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A study of the physiology and genetics of rapid rooting traits in lettuce (*Lactuca sativa*).

A thesis submitted in partial fulfilment of the requirements of Harper Adams University for the degree of Doctor of Philosophy.

by

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Publications.

Roberts, J., Broadley, M.R., Pink, D., Hand, P., Lynn, J., & Monaghan, J.M. (2020). Quantitative trait loci (QTLs) linked with root growth in lettuce (*Lactuca sativa*) seedlings. *Molecular Breeding*,**40**(8).

Roberts. J, Pink. D, Broadley. M, Hand. P & Monaghan. J, (2017), Quantitative trait loci (QTL) analysis and RNA expression linked to a rapid rooting phenotype in *Lactuca sativa* seedlings, Abstract in; *8th International Symposium on Root Development*, Umeå, Sweden.

Declaration.

I declare that the following thesis is the work of myself and as not been submitted or accepted for the award of any degree at any other academic institute. The research undertaken in this thesis was conducted primarily at Harper Adams University with the support of the collaborative funding bodies; Syngenta, the University of Nottingham and the Vegetable Genetic Improvement Network who provided plant material to use in this work.

Justin Roberts.
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Acknowledgements.

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Abstract.

Lettuce is usually germinated and grown for a short period in nurseries before planting (transplanted) in the field in the UK and Europe. Plants that are transplanted are more uniform in maturity due to more uniform germination, avoid early environmental stresses and usually mature earlier than direct sown crops. Lettuce transplants need to establish quickly in the field to optimise growth and uniformity and can be exposed to stresses that include mild initial drought, variable soil and environmental conditions and root pruning. These stresses are only likely to be exacerbated with increasing pressures on growers to reduce inputs. Identification of phenotypic and genotypic variation for a “rapid rooting” trait in the lettuce gene pool therefore may enable faster establishment and has the potential to improve the performance of lettuce transplants if integrated into a marker assisted breeding programme for lettuce varieties.

The following work optimised a 2D high-throughput assay to screen 14-day old seedlings of a lettuce diversity fixed foundation set (DFFS) and identified phenotypic variation for key rapid rooting traits that could prove important for future breeding programmes. Phenotypic variation was also observed within the DFFS for deeper rooting potential of lateral roots and for root hair traits. The 2D assay was also utilised to identify 16 significant quantitative trait loci (QTL) associated with the rapid rooting phenotype, of which six were associated with increased primary root growth, three with increased lateral root growth, two were associated with lateral root length density, three with lateral root number density and two with mean lateral root length.

A targeted transcriptomic analysis utilising extreme lines identified nine candidate genes located under five of the reported QTL for the “rapid rooting” phenotype. The genes coded for proteins involved in various pathways involved with root growth including cell proliferation, cell expansion, cell wall synthesis and ABA synthesis. These genes may offer a promising approach for the improvement of lettuce establishment in a commercial breeding programme.

The extreme lines were then analysed in a 3D transplant sand assay to assess the effect of altering the root:shoot ratio through controlled root pruning had on the rapid rooting phenotype and identified that although some of the lines behaved differently some of the lines maintain a rapid rooting phenotype at transplant maturity and recovered a larger proportion of the root:shoot ratio compared to the slower rooting lines and commercial controls.

Chapter 1 - Introduction.

It is estimated that the world population will reach between 9.1 (Alexandratos, 2005) and 9.3 billion by 2050 (Lee, 2011). To meet this demand global crop yields must increase by around 57% (Rengasamy, 2006). In developed countries, such as the UK, the total area attributed to crop production has been in steady decline since the mid-1980s with yield increase accounting for all production growth whilst compensating for the reduction in land availability (Alexandratos & Bruinsma, 2012) through a process known as agricultural intensification. The large increases in yield seen were achieved through strategies that included the use of high yield varieties, irrigation, pesticides, and fertilizers that were collectively referred to as the 'green revolution'. However, all strategies except high yield varieties can have serious negative impacts on the environment, such as the pollution of ground water, reduction in soil quality, and climate change (Matson *et al*., 1997). For example, soil salinity was estimated to have already affected 10% of all global cropland, including 27% of all irrigated land in 2006 (Barassi *et al.,* 2006). This figure has recently been raised to an estimated 50% of all global irrigation schemes (Fasciglione *et al.,* 2012). The vast reduction in land availability and pressure to reduce inputs means the greatest strategy for future agricultural intensification will come from producing high yielding crops that require less inputs.

Lettuce, due to the fragile nature of the leaves, is a manually harvested crop (Ryder, 1999) and harvesting is completed in a single pass of the field. Any lettuce heads that are not within a pre-determined specification for weight and size are lost. Lettuce in Europe are germinated and raised in glasshouse nurseries and transplanted at around the 5-7 true-leaf stage and require inputs of water and fertilizer. If lettuce production is to carry on at the same or increased levels but with reduced inputs and land this will require increased productivity to come from a combination of agronomy and plant genetics. Genetic improvement of the crop to allow the capture of nutrients and water more efficiently from the soil following transplanting offers a novel area to investigate. Lettuce peat blocks are joined to adjacent blocks and the separation of the blocks inevitably causes root pruning as the roots grow into the adjacent blocks, therefore the ability of the crop to replace roots lost at transplanting and then to produce a large root system in the soil quickly could reduce the time taken to recover the root:shoot ratio following transplanting and allow the crop to access nutrients and water in periods of mild drought or reduced inputs via a larger root surface area in the soil. The ability of the crop to rapidly produce a large functional root system relies

on genetic regulatory processes of the plant to produce a longer, deeper primary root along with an increased growth rate of lateral roots and an increased number of lateral roots in to the surrounding soil (Slovak *et al*., 2016). These rooting traits are defined in this thesis as contributing to a “rapid rooting phenotype”.

The overall aim of this work is to establish whether a rapid rooting phenotype in *Lactuca* seedlings is under genetic control and if it is maintained in commercially relevant transplant production systems.

This overall aim was pursued through a series of experiments with the following objectives:

a) Quantify phenotypic variation in rapid rooting traits in seedlings of a lettuce diversity fixed foundation set (DFFS) consisting of 96 genetically fixed lines representing a large portion of gene pool of the *Lactuca* spp. (*Lactuca sativa, Lactuca serriola, Lactuca saligna & Lactuca virosa*).
b) Identify quantitative trait loci (QTL) and the associated flanking DNA markers associated with rapid rooting traits, by phenotyping the Saladin x Iceberg mapping population.
c) Utilise a targeted transcriptomic approach to identify candidate genes under the QTL that may contribute to the phenotype.
d) Identify if the rapid rooting phenotype observed in extreme lines of seedlings is maintained at post-transplant maturity
e) Establish whether the rapid rooting phenotype hastens recovery of the root:shoot ratio in a transplant system.

The work optimised a high through-put system that allowed the analysis of over a thousand lettuce seedlings along with greenhouse grown transplants in small pots and sand culture.

1.1 Thesis map.

The structure of this thesis is in a journal paper format that will allow the intended extraction and submission of chapters 4-7 to research journals.

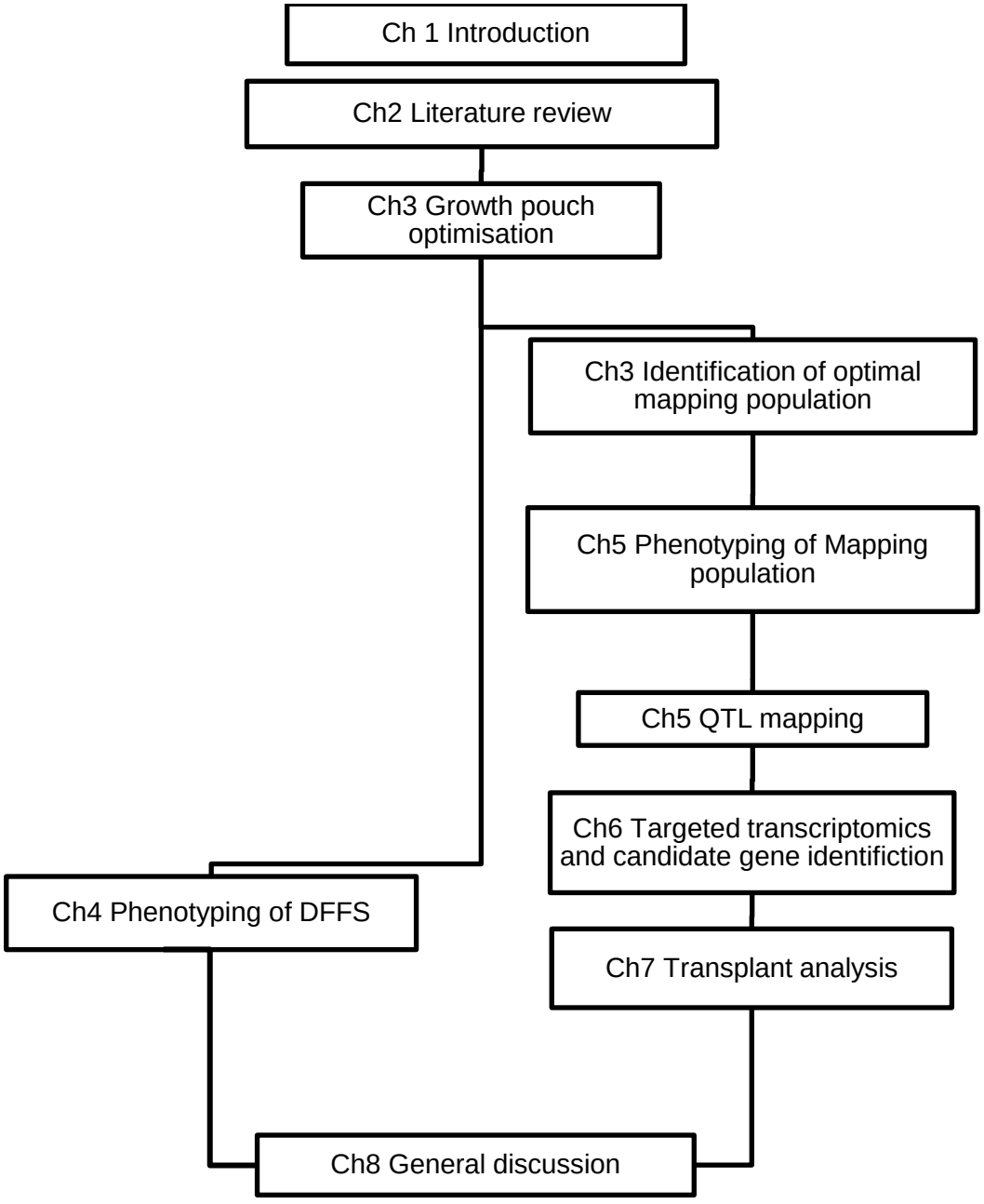

Figure 1.1: Thesis map of structure.

