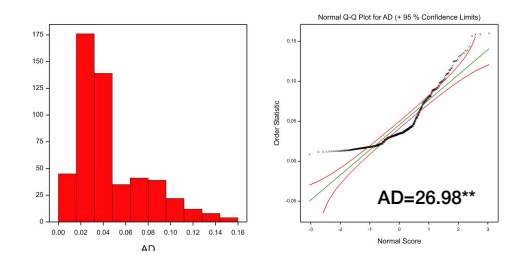
```
#visualisation of corrplot() and its modifications
corrplot.mixed(Mcor1,
                upper = "circle", order = "AOE",
                lower = "number", p.mat = Mcor2, sig.level = 0.05
                )
```

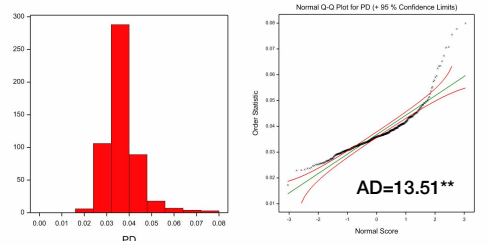
Code AI.2 Coding lines to test Pearson's product-moment correlations coefficient (r_p) between root traits and correlogram visualisation in R language.

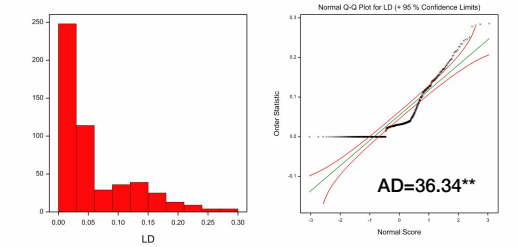
```
# loading files into the R environment, specifying the header is present,
files located on desktop file
Pearson_Dataset <- read.csv(
                                file.choose(),
                                header=T
                                )

# attach the data
attach(Pearson_Dataset)

# review Pearson_Dataset
Pearson_Dataset

# review variable names
names(Pearson_Dataset)

# loading packages
library(Hmisc)
library(corrplot)

# correlations using rcorr() with p-values
# X being selected columns in dataset
Mcor <- rcorr(as.matrix(
                        Pearson_Dataset[,X]),
                        type="pearson"
                        )

# flattenCorrMatrix - obtained from sthda.com website
# cormat : matrix of the correlation coefficients
# pmat : matrix of the correlation p-values
flattenCorrMatrix <- function(cormat, pmat) {
  ut <- upper.tri(cormat)
  data.frame(
    row = rownames(cormat)[row(cormat)[ut]],
    column = rownames(cormat)[col(cormat)[ut]],
    cor  =(cormat)[ut],
    p = pmat[ut]
  )
}

#table of R and p values
flattenCorrMatrix(Mcor$r, Mcor$P)

#printing the correlation matrix
Mcor1 <- signif(Mcor$r, 2)

#printing the p-values of the correlations
Mcor2 <- signif(Mcor$P, 2)
```

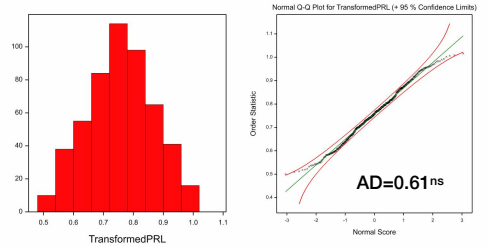
```
#visualisation of corrplot() and its modifications
corrplot.mixed(Mcor1,
                    upper = "circle", order = "AOE",
                    lower = "number", p.mat = Mcor2, sig.level = 0.05
                    )
```

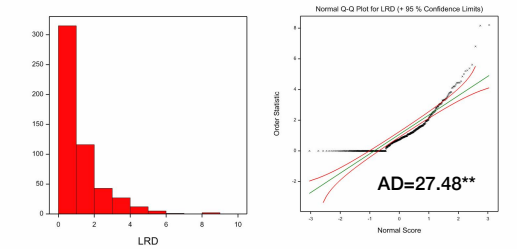
APENDIX II

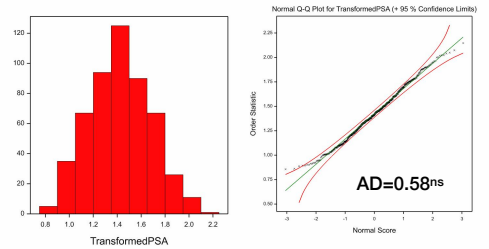

Normal Q-Q Plot for AD (+ 95 % Confidence Limits)
AD=26.98**
Order Statistic
Normal Score
AD