2 Chapter 2 - Literature Review.

Lettuce (*Lactuca sativa*) is a high value vegetable crop that would be affected by the global increase in abiotic stresses, such as mild drought and salinity. Lettuce is part of the *Lactuca* genus within the *Asteraceae* (also known as *Compositae*) family. Lettuce is a diploid species with 9 chromosomes (Truco *et al.,* 2007) and a genome size of 2.7 Gb (Truco *et al.,* 2013). Its closest relative is the wild lettuce *L. serriola*, which is believed to be the progenitor of modern lettuce cultivars, although the primary gene pool consists of *L.saligna*, *L. virosa* (Truco *et al.,* 2007), *L. altaica* and *L. alculeata* (Wei *et al.,* 2014).

2.1 Global production.

The world's largest producer of lettuce is mainland China, where in 2013 13.5 million tons (mt) of lettuce was produced, which was a large increase from the 5.6 mt produced in 1998. The overall yield in China however, only increased marginally from 21.7 t ha^{-1} in 1998, to 23.7 t ha^{-1} in 2013 highlighting most of the increase in production came from increasing the land dedicated to lettuce production, which increased by over 8% annually (FAOSTAT, 2015).

Of the 3.17 mt of lettuce produced in Europe in 2013, Spain was the largest producer (FAOSTAT, 2015). In 1990 Spain produced 0.98 mt of lettuce (Instituto Nacional de Estadística, 2015), which fell slightly to 0.90 mt in 2013. Yield in Spain in 1990 was 28 t ha^{-1}, which was higher than the 26.8 t ha^{-1} in 2013 (FAOSTAT, 2015). In 1988-89, in the UK there was 8,193 ha of field area used for lettuce production, providing the UK market with over 0.21 mt of lettuce at an average yield of 25 t ha^{-1}. In 2018-19 the total land area estimated for lettuce production in the UK was 4,395 ha (DEFRA, 2019), producing 0.1 mt of lettuce at an average yield of 21 t ha^{-1} which highlights the need to increase crop yield on ever diminishing land availability. These figures along with those from Spanish, US and Chinese produced lettuce emphasise the opportunity to greatly increase yields of lettuce without increasing and possibly being able to reduce the land area dedicated to the crop.

2.2 Production of lettuce.

2.2.1 Planting.

Field grown lettuce can either be direct drilled in the field as seed or seedlings can be produced in blocks and transplanted into the field as young plants. There are benefits and disadvantages to both approaches.

2.2.2 Direct drilling.

In the US most of the lettuce is sown directly into the field, by precision planters, contained within a polymer-based pellet. The pellets are sown at around 5 -7.5 cm apart along a 1 or 2-metre-wide bed consisting of either 2 or 4 seed lines, respectively. Following emergence, the seedlings are thinned according to the variety, which can be up to 30cm apart for large lettuce, such as Iceberg. The obvious advantage of direct drilling is costs. Plant raisers are not needed to raise the plants, or transport costs associated with moving the plants (Sharma *et al.,* 2005). Furthermore, as peat is a non-renewable resource, and its use is critical to wetland ecosystems there is growing pressure on producers to reduce its use in transplant production (Mininni & Santamaria, 2012)

However, direct drilled seed can be susceptible to thermo-dormancy in high temperatures if it has not been subjected to osmo-conditioning (also known as priming) (Smith *et al.,* 2011), which causes delayed and staggered germination and emergence that reduces the uniformity of crop development and diminishes yield and profit margins (Cantliffe, Shuler & Guedes, 1981). Seed priming is the osmotic treatment of the seed pre-sowing to allow imbibition and the first stages of germination to take place and pausing the process before the radicle protrudes the seed coat (Parera & Cantliffe, 1994). Furthermore, lettuce seedlings are not very competitive against weed infestation during the first 2 weeks following emergence, which can cause severe losses to yield and therefore require herbicide treatment and/or manual/mechanical weed control (Lanini & Le Strange, 1991) further driving down profitability.

2.2.3 Transplants.

Within Western Europe, lettuce are commonly grown as transplants in compact peat blocks prior to planting to fields, which provides several benefits to the farmer including

prevention of thermo-inhibition and cost of seed priming, faster germination, a more competitive crop against early weed infestation and importantly a more uniform crop (Maltais *et al*., 2008), which is essential as lettuce requires individual harvesting on a single pass through the field..

Several studies have been conducted on the optimum size of transplants of various crops and there is a balance between producing a mature seedling to reduce the time to marketable yield and good uniformity, and a limitation on space for propagation and transport (Cantliffe, 1993). Lettuce crops have been reported to have greater yield uniformity when produced from 13 – 19-day old transplants compared to 25-day old transplants (Wurr *et al*., 1987). However, Wang & Kratsky (1976) highlighted that earlier yields are obtainable from older, larger transplants, although these results were found in a glasshouse study they confer with the findings of the study by Kerbiriou *et al*., (2013), that found older, more mature lettuce transplants performed better in the field.

One disadvantage of transplanting lettuce is transplant shock (van Iersel, 1998). This is a response to damage of the roots when handling and planting transplants, which can halt shoot growth whilst the plant recovers (Biddington & Dearman, 1983). This will be discussed in section 2.4.3.

2.3 Establishment.

Cultivated lettuce plants have relatively shallow roots with the majority of the root system architecture functioning within the top 20cm of the soil and has a very high water content of more than 95% water, which makes the crop sensitive to drought conditions (Johnson *et al.,* 2000). The high water content also makes lettuce relatively sensitive to soil salinity compared to other vegetable crops such as cucumber, spinach, cabbage, and broccoli (Barassi *et al.,* 2006).

2.3.1 Crop growth and harvesting.

Wholehead lettuce, such as crsiphead, butterhead and cos types are sown into 4x4x4 cm (64 cm^3) peat blocks within nurseries and then transplanted to the field with a mechanical planter when the crop reaches the 5-7 true leaf stage (Kerbiriou *et al*., 2013) avoiding the thinning required in direct drilling. For crisphead lettuce, the plant develops overlapping leaves that form the head, confining the terminal bud. As more leaves develop,

the head size increases along with solidity, which is used to define the maturity of harvest (Garrett *et al*.,1969). Harvesting usually occurs approximately 55 - 65 days after transplanting, dependent on weather conditions and seasonal timings (Ryder, 1999), and harvesting is a one-pass manual process undertaken on mobile harvesting rigs due to the fragile nature of the crop.

2.3.2 Agronomic and physiological factors influencing young plant establishment.

The term establishment equates to the ability of a crop to produce root growth into the surrounding soil following transplanting, providing support for shoot growth. The establishment of a transplanted crop including lettuce is influenced by many agronomic and physiological factors that can impede or promote root growth into the surrounding soil. Lettuce establishment and subsequent optimal growth through the crops ability to access the water and nutrients relies on these factors to be at optimal conditions.

2.3.3 Water.

Water is essential for photosynthesis, respiration, and nutrient up-take in plants (Kizil *et al*., 2012). In periods of drought keeping all areas of field production can be a difficult task. In a study by Kizil *et al*., (2012) an unknown lettuce variety head fresh weight was reduced by more than 5% and 20% in treatments that provided 66% and 33% of pot capacity of water respectively over a period of 24 days after transplanting. These findings were supported by Sayyari *et al*., (2013), who found water stress to reduce lettuce yields by more than 10% and 25% in treatments that provided 60% and 30% of pot capacity of water, respectively. However, the cultivar and length of the experiment was not disclosed. The findings of these studies were corroborated in the study by Kerbiriou *et al*., (2013), which observed a reduction in shoot growth and root growth when exposed to a period of water stress. Root growth relies primarily on cell division and cell expansion and during cell expansion osmotic regulation to keep the cell turgid, while expanding, is required (Péret *et al.,* 2014).

The shallow root systems of *L. sativa* make the crop susceptible to mild drought and therefore regular applications of water are required for young lettuce plants following transplanting to ensure optimal yields (Gallardo *et al*., 1996). During mild drought the drying soil not only increases the water stress inflicted on the crop but also adds the stress of mechanical impedance on the root system resulting in a reduction in root elongation from increasing soil strength (Bengough *et al*., 2006) due to the increasing pressure required for

the root to push through the soil. Drought conditions have a direct effect on the gravitropism of plant roots, where root hydrotropism, which is the growth of the root towards the moisture gradient controlled within the root cap occurs through the suppression of gravitropism (Taniguchi *et al*., 2010).

2.3.4 Soil.

Soil compaction is known to affect root growth (Tracy *et al*., 2011). Increasing soil compaction has been shown to reduce root growth, affect rooting depth and the number of lateral roots in tomato lines (Tracy *et al*., 2015). Many relatively moist soils have penetrometer resistance of over 2 MPa, which is enough to reduce root elongation by more than half of that of unimpeded roots (Bengough *et al*., 2011).

Lettuce root elongation was reported to be reduced by more than 29% in soil with a density of 1.4 g cm^{-3} compared to a soil density of 1.2 g cm^{-3}, however the dry weights of the roots were increased by more than 30% in the higher soil density (Azzi *et al*., 2017), which may be a result of radial expansion of the roots leading to shorter, thicker roots (Bengough *et al*., 2006).

2.3.5 Soil pH.

Another factor influencing lettuce growth in soil acidity (pH), a study by Hemphill & Jackson, (1982) identified the optimal growth pH for lettuce was between 6.1 and 6.6. The study found that head weight below a pH of 5.7 was significantly reduced with the application of nitrogen. In soils above the 6.1 – 6.6 optimum the application of nitrogen increased head weight (Hemphill & Jackson, 1982). This was later confirmed by Ryder (1999) who suggested lettuce prefers fertile soils with a pH level that ranges between 6.5 - 7.2. When soil pH levels are neutral or slightly acidic aluminium is generally insoluble, but as soil pH levels drop below 5.0 toxic aluminium ions are released into the soil, which reduces root growth and nitrogen uptake efficiency as a result. The aluminium ions cause phytotoxicity by interfering with the plant cell walls, plasma membrane and auxin polar transport, which inhibits cell elongation and cell division (Zhao & Shen, 2018).

2.4 Nutrients.

Transplants initially have very little access to nutrients from the field as they do not have established roots. The transplanted crop must produce roots in the soil profile to access available nutrients for growth. The ability of the transplanted crop to produce roots rapidly would be adventitious in supplying the necessary nutrients for healthy and rapid growth.

2.4.1 Nitrogen.

Plants including lettuce require nitrogen (N) to produce amino acids including glutamine, glutamate, asparagine and aspartate, which are required for healthy plant growth and development (Lam *et al*., 1996). The main source of N for plants is inorganic nitrogen. These forms of N account for less than 5% of the total nitrogen in the soil (Liu *et al*., 2014). N deficiency can reduce chlorophyll and Rubisco content of the leaves reducing the photosynthetic capacity of the crop, which leads to reduced vegetative growth (Delgado *et al*., 1994), which is a detrimental outcome for growers as the mature head of the lettuce is the marketable product. An issue with over supply of N is the high concentrations of nitrate accumulation in the leaves can be detrimental to human health (Liu *et al*., 2014).

A study by Walworth *et al*., (1992), described 112 kg ha^{-1} of N to be the optimal application for transplanted crisphead lettuce (cv. Salinas) throughout the growing season. However, the increased photosynthetic activity of the summer months is associated with reduced nitrate accumulation in the heads of lettuce (Buwalda & Warmenhoven, 1999). Growing concerns over water and air pollution from fertilizer N use are leading to pressures on growers to reduce the amount of N used (Li *et al*., 2016). In the UK agriculture has been suggested to contribute to 70% of the N pollution in UK rivers (Edwards & Withers, 2008). A rapid establishment phenotype may reduce the needs for inputs and therefore reduce pollutants.

2.4.2 Phosphorus & Potassium.

Phosphorus (P) is an important mineral in plants as it is a key component of nucleic acids, carbon phospholipids and energy transfer reactions in the form of ATP and accounts for around 0.2% of a plants dry weight (Schachtman *et al*., 1998; Shin *et al*., 2004). Available phosphate levels in soils are low and more than 80% of applied phosphate as fertilizer in

agriculture systems becomes unavailable to plants through conversion to the organic form (Schachtman *et al*., 1998). Potassium (K) is the most important cation in plants and plays a key role in many cellular processes from enzyme activation, osmoregulation, and membrane transportation (Wang & Wu, 2013). K is the most abundant mineral taken up by plants and plants can accumulate K to levels above 100 mM from a host of soil types, through low-affinity and high affinity transport methods based on the soil K concentrations. The role of K in osmoregulation of cells plays a key role in root cell elongation. During cell elongation cell turgor pressure is essential and K transporters in the plasma membrane and vacuole regulate the osmotic influx which balances the osmotic pressure as the cell elongates (Rigas *et al*., 2001). Plants are adapted to a high intercellular ratio of K^+ compared to Na^+ with the K^+ counteracting the inhibitory effects of Na^+. During cell elongation intercellular levels of K^+ are increased through active transport of K^+ through K transporters and this causes water to enter the cell via aquaporins to keep the cell turgid (Cushman, 2001). Lettuce root growth was increased with increasing concentrations of K in hydroponically grown lettuce (cv. South Bay) 28 days after sowing (Soundy *et al*., 2001).

A study by Smith & Scaife (1973) observed optimal lettuce growth requires between 120 ppm of P application in a sandy soil to 300 ppm in a moss peat soil and the required concentration of P in young lettuce leaves for optimal growth was ~0.6%. Due to the low diffusion of phosphate in the soil plants quickly use the phosphate within the rhizosphere and must produce greater root mass to continuously acquire the nutrient (Schachtman *et al*., 1998). A cause for concern is the diminishing availability of phosphate for fertilizers and reserves have been predicted to have only another 100 years of supply left. However, this figure could be an overestimate if fertilizer demand continues to grow (Gilbert, 2009). Further to this concern is the pollution of UK waterways with P, which agriculture contribute approximately 13% of the overall levels of P (White & Hammond, 2008).

2.4.3 Transplant shock.

Transplant shock is a response to damage of the roots when handling and planting transplants, which causes an imbalance to the root:shoot ratio which halts shoot growth until the root:shoot ratio is restored by root growth during a recovery phase (Biddington & Dearman, 1983). In a study into the effects of root pruning, where root tips of the primary root and lateral roots were pruned on a *L. sativa* cv. Arctic King, Biddington & Dearman (1983) suggested that overall root length had still not recovered after 8 days in a root pruning treatment similar in extent to that which may occur in commercial handling of a transplant,

compared to controls. Transplant shock has also been observed in bell peppers, where the reduction in water uptake due to the destruction of root hairs during transplantation leads to stress lasting around 2 days through reduction in the plants ability to uptake water for transpiration replacement (Berkowitz & Rabin, 1988).

Root pruning, as seen in transplanting, has also been shown to reduce the uptake of N in tomato plants (Bar-Tal *et al*., 1994) and make tobacco plants more susceptible to disease (Moss & Main, 1989). Interestingly, a development stage orientated study of lettuce transplants by Kerbiriou *et al*., (2013) concluded that the age of transplanting, ranging from 2-7 true leaves had no effect on overall yield in “optimal conditions”. The study sampled 3 plants per plot for shoot weight at final harvest in each treatment and no comment was made on crop uniformity in the different transplant treatments. The study by Kerbiriou *et al*., (2013) did find a larger root mass was linked with greater shoot weight and further suggested a trade-off could exist in a more robust crop between high root regeneration capability and shoot growth, however if a robust crop increases crop uniformity across a field it would be beneficial to know if the reduction in shoot weight at harvest is compensated for by increased uniformity and therefore the number of mature heads produced.

The hierarchical order of roots which are pruned may influence the extent of transplant shock. Biddington & Dearman, (1983) observed that when the primary laterals are pruned, secondary lateral growth ceased along these laterals. This suggests that when the primary root and primary laterals are pruned, establishment would be dependent on how quickly further undamaged primary laterals can grow from inside the transplant block and from the remaining portion of the primary root, as these would be needed to produce secondary and tertiary laterals in the surrounding soil (Figure 2.1), therefore a rapid rooting phenotype would reduce stand establishment time and recovery phase, restoring the root:shoot ratio more quickly and improving crop uniformity and yield. However, it has also been reported that a larger transplant and therefore a larger root system can improve stand establishment in lettuce by decreasing transplant shock but has no effect on the final head weight in lettuce (Nicola & Cantliffe, 1996).

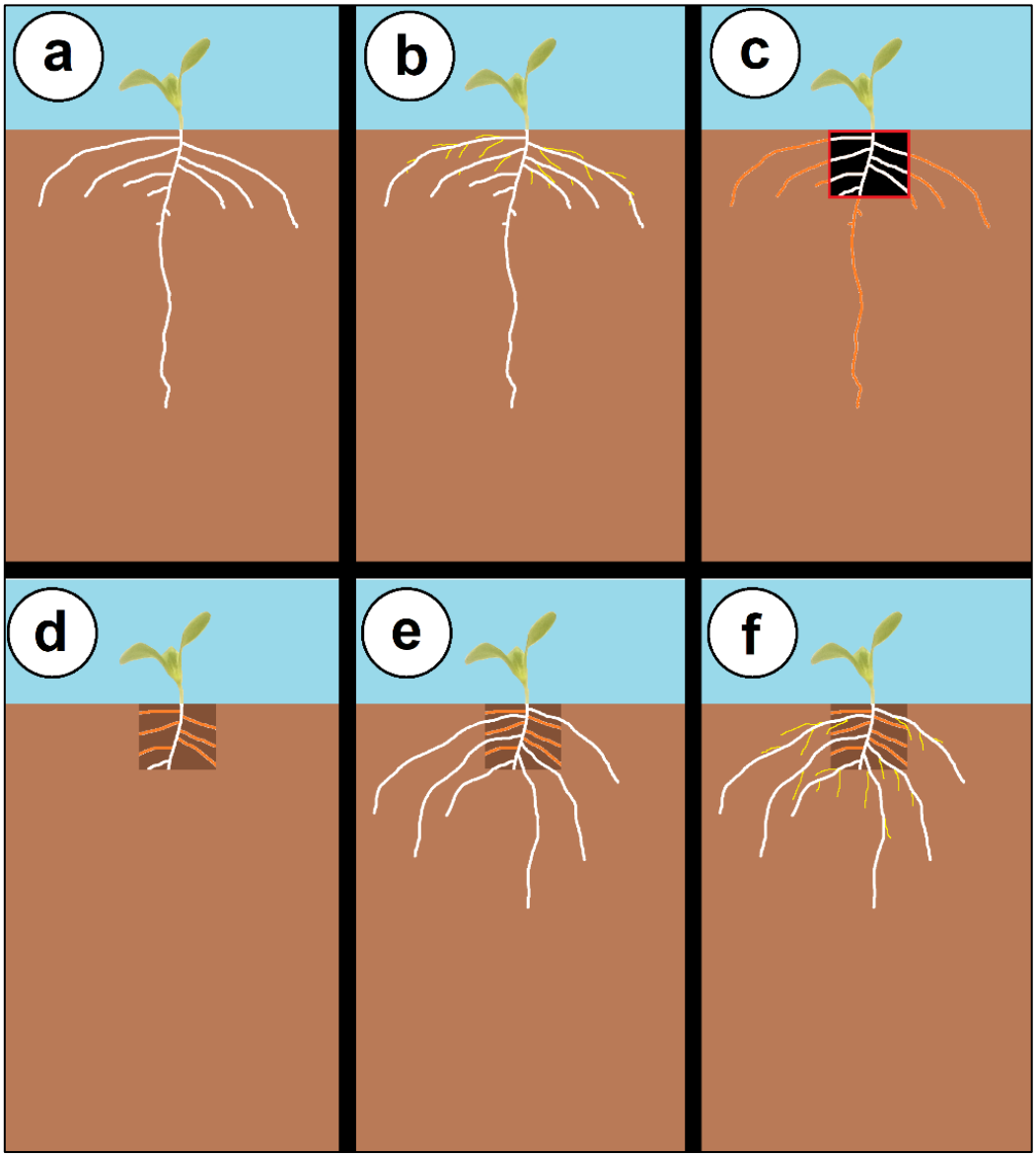

Figure 2.1: Schematic representation of pruned roots in transplanting.
a) Primary root and primary lateral growth; b) the development of secondary laterals highlighted yellow; c) representation of pruning from transplanted lettuce (pruned roots highlighted orange; d) highlights all the primary laterals that will not produce secondary laterals following root pruning at transplanting; e) development of new primary laterals from the primary root; f) development of secondary lateral roots from the new primary laterals (highlighted yellow).

Another factor that may influence the extent of transplant shock is the size of the block or module that the seedling is rooted in and hence the seedling age/size. There is a new, small volume, plant-tape system that enables automated planting and reduce volumes of peat used. This system uses a much smaller transplant in a volume of ~3 cm^3 than the typical block system which has a volume of 64 cm^3. The plant-tape used in the system reduces the number of roots growing into adjacent blocks causing most of the roots to orientate in a vertical position. Kerbiriou *et al.*, (2013) reported that the transplanting of

underdeveloped seedlings, as in the plant-tape system, has a negative effect on plant development and that underdeveloped seedlings were less able to recover from transplant shock compared to larger seedlings. The report also suggested that the time to harvest of under-developed seedlings was increased placing a financial loss on the growers and observed that the cultivars that developed a larger root system were able to produce greater shoot growth. The study stated that the root system was much smaller due to disturbance of root initiation without an increase to nitrate uptake efficiency. Notably, in this study the primary root was damaged as root development in lettuce under ideal conditions takes place rapidly with lateral roots appearing on the initial 2.5 – 3.5 cm of the primary root after just six days following seed germination (Weaver & Bruner, 1927).

One of the key physiological factors impacting on lettuce transplanting is the age of the transplant, as younger plants tend not to recover as well as older transplants from environmental stresses (Kerbiriou *et al*., 2013). The amount of stress is impacted by the size of the transplant container, an adequate root system developed within the container allows a larger transplant and reduces shock from environmental factors and pruning. Root pruning and ultimately the amount of root pruned will have an impact on shoot growth, in the short- or longer-term dependent on how optimal the field conditions are after transplanting.