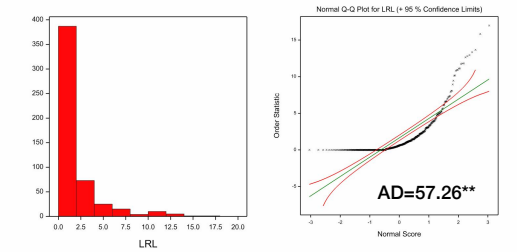

Normal Q-Q Plot for PD (+ 95 % Confidence Limits)
AD=13.51**
Order Statistic
Normal Score
PD

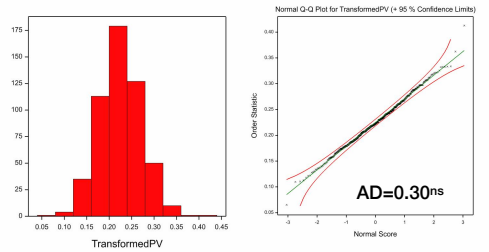

Normal Q-Q Plot for LD (+ 95 % Confidence Limits)
AD=36.34**
Order Statistic
Normal Score
LD

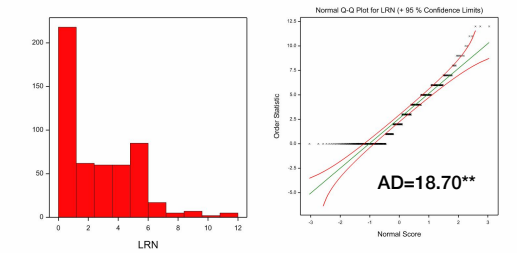

Normal Q-Q Plot for TransformedPRL (+ 95 % Confidence Limits)
AD=0.61ns
Order Statistic
Normal Score
TransformedPRL

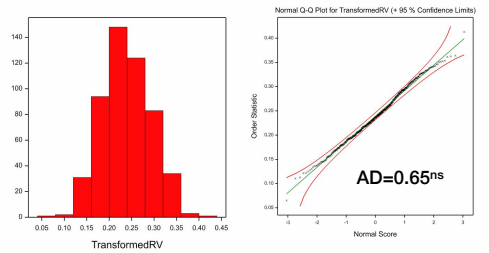

Normal Q-Q Plot for LRD (+ 95 % Confidence Limits)
AD=27.48**
Order Statistic
Normal Score
LRD

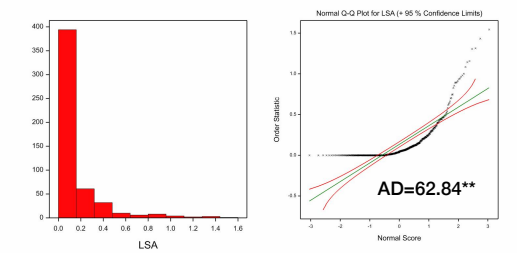

Normal Q-Q Plot for TransformedPSA (+ 95 % Confidence Limits)
AD=0.58ns
Order Statistic
Normal Score
TransformedPSA

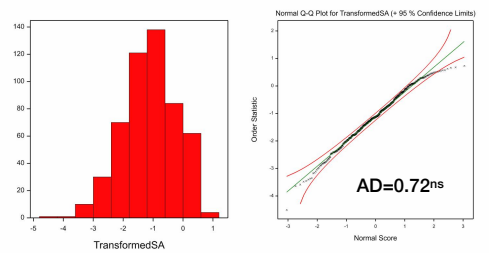

Normal Q-Q Plot for LRL (+ 95 % Confidence Limits)
AD=57.26**
Order Statistic
Normal Score
LRL

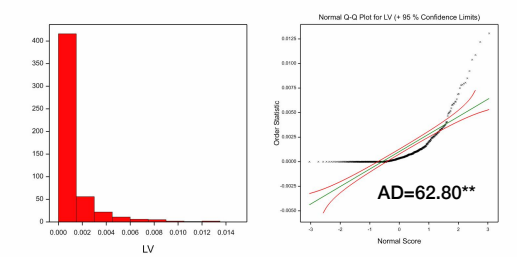

Normal Q-Q Plot for TransformedPV (+ 95 % Confidence Limits)
AD=0.30ns
Order Statistic
Normal Score
TransformedPV