Establishment of lettuce is influenced by the balance of nutrient and water at transplanting. Karchi *et al*., (1992) observed the root:shoot ratio was increased in treatments containing additional water or a high phosphate-low N regime. For the high phosphate-low N regime this would be a direct result of increased lateral root number, as nitrate has been shown to directly stimulate cell proliferation of roots in contact with high concentrations of nitrate within the soil in what is described as an overlap with the auxin response pathway (Zhang *et al*., 1999), which will be discussed in greater deal in section 2.6. Low P availability has been shown to increase lateral root development at the expense of primary root growth in *Arabidopsis thaliana* (Sánchez-Calderón *et al*., 2006), which is described as a survival mechanism to avert the poor diffusion of soil P previously mentioned (Schachtman *et al*., 1998). The study by Kerbiriou *et al*., (2013), was the first report of phenotyping the root system of transplanted lettuce to try and identify cultivars that would be able to provide some tolerance to reduced inputs.

The capability of the crop to overcome root pruning and agronomical and physiological factors would be beneficial in allowing the crop to establish faster. It is proposed here that a “rapid rooting phenotype”, defined by an increased cell elongation and

division rate (increased primary root length and lateral root length) accompanied by an increase in lateral root number, would allow better establishment of lettuce transplants and reduce the impact of abiotic stresses, such as mild periods of drought and nutrient deficiency by allowing access to a greater area of the soil profile.

2.5 Genetic factors influencing root growth and development during young plant establishment.

2.5.1 Root system architecture in the model plant *Arabidopsis thaliana*.

Dicot and monocot root systems differ greatly, dicots consist of a primary root with lateral roots repeatedly branching in several orders (Péret *et al.,* 2009a), whereas monocots, such as maize (*Zea mays*) consist of both an embryonic system of primary and seminal roots along with a post-embryonic system with shoot-borne and lateral roots (Tian, De Smet & Ding, 2014). In the model plant species *A. thaliana,* the formation of the primary and lateral roots, the basic components of a taproot system of dicot plants, have been studied extensively, and other dicot species usually have a largely comparable developmental and morphological root system (De Smet *et al.,* 2012) including lettuce (Jackson, 1995).

2.5.2 Dicot lateral root formation and emergence.

The emergence of lateral roots occurs in three definitive stages, pre-initiation, initiation, and post-initiation (Péret *et al*., 2009a). During the pre-initiation stage pericycle cells, which are cells that surround the vascular tissue are produced in the root apical meristem and form two distinct groups: those at the phloem poles and those at the xylem poles (Beeckman & De Smet, 2014). At the phloem poles the pericycle cells remain in the G1 phase of the cell cycle and cell division is prevented. At the xylem poles individual pericycle cells progress to the G2 phase and retain the ability to undergo cell division, these cells are known as founder cells (Beeckman *et al*., 2001) (Figure 2.2)

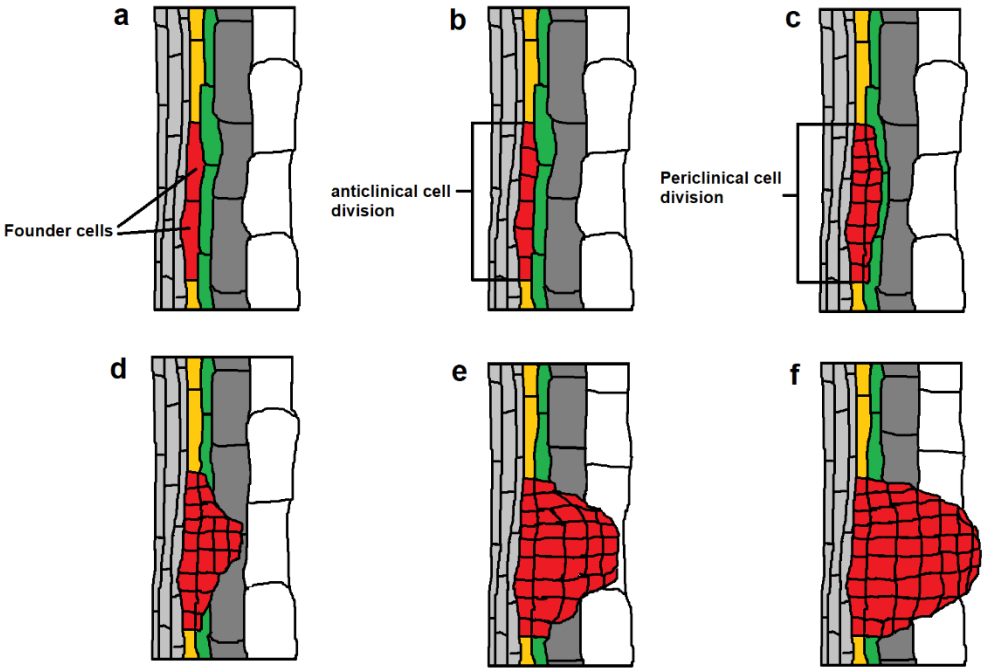

Figure 2.2: Lateral root development and emergence.
At the pre-initiation stage founder cells (red cells) within the pericycle cells (orange cells) enter G2 phase (a) these cells then begin to divide anticlinically during the initiation phase (b). The central lineage founder cells then divide periclinically (c) to begin to form the dome shape (d). Endodermal (green cells), cortex (grey cells) and epidermal cells (white cells) exterior to the founder cells undergo cellular remodelling (e) to allow the forming lateral root to emerge (f).

In a region between the root apical meristem and elongation zone, known as the basal meristem, the founder cells are exposed to the hormone auxin, which triggers the initiation stage of lateral root formation and lateral root position (De Smet *et al.,* 2007). Firstly, the initiation stage sees founder cells, mainly from the central file lineage (Kurup *et al.,* 2005) undergo a series of anticlinical divisions. These founder cells divide up to approx. 10 cells perpendicular to the root axis, forming a single layer primordium (Péret *et al.,* 2009b). These central file lineage founder cells then divide periclinically, parallel to the root axis and begin to form the inner and outer layers of the lateral root primordia, where a distinct dome shape begins to form (Malamy & Benfey, 1997). The central file lineage founder cells continue to divide forming a more pronounced dome at which point, during the post-initiation stage, modification to the tight junctions between the cell walls of outer tissues occurs via the accumulation of auxin at the apex of the root primordia which triggers the LAX3-dependent auxin induction of cell wall remodelling enzymes (Swarup *et al*., 2008), that will allow emergence of the primordium from the parent root (Péret *et al.*, 2009b).

2.6 The genetic control of root growth.

The expression of the ABERRANT LATERAL ROOT FORMATION 4 (ALF4) nuclear protein prevents the founder cells from terminally differentiating (DiDonato *et al.,* 2004), which allows lateral root initiation to occur and provides root plasticity to environmental triggers (Beeckman & De Smet, 2014). Lateral root initiation begins at the cortex-steele junction (Baluška *et al*., 2010) and is said to be under the hormonal control of shoot-derived auxin, which mediates root growth through the degradation of AUX/IAA proteins. When the shoot-derived auxin is transported to the root system (polar auxin transport) it triggers pericycle cells to begin to rapidly divide (Casimiro *et al*., 2001). In *A. thaliana* shoot-derived auxin binds to the auxin receptor, known as TRANSPORT INHIBITOR RESPONSE 1 (TIR1), which triggers the degradation of auxin transcriptional repressors (Dharmasiri *et al*., 2005), also known as AUX/IAA proteins, such as SHORT HYPOCOTYL 2 (SHY2) (Baluška *et al*., 2010). The SHY2 protein forms a heterodimer with TIR1 preventing expression of auxin response factors (ARF) (Dello loio *et al*., 2008). Auxin binds to TIR1 via the ubiquitin protein ligase SKIP-CULLIN-FBOX (SCF) forming the SCF^{TIR1} ubiquitin ligase complex, which triggers the degradation of *SHY2* and induces the expression of an auxin response factor (*ARF*), a transcription factor known as NAC1 (Xie *et al*., 2000). NAC1 belongs to the NAC domain family of transcription factors that are involved in various roles in plant development (Hu *et al*., 2010). The transcription factor *NAC1* consists of NAC-DNA binding domain and C terminal activation domain. *NAC1* is an early auxin responsive gene and during lateral root development mediates expression of genes involved with auxin signalling, namely the genes *DBP* and *AIR3* (Xie *et al*., 2000).

Following lateral root emergence, the growing apex of the lateral root, and that of the primary root for that matter, consists of four distinct zones of activity. The first lies behind the root cap and is known as the meristematic zone and houses all cells that undergo mitotic division (Verbelen *et al*., 2006). Auxin regulates the rate of cell division in the meristematic zone through the SCF^{TIR1} ubiquitin ligase complex previously described (Dello Loio *et al*., 2008). Directly behind the meristematic zone is the transition zone, where some cells differentiate, while others remain able to undergo cell division through the expression of the cell cycle control gene *CDC2*. (Verbelen *et al*., 2006). Within the transition zone cell fate is undertaken and is where adaptation to endogenous and exogenous stimuli occurs (Baluška *et al*., 2010). Cells in the transition zone undergo three-dimensional slow growth (Verbelen *et al*., 2006).

The plant hormone, cytokinin specifically acts upon vascular tissue within the transition zone where it controls the differentiation rate of other root cells (Dello Loio *et al*., 2008). Cytokinin binds to the sensor kinase *A. thaliana* histidine kinase 3 (AHK3) (Ueguchi *et al*., 2001) which through a phosphorylation cascade upregulates the transcription factor *A. thaliana* response-regulator 1 (*ARR1*) (D'agostino & Kieber, 1999), which in turn upregulates the expression of *SHY2*. SHY2 inhibits the expression of PIN-FORMED (PIN) proteins. PIN proteins are plant-specific transmembrane proteins that mediate the directional transport of auxin by the accumulation of PINs at plasma membrane domains where the auxin is transported (Křeček *et al*., 2009). The upregulation of *SHY2* expression thus supresses the expression of *ARF*s (Baluška *et al*., 2010) within the transition zone, inhibiting the effect of polar auxin transport and drives cell differentiation (Dello Loio *et al*., 2008). The cell differentiation of the root epidermis into trichoblasts or atrichoblasts is controlled by the expression and interaction of the *MYB*-like genes *WEREWOLF* (*WER*) and *CAPRICE* (*CPC*), which regulate the position-dependent expression of the homeobox gene *GLABRA2* (*GL2*). In cells where the WER protein is preferentially expressed the transcription of GL2 drives differentiation into atrichoblast cells, whereas higher expression of *CAPRICE MYB* drives differentiation into the root hair forming trichoblast cells (Lee & Schiefelbein, 1999).

After the transition zone cells enter the elongation zone and undergo a period of rapid cell growth along the apical-basal polarity axis (Verbelen *et al.,* 2006). Auxin regulates root cell elongation via the previously mentioned SCF^{TIR1} ubiquitin ligase complex. In the presence of auxin, the rapid expression of SMALL AUXIN UP RNA (*SAUR*) genes is activated, which are short lived proteins that localize to the cell membrane (Spartz *et al.,* 2012) activating plasma membrane H+-ATPases through phosphorylation of the C-terminus. The plasma membrane H+-ATPases, which pump H+ ions out of the cell causes a hyperpolarization of the plasma membrane leading to the influx of ions and water into the cell that in turn keeps the cell turgid during elongation (Spartz *et al.,* 2014).

A restriction to cells undergoing cell elongation is the cell wall. For cell elongation to take place the xyloglucan cross linkages that bind the cellulose microfibrils within the cell wall need to be broken (Vissenberg *et al*., 2000). This is achieved by enzymes known as xyloglucan endotransglycosylases (XETs), which endolytically break the xyloglucan polymer (Campbell & Braam, 1999), allowing the cellulose microfibrils to move apart before newly synthesized xyloglucan bridges are formed (Vissenberg *et al*., 2000). Another key group of proteins involved with cell elongation are the expansin proteins. Expansins are activated via the H+-ATPase activation by the SAUR protein within the plasma membrane and the

resulting increase in acidity within the cell wall (Spartz *et al*., 2014). Two major groups of expansins have been documented; the α-expansins (EXP) and the β-expansins (EXPB)(Cosgrove *et al*., 2002) and both assist in the weakening of the cell wall to allow elongation by making the cell wall acidic allowing a process known as wall creeping to occur. It is understood that EXPs act on dicot cell wall elongation whereas EXPB act on monocot cell walls (Cosgrove, 1998). The nuclei within the expanding cells are forced to the side of the cell by the development of very large central vacuoles. The rapid elongation of cells is accompanied by the noticeable bulging of cell walls of trichoblast cells during the root hair initiation phase (Verbelen *et al*., 2006).

Other genetic pathways play a key role in root growth, such as the genes involved with cell elongation. The homeostasis of auxin within the *A. thaliana* cell is important for root development and evidence suggests auxin is actively removed from the vacuoles via the tonoplast-localised auxin transporters; WALLS ARE THIN1 (WAT1), which has over 46 family members (Ranocha *et al*., 2013). WAT1 has been shown to be an essential component during secondary cell wall development (Ranocha *et al*., 2010) and light-independent cell elongation (Denancé *et al*., 2010) such as that of roots. Cell elongation takes place over key steps. Firstly, the xyloglucan cross linkages that bind the cellulose microfibrils within the cell wall need to be broken (Vissenberg *et al*., 2000) by enzymes known as xyloglucan endotransglycosylases (XETs) (Campbell & Braam, 1999). This allows the cellulose microfibrils to move apart before newly synthesized polysaccharide bridges are formed (Vissenberg *et al*., 2000) through enzymes such as *the IRREGULAR XYLEMs (IRXs), such* as IRX9, IRX10 and IRX 14, which synthesise xylan (Ren *et al*., 2014). *A. thaliana* mutations to the *IRX9* gene displayed a retarded growth trait (Lee *et al*., 2010), however no growth retardation was observed in *A. thaliana* wat1 mutant plants until around 4 weeks, where after a cell elongation defect was identified (Ranocha *et al*., 2010).

Plant growth depends not only on cell elongation, but also cell division and the balance of the two factors is essential for plant growth (Mar Castellano *et al*., 2001). Auxin regulates the rate of cell division in the meristematic zone through the SCF^{TIR1} ubiquitin ligase complex described earlier (Dello Loio *et al.,* 2008). Directly behind the meristematic zone is the transition zone, where some cells differentiate, while others remain able to undergo cell division through the expression of the *CELL DIVISION CONTROL 2* (*CDC2*) gene, (Verbelen *et al.,* 2006). In the G1 phase of cell division the genes CDC6A and CDC6B associate with the other origin recognition complex (ORC) subunits (ORC1-4, ORC6, CDT1 and PCNA) (Gutierrez, 2009) and confirms a cells ability to replicate its DNA during the S-

phase of cell division (Mar Castellano *et al*., 2001). Some of these DNA replication factors have other functions, such as CDT1 (Caro *et al*., 2007). Cell differentiation of the root epidermis into trichoblasts or atrichoblasts is controlled by the expression of the homeobox gene *GLABRA2* (*GL2*) (Lee & Schiefelbein, 1999). CDT1 interacts with the GL2 EXPRESSION MODULATOR (GEM) (Caro *et al*., 2007) and plays a role in deciding cell fate in the differentiation into atrichoblasts or into the root hair forming trichoblast cells (Lee & Schiefelbein, 1999).

A key promoter of rapid cell expansion within the meristem is cell division control protein CDC48, which interacts with P97 adapters to form the CDC48/P97 protein complex (Gallois *et al*., 2013). The abundance of CDC48 is negatively regulated by the PLANT UBX DOMAIN CONTAINING protein family (PUX1-4), which bind to the CDC48 protein via the UBX domain, preventing phosphorylation of the protein and targeting it to degradation via the ubiquitin pathway (Rancour *et al*., 2004).

The primary organic carbon source utilised during cellular processes in plants including cell division and expansion is sucrose (Zhang *et al*., 2014). Sucrose has been shown to act as a signalling molecule and varying levels of sucrose, and ultimately carbon availability can affect growth rates (Smeekens *et al*., 2009). An important secondary messenger of signal-transduction pathways is the ion calcium (Ca^{2+}) (Felle *et al*., 1992). Changes to cellular levels of Ca^{2+} affect developmental process, such as cell division and the polar growth seen in cell elongation of root cells. Key regulators of cellular Ca^{2+} homeostasis are the plasma membrane Ca^{2+} ATPases (ACA), such as ACA8, which has been observed to play an important role in sucrose signalling in *A. thaliana* seedlings and mutant knock-outs displayed arrested root growth due to loss of cell division in the root meristem (Zhang *et al*., 2014).

A further hormone involved with regulation of plant development including root growth is the extrinsic signalling stress hormone abscisic acid (ABA) (Hong *et al*., 2013). The addition of a 10 µM concentration of ABA was reduced root growth in *A. thaliana* plants to around 20 % of that of a control (Leung *et al*., 1997) and inhibited maize root cell elongation (Pilet & Saugy, 1987). Inversley, ABA has been shown to improve root growth in compacted soils, where the stress of soil strength is encountered, when compared to an ABA deficient mutant (Tracy *et al*., 2015) and plays a key role in the hydrotropism response through the up-regulation of the *MIZU-KUSEI 1* (*MIZ1*) gene via the ABA signalling kinase SnRK2.2 (Dietrich *et al*., 2017). ABA positively regulates the expression of a transcription factor known

as ABSCISIC ACID INSENSITIVE 4 (ABI4), which reduces polar auxin transport through the inhibition of expression of PIN-FORMED (PIN) proteins (Shkolnik-Inbar & Bar-Zvi, 2010). Increased expression of ABI4 was shown to inhibit lateral root development in *A. thaliana* (Shkolnik-Inbar & Bar-Zvi, 2010).

Key negative regulators in *A. thaliana* for the expression of ABA induced genes are the *C*-TERMINAL DOMAIN PHOSPHOTASE LIKE 1 (CPL1), CPL3 and SUPERSENSITIVE TO ABA AND DROUGHT 1 (SAD1) (Seifert *et al*., 2014). CPL1 is a negative regulator of the expression of *RESPONSIVE TO DESSICATION 29A* (*RD29A*) (Koiwa *et al*., 2002). The gene *RD29A* contains a dehydration responsive element (*DRE*) and an ABA-responsive element (*ABRE*) that can trigger expression of *RD29A* independently following the activation of *DRE* or *ABRE* binding proteins by ABA (Narusaka *et al.,* 2003). *CPL1* acts as a negative regulator of *RD29A* through the biogenesis of miRNA. CPL1 interacts with the protein SERRATE (SE), which allows the complex to then dephosphorylate the RNA binding protein HYPONASTIC LEAVES 1 (HYL1), which is required for the accurate strand selection and targeting of the DICER-LIKE 1 (DCL1) during miRNA gene silencing (Manavella *et al.,* 2012).

There are two conjunctive explanations for the initiation of lateral root development from the pericycle cells. The first is the initiation through root bending, where lateral root initiation takes place unilaterally at places of bending along the root axis. The second is where lateral roots are initiated through a regulated oscillatory pattern along the main root axis (Kircher & Schopfer, 2015). Lateral root initiation through root bending is caused by the process of the root bending itself. Auxin is translocated to the site of elongation and to pericycle cells, on the outside of the bend, resulting in an increase of auxin levels in these cells and the abundance of the AUX1 transporter within one or more pericycle cells in the region. The increase in auxin and AUX1 correlates with the reduction of *PINs* expressed in the cells and therefore the pericycle cell is pushed towards cell division and the formation of founder cells (Laskowski *et al.,* 2008).

The second explanation is that lateral root initiation occurs through the regulatory oscillating expression pattern of genes involved with the process within the basal meristem which lies before the elongation zone. This response was studied following the development of an artificial auxin response element known as *DR5*, which was developed to include the auxin response element (TGTCTC element), which usually lies upstream of start codons of auxin response genes such as *INDOLE-3-ACETIC ACID 7* (*IAA7*) and *IAA19*, and was fused

to the β-Glucuronidase reporter gene (*DR5::GUS*) allowing the observation of auxin mediated expression (Nakamura *et al.,* 2003). A study using this approach by De Smet *et al*., (2007) identified *DR5::GUS* expression oscillates within the basal meristem of *A. thaliana* roots that correlated with lateral root primordia spacing and preceded the expression of the auxin response gene *IAA14* (*SOLITARY ROOT*), which has been identified to be essential in the control of lateral root development via the transcriptional repression of *ARF*s (Fukaki *et al.,* 2002; Fukaki *et al.,* 2005). In a study by Moreno-Risueno *et al*., (2010) the oscillating expression of *DR5::GUS* was also found to be correlated with the oscillating expression of *ARF7*, which is an upstream regulator of *LOB-DOMAIN 16 (LBD16)*, a member of the *LATERAL ORGAN BOUNDARIES DOMAIN/ASYMETRIC LEAVES2-LIKE (LBD)* genes family. *LBD* genes have a conserved amino acid domain found only in plants and *LBD16* has been identified to be involved with lateral root emergence (Lee *et al.,* 2009).

2.6.1 Root hair development.

Root hairs form within trichoblasts through a process known as cytodifferentiation. Firstly, trichoblasts undergo bulge formation, which is associated with the localised accumulation of root hair specific EXPs, specifically *at*EXP7 and *at*EXP18 in *A. thaliana* (Cho & Cosgrove, 2002). These specific EXPs carry root hair specific *cis*-elements (RHEs) in the proximal promoter region (Lin *et al.,* 2011). The EXPs weaken the bonds of the cell wall cellulose of the trichoblast cell as previously mentioned. This process is followed by the reorganization of the actins known as F-actin and profilin to form mesh-works within the bulge (Baluška *et al.,* 2000). The reorganization of these actins is regulated by proteins known as actin depolymerizing factor (ADF) proteins, where the actins are localised to the site of polymerization by the ADF proteins (Jiang *et al*.,1997). The polymerization of the actins by ADFs drive root hair tip growth (Lin *et al.,* 2011) and the final size and elongation of the root hair is determined by a basic helix-loop-helix transcription factor, known as ROOT HAIR DEFECTIVE 6-LIKE 4 (RSL4) (Yi *et al.,* 2009). *RSL4* controls the transcription of genes involved in cell elongation, such as SUPPRESSOR OF ACTIN (SAC1), EXOCYST SUBUNIT 70A (EXO70A1), PEROXIDASE7 (PRX7) and CALCIUM-DEPENDENT PROTEIN KINASE 11 (CPK11) (Vijayakumar *et al.,* 2016).