Normal Q-Q Plot for LRN (+ 95 % Confidence Limits)
AD=18.70**
Order Statistic
Normal Score
LRN


Normal Q-Q Plot for TransformedRV (+ 95 % Confidence Limits)
AD=0.65ns
Order Statistic
Normal Score
TransformedRV


Normal Q-Q Plot for LSA (+ 95 % Confidence Limits)
AD=62.84**
Order Statistic
Normal Score
LSA


Normal Q-Q Plot for TransformedSA (+ 95 % Confidence Limits)
AD=0.72ns
Order Statistic
Normal Score
TransformedSA


Normal Q-Q Plot for LV (+ 95 % Confidence Limits)
AD=62.80**
Order Statistic
Normal Score
LV

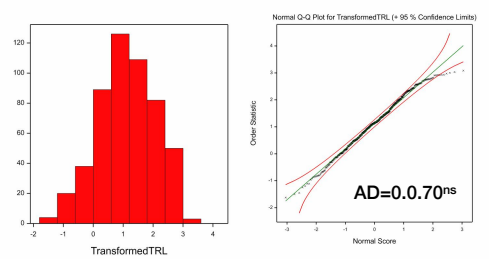

Normal Q-Q Plot for TransformedTRL (+ 95 % Confidence Limits)
AD=0.0.70ns
Order Statistic
Normal Score
TransformedTRL

Figure AII.1 Distribution of root traits screened of the mapping population with Anderson-Darling normality test results. Anderson-Darling test threshold was set at 5%. AD, Anderson-Darling value; ns, not significant; **, *P*=0.001.

Code AII.1 Coding lines of principal component analysis (PCA) using R.

```
#define and locate file path
path <- file.path("~", "Desktop", "PCA.csv")
path

#load file into R
PCA <- read.table(path,
                  header = T,
                  sep = ","
                  )

#read file
PCA

#Applying PCAnalysis on data
dataPCA <- princomp(PCA)
dataPCA$loadings
screeplot(dataPCA, type = 'line', main = 'Scree Plot for SerxSal')

#Apply Kaiser-Guttman criterion
ev <- dataPCA$sdev^2

# Plot eigenvalues and percentages of variation of an ordination object
# Kaiser rule and broken stick model
# Usage:
# evplot(ev)
# where ev is a vector of eigenvalues

# License: GPL-2
# Author: Francois Gillet, 25 August 2012

evplot <- function(ev)
{
 # Broken stick model (MacArthur 1957)
 n <- length(ev)
 bsm <- data.frame(j=seq(1:n), p=0)
 bsm$p[1] <- 1/n
 for (i in 2:n) bsm$p[i] <- bsm$p[i-1] + (1/(n + 1 - i))
 bsm$p <- 100*bsm$p/n
 # Plot eigenvalues and % of variation for each axis
 op <- par(mfrow=c(2,1))
 barplot(ev, main="Eigenvalues", col="bisque", las=2)
 abline(h=mean(ev), col="red")
 legend("topright", "Average eigenvalue", lwd=1, col=2, bty="n")
 barplot(t(cbind(100*ev/sum(ev), bsm$p[n:1])), beside=TRUE,
       main="% variation", col=c("bisque",2), las=2)
 legend("topright", c("% eigenvalue", "Broken stick model"),
      pch=15, col=c("bisque",2), bty="n")
 par(op)
}
evplot(ev)
```

```
#Plot PCA
library(ggplot2)
library(ggfortify)
autoplot(princomp(PCA), data = PCA, colour = 'grey', loadings = T,
loadings.colour = 'blue', loadings.label = T, loadings.label.size = 5, label.size
= 3, xlab = "PC1: 75.2% of variation", ylab = "PC2: 15.6% of variation”)
```

Code AII.2 Coding lines of Benjamini-Hochberg false discovery rates (FDR) multiple comparison test for non-parametric QTL analysis using R.

```
# Data input
# X is P-values of K* values from KW analysis
Input = ("X")

# Data into table object
Data = read.table(textConnection(Input),
                  header=T)

# review data
headtail(Data)

# load package
library(FSA)

# Obtained adjusted P value
Data$BH = signif(p.adjust(Data$Raw.P,
                          method = "BH"),
                          n = Y)  # Y = length of data

# Show locus with adjusted P values
View(Data)
```