2.6.2 GRAS-domain transcription factors and DELLA proteins.

Root growth is controlled by auxin, which regulates the cellular response to the phytohormone gibberellin, through the previously mentioned degradation of AUX/IAA

proteins (Xiangdong & Harberd, 2003). Another key player in the regulation of root growth are the DELLA proteins, which have been identified to be involved with the plasticity of roots to various environmental responses along with cell elongation (Weston *et al*., 2008). DELLA proteins are regulators of plant-specific GRAS-domain transcription factors, which were named after the first three identified family members; GIBBERELLIC-ACID INSENSITIVE (GAI), REPRESSOR OF GAI (RGA) and SCARECROW (SCR) (Hirsch & Oldroyd (2009) and their abundance is regulated by the levels of gibberellin.

Gibberellin destabilises DELLA proteins through SCF/26S proteasome targeting allowing root growth to be triggered (Fonouni-Farde *et al.,* 2016). Gibberellin first binds to the nuclear receptor protein, known as GIBBERELLIN INSENSITIVE DWARF1 (GID1), which is a soluble receptor with a high affinity to biologically active gibberellins (Ueguchi-Tanaka *et al.,* 2005). Upon binding with gibberellin the GA- GID1 changes ternary structure (Hirsch & Oldroyd, 2009) and binds with DELLA proteins, which in turn triggers a conformational change leading to the recruitment of the F-box proteins SLEEPY1 and GA-INSENSITIVE DWARF2 (GID2). SLY1 binds to the DELLA protein and the formation of this complex leads to degradation via the SCF^{SLY1}E3 ubiquitin ligase through the 26S proteasome (Xiangdong *et al.,* 2004). DELLA proteins have also been shown to be self-regulating, and a high abundance of DELLA proteins have been linked to the expression of gibberellin synthesis genes (Weston *et al*., 2008).

GRAS-domain transcription factors are involved with many processes of plant growth and development, including but not limited to radial organisation of the root, gibberellin signal transduction, and axillary meristem initiation. There are five conserved regions within the C-terminus of almost all GRAS-domain transcription factors and the GRAS-domain proteins are categorised into eight sub-families based on members or the common motifs within these regions. The subfamilies are DELLA, LISCL, HAM, PAT1, LS, SCR, SCL3 & SHR (Hirsch & Oldroyd, 2009). The first is the Leucine heptad repeats 1 (LHR1) region which includes a nuclear localization signal (NLS) relative to an NLS conserved in the DELLA proteins. The LHR domains are essential for protein homodimerization. Secondly, a VHIID domain, which consists of a highly conserved region within each group. Thirdly, there is the LHRII domain, which has a motif that has been linked to protein-protein interactions. The fourth is the PFYRE motif and the fifth is the SAW motif (Chaoguang *et al.,* 2004), which act as regulatory domains (Hunag *et al*., 2006).

Within the DELLA subfamily the N-terminus houses the conserved DELLA domain, which is needed for gibberellin regulation and deletion of this region causes mutant plants to display dwarfed phenotypes and are non-responsive to gibberellin (Tyler *et al.,* 2004). A total of five DELLA proteins have been identified in *A. thaliana* that are involved with repression of gibberellin-responsive plant growth. *GA INSENSITIVE* (*GAI*), *REPRESSOR OF gal-3* (*RGA*) and three *REPRESSOR OF gal-3-LIKE* genes (*RGL1, RGL2* & *RGL3*). *RGA* and *GAI* are reported to be involved with leaf expansion, stem elongation, leaf development from juvenile to adult phase apical dominance and vegetative to reproductive transition. Both *RGA* and *GAI* exhibit redundancy in that both been reported to have similar functions in the gibberellin-signalling pathway with loss-of-function of one gene having little to no impact versus wild types, however when both genes are knocked down complete reversal of repression of the gibberellin-signalling pathway is observed and specific phenotypes observed (Dill & Sun, 2001). RGL1 has been identified to be involved with floral development *and RGL2 has* been identified to be the major repressor in seed germination (Tyler *et al.,* 2004). In a study by Sawada *et al*., (2008) DELLA proteins with 62% and 61% similarity to *A. thaliana GAI* were identified in lettuce seeds and named *Ls*DELLA1 and *Ls*DELLA2, respectively.

Within *Pisum sativum*, a study identified two DELLA proteins that promote the biosynthesis of gibberellin and the depression of other deactivating proteins. The LA and CRY DELLA proteins were shown to upregulate the biosynthesis of gibberellin through loss-of-function mutations, interestingly LA was found to overcome the null-mutation to CRY and a wild type phenotype was observed indicating the DELLA protein LA is the main functioning DELLA protein in root growth of *P. sativum* (Weston *et al.,* 2008).

In summary the auxin hormone response pathway promotes lateral root initiation through cell division of founder cells and root growth through promotion of cell elongation. The cytokinin hormone response pathway acts upon genes associated with the auxin pathway and promotes cell differentiation to trichoblasts or atrichoblasts. The gibberellin response pathway plays a key role in the degradation of the DELLA proteins, which in turn inhibit expression of genes associated with the auxin response pathway. Abscisic acid plays a key role in stress response including root growth in compacted soils.

2.7 Identifying breeding targets for rooting traits.

Crops, except for tubers and tap root vegetables, such as potatoes and carrots, have traditionally been bred to improve above ground characteristics and predominantly yield (Zhu

et al., 2011). This includes lettuce, which has been historically bred for phenotypes that confer a large yield in high input systems or pest resistance ignoring the root system architecture (RSA) (Johnson *et al.,* 2000); however, future crop production in some areas will most likely be constrained by increasingly nutrient deficient soils and drought conditions due to lower fertilizer and water availability (Zhu *et al*., 2011) and increased prices as nutrients such as phosphorus diminish (Le Marié *et al.,* 2014).

2.7.1 Desirable transplant rooting traits for breeding.

By identifying genetic control and hence breeding potential for desirable rooting traits it may be possible for breeders to develop lettuce varieties that can establish more rapidly in more variable conditions whilst maintaining commercial uniformity and yield. This can be achieved by: a) identifying phenotypes of interest in diversity collections; and/or b) locating regions of the genome that carry genes of interest by mapping quantitative trait loci (QTL); and/or c) identifying candidate genes, through gene expression studies, involved in development of a root system architecture (RSA) that optimizes water and nutrient uptake.

Fitter & Strickland, (1991) describe RSA as being defined by the distribution of lateral root branching of the root system (Topology) combined with internodal length between lateral roots and the angle of the roots (geometry). RSA have been identified for such traits, such as a deep root RSA phenotype, such as that displayed by the wild lettuce *Lactuca serriola*, to acquire water in drought conditions (Johnson *et al.,* 2000; Uga *et al.,* 2013) or nitrogen deficient soils (Kerbiriou *et al.,* 2013) and an optimal root system architecture phenotype for phosphate acquisition (Lynch, 2011); (Shi *et al.,* 2013). Following identification of a beneficial RSA the phenotype could be introduced into commercial lines (de Dorlodot *et al*., 2007). This practice can be applied to directly drilled lettuce seedlings, which develop a strong primary root, however; transplants usually develop a specific root system, caused by the destruction of the primary root in the container (Leskovar & Cantliffe, 1993) (Figure 2.1) or during transplanting and therefore the identification of a genotype with the ability to develop a high number of adventitious lateral roots at variable depths could be beneficial in stress conditions.

2.8 Diversity fixed foundation sets (DFFS).

Diversity fixed foundation sets (DFFS) are an informative set of fixed (homozygous) lines representative of a gene pool (Pang *et al*., 2015). DFFS have been developed to assist

breeders, who would usually have to screen very large numbers of lines from Genebank collections for the target phenotype, by capturing the majority of the genetic variation accessible to breeders in smaller more manageable collections (Whalley *et al*., 2012).The modern domesticated crops have been bred to have great morphological differences, although many hidden genetics, such as disease resistance and rooting traits, are lost though the breeding process of selecting for visible traits and culminate in a restricted gene pool (Whalley *et al*., 2012).

The lettuce DFFS used in this work was developed by the Vegetable Genetic Improvement Network (VeGIN) and consists of 96 accessions with accessions from *Lactuca sativa* (79 accessions), *Lactuca serriola* (12 accessions), *Lactuca saligna* (3 accessions) and *Lactuca virosa* (2 accessions). The lettuce DFFS has previously been used to identify phenotypic variation for postharvest discolouration (Atkinson *et al*., 2013a).

2.9 Quantitative traits and discrete traits – QTL analysis.

Traits are controlled either by a single gene, referred to as a discrete or qualitative trait, which follows a simple Mendelian pattern of inheritance, such as wrinkled or smooth pea seed, or they can be quantitative traits controlled by many interacting genes located within regions of the genome, known as quantitative trait loci, and have complex patterns of inheritance (Jeuken & Lindhout, 2004). Many important traits for agriculture, such as those for yield, quality, some disease resistance (Collard *et al*., 2005) and root development are quantitative traits.

2.9.1 Mapping populations.

Mapping populations consist of a genetically defined plant population, usually consisting of between 50 and 250 individuals that are segregated. The segregation of modern mapping populations is based on the identification of DNA markers, such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), microsatellites or single sequence repeats (SSRs) and more recently single nucleotide polymorphisms (SNPs), within the parent lines. The markers once identified must be screened across all the population lines including the parents in a process known as marker genotyping (Collard *et al*., 2005).

To identify the most appropriate mapping population to use, the parents must first be analysed, and a difference observed for one or more of the traits of interest. Mapping populations can be developed in a number of ways and the goal in each method is to produce a population that is genetically defined (i.e. the markers and genes will constantly remain the same), which also benefits from allowing comparison or replication studies on the same population at different locations and years (Collard *et al*., 2005).

The first method is to produce a fixed population by several rounds of selfing or sibling mating of the first filial (F1) generation produced from the parents to create what are known as recombinant inbred lines (RILs) (Jones *et al*., 2009). RILs can be employed in varying generations but not usually before the F_5 (van Ooijen, 1999) as selfing causes a high level of recombination due to the repeated occurrence of meiotic events (Jones *et al*., 2009) but most recombination occurs before the F_5, after which fixation (high levels of homozygosity) ensures recombination has little effect (van Ooijen, 1999). The results are individual RILs with a unique genetic combination from the two parents.

The second method is the production of back cross inbred lines (BILs) where an individual RIL is repeatedly crossed with one of the parent lines resulting in lines with single introgressions, these fixed populations are also known as introgression lines (ILs) or near-isogenic lines (NILs) (Jones *et al*., 2009).

A relatively new strategy for producing homozygous lines without the need for several generations of selfing is the double haploid method. When geneticists identified spontaneously occurring haploid plants, a short cut to homozygosity from heterozygous parents was realised. The modern technique, termed genome elimination, includes the wide crossing of a distant relative or intra-species, such as the maize haploid inducers derived from the Stock6 line, where the distant related genome is lost during embryogenesis, which usually means the haploid seed, is not viable and requires embryo rescue (Chan, 2010). The haploid seedlings then undergo artificially triggered chromosome doubling through the addition of colchicine to produce a double haploid (100% homozygous) plant (Eder & Chalyk, 2002). Double haploids have also been used in the production of diversity fixed foundation sets in brassica species and the lettuce DFFS, which are an informative collection of lines of a gene pool (Pink *et al*., 2008).

2.9.2 Linkage maps (Genetic maps).

A linkage map or genetic map is the assembly of polymorphic molecular markers on chromosomes derived from two different parents, which display the relative genetic distance between markers and their position along the chromosomes. Markers can be either co-dominant, where many sized bands may be present on a gel and discrimination can be accomplished between homozygotes and heterozygotes, such as RFLPs, or dominant, where the marker is either present or absent and no discrimination between homozygotes and heterozygotes can be made, such as RAPDs.

In a segregating population (mapping population) recombination occurs more frequently between markers that are far apart or 'unlinked' compared with those that are closer together or 'linked' (Collard *et al*., 2005). Linkage maps are developed based on the principle that during the diplotene stage of meiosis chromosomal crossover, also known as chiasma, occurs between homologous chromosomes resulting in recombinant chromosomes with segregated genes and markers. The frequency of 'crossing over' is proportional to the genetic distance between the markers and can therefore be used to order the markers along the chromosome. One percent recombination is equal to one centimorgan (cM) in distance. For example, if recombination is displayed between marker A and B in 2 recombinants and between marker B and C in 4 recombinants of a total mapping population of 20 lines then the genetic distance between marker A and B is 10 cM and between marker B and C would be 20 cM. What must be noted is the frequency of recombination can only be a maximum of 50% for genes that are at opposite ends of the chromosomes due to only two of the four chromatids undergoing cross over (Jones *et al*., 1997).

2.9.3 Mapping functions.

As linkage maps are constructed based on genetic distances (cM) that are based on recombination frequency, clustered regions of crossover events rather than random distribution will cause a distortion of the physical distances between loci (Jones *et al*., 1997) when map distances are greater than 10 cM, therefore mapping functions are employed to convert recombination fractions in to cM. The Haldane mapping function assumes no interference from recombination has occurred whereas the Kosambi and Carter-Falconer mapping functions assume there is interference (Collard *et al*., 2005).

2.9.4 QTL analysis.

There are several methods for detecting QTL, the first and most simple is single-marker analysis, which detects QTL associated with single markers and statistical analysis is usually through linear regression. The further a QTL is from a marker the less likely it is the QTL will be detected due to recombination (Collard *et al*., 2005) and the method cannot tell if the marker is associated with one or more QTL or the relative position of the QTL (Zeng, 1994). The second and most used method is simple interval mapping developed by Lander & Botstein (1989). This method uses linkage maps and analyses the intervals between linked markers to compensate for recombination between the markers and the QTL (Collard *et al*., 2005), although a bias of estimation and identification of QTL can take place when multiple QTL are located on the same linkage group. A more recently employed method to alleviate the issue with interval mapping is the use of composite interval mapping, which combines multiple regression analysis with interval mapping. The analysis includes additional markers as covariates to control for other QTL whilst analysing for QTL in an interval with the linked markers of interval mapping (Kao, Zeng & Teasdale, 1999).

2.9.5 Lettuce mapping populations.

There are several published genetic maps of lettuce (Truco *et al.,* 2013) three of these were produced from crossing *L. sativa* cultivars, the first was produced by crossing the cultivars 'Kordaat' (Butterhead) and 'Calmar' (crisphead), which utilised an F_2 population of 222 individuals to create a genetic map consisting of 53, then newly developed, RFLPs, which distributed along 9 linkage groups (Landry *et al*., 1987). Later the population was increased to 309 individuals and a further 229 DNA markers were added to the genetic map and the number of linkage groups increased to 13, however; as there are only nine chromosomes in *Lactuca* spp. large marker-sparse regions were predicted to still be present (Kesseli *et al*., 1994).

The second was the crossing of the 'Dwarf-2' and 'Saffier' cultivars, which was for the purposes of mapping the *dwf-2* locus involved with a loss of response to gibberellic acid (GA) and loss of reproductive growth (known as bolting) that causes loss of heading and loss of the crop (Waycott *et al*., 1995), and similarity to the Kordaat X Calmar cross, which would allow comparability and integration of linkage data. However, this map also suffered from more linkage groups than actual chromosomes (Waycott *et al*., 1999). The third intra-specific cross, developed by Atkinson *et al.,* (2013b) at the University of Warwick, was between a Batavian cultivar (Iceberg), bred in France and the crisphead variety Saladin, bred in the US. Most of the European and US crisphead cultivars today are derived from this

Saladin variety. The Saladin X Iceberg cross was initially developed at the University of Warwick by Hand *et al*., (2003) for the identification of molecular markers for marker assisted selection (MAS) of resistance against the peach-potato aphid (*Myzus persicae*) and downy mildew (*Bremia lactucae*) and later used for the study of post-harvest discolouration (Atkinson *et al.,* 2013b).

Further lettuce mapping populations have been produced from crossing *L. sativa* (cv Salinas syn. of Saladin) with the wild lettuce *L. serriola* (Johnson *et al.,* 2000; Syed *et al.,* 2006) and *L. sativa* with *L. saligna* (Jeuken & Lindhout, 2004). The majority of mapping studies in lettuce have focused on the segregation for disease resistance (Waycott *et al*., 1999; Jeuken *et al*., 2008; McHale *et al.,* 2008), although lettuce mapping populations have been successfully used in the identification of QTL for seed germination (Argyris *et al.,* 2005; Hayashi *et al*., 2008), seed storage longevity (Schwember & Bradford, 2010), shelf life (Zhang *et al.,* 2007), and tipburn (Jenni *et al*., 2013).

Only two papers have been published to date that focuses on identifying QTL based on the segregation for lettuce root traits, the first was for drought tolerance, through deep soil water exploitation (Johnson *et al.,* 2000) and the second was for salt tolerance in seedlings through changes in RSA (Wei *et al.,* 2014) and both studies employed a *L. sativa* and *L. serriola* cross mapping population. The study by Johnson *et al.,* (2000) identified 13 QTL involved in RSA and soil water extraction and the later study by Wei *et al.,* (2014) confirmed two of these locations were major QTL involved with root development. The first was qRC9.1 (qRS9.2) on linkage group 9 that was shown to be involved with lateral root length and lateral root number and the second was qRS2.1 on linkage group 2, which was identified to be involved with lateral root length.

2.9.6 Bulk segregant analysis.

The procedure of bulk segregant analysis is a rapid way of identifying markers linked with a specific gene or genetic region. The method works by bulking DNA from individuals that have the same phenotype or genetic region into bulks. The bulks differ for everything but the phenotype or genetic region of interest, which following marker analysis identifies the DNA markers that differ between the two groups highlighting the genomic region of interest (Michelmore *et al*.,1991).

2.10 Transcriptomics & RNA-seq.

The transcriptome is the complete set and quantity of transcripts within a cell at a given development stage or physiological condition (Wang *et al.,* 2009). The term transcriptome was first termed in 1997 in a gene expression study of yeast cells using a method known as serial analysis of gene expression (SAGE) (Velculescu *et al*., 1997). The methodology of SAGE was the binding of short 9-10 bp nucleotide tags binding to the RNA and cleaved using a specific anchoring enzyme (restriction endonuclease). The cleaved sequences were then bonded to streptavidin beads, sequence tags added and amplified. The abundance of the tags that bind to the strands is reported to be proportional to the differential gene expression of the transcriptome. The sequence strands were then concatenated together and sequenced using Sanger sequencing (Velculescu *et al*., 1995). Other tag-based methods were also developed, but these tag-based methods all relied on Sanger sequencing, which is relatively expensive and they only analysed a proportion of the transcriptome and were unable to differentiate between splice variants (Wang *et al.,* 2009).

The most utilised modern method is RNA sequencing (RNA-seq), which uses deep-sequencing platforms. The population of RNA is converted to a complementary DNA (cDNA) library with a reverse transcriptase with adaptors attached to one or both ends. The sequences are then sequenced using a high through-put sequencer in a single (one end) or paired end (both ends) orientation (Holt & Jones, 2008). Once sequenced the reads can then be aligned to a reference genome (mapping first) or to each other in de novo assembly (assembly first) if a reference genome is unavailable (Grabherr *et al*., 2013; Wang *et al.,* 2009). De novo assembly requires transcript assembly programs, such as Trinity which assemble the reads into transcripts through a defined fixed length of k nucleotides overlapping, known as k-mers (Grabherr *et al*., 2013). De novo assembly is error prone as many genes have paralogues and isoforms with regions that overlap with one another (Kovaka *et al*., 2019).

There are various tools for the alignment of transcript reads to a reference genome, including the hierarchical indexing for spliced alignment of transcripts 2 HISAT2 program. HISAT2 is a spliced alignment program based on the indexing by the Burrows-Wheeler method and the FM index and was reported to be the most accurate alignment program to date (Kim *et al*., 2015). HISAT2 aligns the reads to the reference genome. Following spliced alignment to the reference genome transcriptome assembler programs are used to assemble the reads, which includes the Sringtie2 program. StringTie 2 assembles spliced

variants of each transcript and based on the number of reads associated with each transcript estimates the transcript abundance. StringTie 2 has been shown to be more accurate than other reference guided assemblers including Cufflinks and Bayesembler (Kovaka *et al*., 2019).

Once assembler programs have been used to assemble and quantify the transcripts differential expression of count analysis programs, such as the Empirical analysis of DGE in R (EdgeR) program can be utilised to identify significant differences in differential expression of replicated groups (Robinson *et al*., 2009). Recently these individual programs have all been brought together in one online, cloud based, useable interface, known as Galaxy (Blankenberg *et al.,* 2011; Afgan *et al.,* 2018).

The use of RNA-seq analysis has been previously used in lettuce to identify genes associated with flowering (Han *et al*., 2016), shoot growth under varying LED light quality and intensity (Kitazaki *et al*., 2018), genes associated with the circadian clock system (Higashi *et al*., 2016) and the evolution of lettuce and flavonoid biosynthesis regulation (Zhang *et al*., 2017). Only one other study was identified that had analysed the differential expression of genes within the roots of lettuce using RNA-seq. The study by Wang *et al*., (2017a) was looking for differences in gene expression associated with the application of nanomaterials for the stimulation of root growth and found differential expression of genes associated with photosynthetic, sucrose, starch and N metabolic pathways.

Several studies have combined QTL mapping and transcriptomic analysis as an approach to identify candidate genes. A study by Liu *et al*., (2016) used fine mapping and RNA-seq to identify genes associated with fibre quality in cotton and suggested the integrative approach is a powerful strategy for the identification of candidate genes. A study by Zheng *et al*., (2003) combining QTL mapping with transcriptomics in a method referred to as cDNA-AFLP analysis identified four candidate genes associated with increased seminal and lateral root length in rice.

In conclusion RNA-seq is a powerful tool in understanding transcriptome differences at given points of development or physiological conditions. The accuracy of RNA-seq is greatly increased with the use of a reference genome compared to that of *de novo* assembly and RNA-seq can be used in combination with QTL mapping to greatly reduce the number of differentially expressed genes needed to be characterized for the identification of candidate genes associated with a given trait.

2.11 Rooting assays.

The obvious difficulty with studying the root system architecture of plants is that the root system is in the soil and root analysis often means disturbance of the soil-plant system (Perret, Al-Belushi & Deadman, 2007). However, several techniques have been developed that can remove this limitation. A summary of rooting assays is presented in Table 2.1 and the most relevant approaches that would allow the phenotyping of seedlings are discussed in the following section.

2.11.1 2D rooting assays.

Rhizotrons, which are systems where roots are grown in a narrow gap between two sheets of material, usually with a window or clear face to allow analysis of root architectural traits (Taylor *et al*.,1970) have been used in various studies to analyse the phenotypic root architecture of different plant species including rice (Price *et al.,* 2002) and lettuce (Schreiter *et al.,* 2014). The plant is placed at the top of a soil filled box containing a transparent observation window down one side. The rhizotron is usually placed at an angle to encourage root growth along the observation window (Neumann *et al.,* 2014). In horhizotrons the plant is placed in the centre of a four-pronged star shaped tub with observation windows along the sides of the tub. In each prong of the tub the soil environment or rhizosphere can be altered to observe how the root system grows in each individual condition (Wright & Wright, 2004). Rhizotrons offer analysis of the root architecture in similar conditions to in-field, where the rhizosphere would be similar, however; rhizotrons are expensive to construct, time consuming and require large areas of space making high-throughput screening difficult. Although the root architecture can be analysed in real time it is only a 2D representative of the entire root system architecture (Shrestha *et al.,* 2014).

Several approaches have been developed where the root system is grown without soil on anchor paper or agar plates, but these approaches are only suited to study root growth from seeds rather than transplants. In agar assays variable concentrations of nutrients can be added to the agar before it sets and then poured to form a thin layer. The seed is usually germinated on germination paper prior to introduction to the agar. Once the seed is placed on the agar the assay is sealed and stored upright to allow root growth (Shi *et al.,* 2013). The anchor paper assay consists of a piece of anchor paper on to which the seed is placed and a sheet of black polyethylene, which is added to minimize light penetration, to

form what is termed a growth pouch. The bottom edge of the growth pouch is placed in a reservoir that can contain a solution consisting of variable concentrations of nutrients that diffuse into the anchor paper (Hund *et al*., 2009).

A technique, known as rhizoslides, has been developed from the anchor paper assay to allow the separation of embryonic and post-embryonic roots in cereals. The seed is placed on the tip of a plexiglass sheet with anchor paper each side of the plexiglass, which in turn is covered with a polyethylene sheet. The embryonic roots grow between the plexiglass and the anchor paper whereas the post-embryonic roots grow between the anchor paper and overlying polyethylene sheet allowing direct analysis of post-embryonic root traits (Le Marié *et al.,* 2014).

Once the root has grown along the 2D plane in the assays cameras and scanners are used to create an image and the images can be analysed using various software packages developed such as RootReader 2D that allow high throughput phenotyping of specific root growth traits and entire root systems (Clark *et al.,* 2013).

2.11.2 3D rooting assays.

A relatively modern, non-destructive, technique for analysis of root system architecture is computed tomography (CT) scanning, which allows an image of the root system to be created without removing the roots from the soil. CT scanning works by passing a 1mm thick X-ray beam (Lontoc-Roy *et al.,* 2006) through the object resulting in the accumulation of CT numbers, which can be used to build 3D matrix images by assembling several consecutive scans (Perret *et al*., 2007). The technique has been successfully used to observe the 3D root system architecture of species with fine roots, such as *A. thaliana* (Tracy *et al*., 2010) along with the impact of soil compaction on tomato (*Solanum lycopersicum*) (Tracy *et al*., 2012b) and wheat (Tracy *et al*., 2012a).

A gel-based growth platform has been developed that uses Phytagel as a media to grow the root system in. The seed is usually germinated on germination paper before being transferred to a transparent tube containing the Phytagel substrate that has had plant nutrients added to it. The tube is covered with a sleeve and the root system is allowed to develop. The root system architecture is then scanned using a 3D laser scanner. The scanner remains in a fixed position and the transparent tube is rotated through 360° to create a 3D image of the root system (Fang, Yan & Liao, 2009). More recently 2D images

have been developed in a similar way but employing a digital camera instead of a laser scanner and using Gelzan CM, which is more transparent than Phytagel. Currently software is being developed that will allow 3D models to be constructed from the digital images produced (Iyer-Pascuzzi *et al.,* 2010).

Gel chambers have been used to analyse root growth phenotypes in *Brassica napus* seedlings (Shi *et al.,* 2013) and barley (*Hordeum vulgare*) seedlings (Bengough *et al.,* 2004). However, no literature could be found relating to the use of agar to analyse root growth from a transplant. Agar is relatively inexpensive, allows a direct 3D observation of the root system architecture and is not lethal to the plant, but root growth in agar may not relate to in-field phenotype displayed in soil. A further issue that needs to be addressed when using gels/agar assays is the contamination of the media by fungal and bacterial species due to the high sugar content. This would be exacerbated when placing a peat transplant block onto the agar, which can be alleviated with the addition of antibiotics and fungicides, although this may have a detrimental effect on the rhizosphere.

Root systems in field trials can be analysed by taking core samples using a cylindrical auger at various positions. The core sample allows the root density at various depths to be recorded and the roots can be washed to remove the soil before being scanned for analysis of root length etc using software packages such as WinRhizo Pro 2007 (Kerbiriou *et al*., 2013). In the Trench profile method, a channel is dug perpendicular to the crop and water can then be used to expose the root system, which allows analysis of root mass etc at various depths (Neukirchen *et al.,* 1999). More recently technology has been developed that allow non-invasive analysis of root systems in plants and trees. In ground penetrating radar (GPR) pulses of high frequency radio waves are used to create images of subterranean objects such as roots around 0.5 cm and larger. Electrical resistivity imaging uses electrode arrays to pass a current through the soil and measure resistance, which can then be used to generate an image. ERI can be used at the soil surface or within boreholes (Zhu *et al*., 2011).

2.11.3 Alternative rooting assays.

Buried herbicide assays have been developed for high throughput screening of root depths. The herbicides are buried at predetermined levels and the time taken for symptoms to appear and the severity are recorded and scored respectively (Grumet et al., 1992; Al-Shugeairy *et al.,* 2014). The herbicide Diuron has been successfully used to detect the root

growth phenotype in rice cultivars (Al-Shugeairy *et al.,* 2014). Diuron is a phenylurea herbicide, which inhibits photosynthesis by binding to the exchangeable quinone (QB) site of the D1 protein of photosystem II and blocks the electron flow from the QA site (Werner *et al.,* 2002). A triazine herbicide with a similar mode of action to Diuron is Simazine (Wilson, Whitwell & Klaine, 2000), which was used in a study by Grumet *et al* (1992) to analyse cucumber root growth. Diuron is only slightly water soluble and is therefore able to be soaked into a filter paper and will not diffuse through the soil (Al-Shugeairy *et al.,* 2014), this makes the diuron assay ideal for analysing both root depth and lateral root growth as the filter paper can be placed around the entire root system at variable distances. The herbicide is resistant to leaching and diffusion through the soil ensuring root growth and position can be analysed.

The herbicide could also be employed in variable soil conditions related to stress, such as low nutrient availability, high salinity, and drought. The herbicides used in the mentioned studies are relatively inexpensive and simple to achieve a high level of screening (Grumet *et al.,*1992). However, there are a number of limitations to herbicide assays: they require systemic herbicides rather than contact herbicides and are therefore lethal to the plant, the symptoms in systemic herbicides typically take approximately 3-7 days to appear, uptake is dependent on water content of the soil and needs to be constant across all lines, and uptake could in fact be dependent on root uptake efficiency phenotypes and not root growth phenotypes (Grumet *et al.,*1992; Al-Shugeairy *et al.,* 2014).

An alternative to using herbicide is the use of fluorescent dyes. Fluorescent dyes have been successfully used to analyse the relatively low translocation time from root to leaf of around 4-24 hours (Donaldson & Robinson, 1971). Fluorophores would easily be observed in a high through-put system requiring only a UV lamp and would be non-destructive to the plant ensuring further analysis would be achievable. However, fluorophores can be expensive. Fluorophores can diffuse through the soil easily and therefore they would need to be made stationary by incorporating it in a gel/agar matrix or similar media that would prevent mobility and diffusion.

Table2.1: List of rooting platforms/assays previously used to measure root system phenotypes and their advantages and disadvantages.

Assay/platform.	Description.	Advantages.	Disadvantages.	Reference.
Rhizotrons.	Roots are grown in a narrow gap between two sheets of material, usually with a window or clear face to allow analysis of root architectural traits.	Inexpensive. Direct observational analysis. Non-destructive.	Only 2D analysis. Require large areas. Low through-put.	Taylor *et al*., (1970). Shrestha *et al.,* (2014).
Mini-rhizotrons/clear pots.	Clear tubes ~51 mm dia. Allow analysis of roots in contact with the side of the pot.	Direct observational analysis. Non-destructive. 3D system.	Restrictive growth due to pot size. Low through-put.	Upchurch & Ritchie (1983). Richard *et al*., (2015).
Rhizoslides.	Used for cereals, where the embryonic roots and post-embryonic roots need to be separated for analysis.	Inexpensive. Direct observational analysis.	Low through-put. Only 2D analysis.	Le Marié *et al.,* (2014). Le Marié *et al*., (2016).
Shovelomics.	A simple method used for cereals, where the roots are simply dug up close to the plant and the number of crown roots counted.	Very inexpensive. High through-put. In-field analysis.	Only partial analysis. Destructive.	Trachsel *et al*., (2011). Arifuzzaman *et al*., (2019).
Magnetic resonance imaging (MRI).	Based on the magnetic moment of nuclei of atoms and the ability of magnets to manipulate them.	High-resolution 3D imaging. Non-destructive.	Expensive. Requires skilled technicians. Low through-put.	Schultz *et al*., (2013). Van Dusschoten *et al*., (2016).
Light sheet tomography (LST).	Use of a laser to provide a light sheet and capture of the scattered light after it hits the object.	High resolution 3D imaging. Non-destructive.	Limited pot size. Low through-put. Expensive.	Yang *et al*., (2013).
Biospeckle imaging	Laser refractions, known as speckles, are used to identify areas of biological activity (biospeckles) within a root system architecture, such as cell elongation or division.	High-resolution 3D imaging.	Expensive. Requires skilled technicians. Low through-put.	Ribeiro *et al*., (2014). Braga *et al*., (2009).
Transparent soil	Matches the refractive index of a solid (Nafion) with an aqueous solution (Sorbitol) to allow the solid to appear invisible.	3D analysis. Non-destructive.	Expensive. Poor lateral root density.	Downie *et al*., (2012).
Horizotron	Like rhizoboxes but allows analysis of horizontal root growth as opposed to vertical growth.	Inexpensive. 3D system	Low through-put. Require large areas.	Wright & Wright (2004).
Growth pouches	Growth of the root system, on a 2D plane, on germination paper covered with a polyethylene sheet.	Inexpensive. Very High through-put. Non-destructive.	Only 2D analysis Difficult to correlate with field.	Atkinson *et al.,* (2015). Thomas *et al*., (2016a, b).
Buried Herbicide assays	The use of systemic herbicides buried at a variety of levels and symptoms scaled and related to root contact time and contact levels.	Inexpensive. 3D system.	No direct analysis. Destructive. Low through-put.	Grumet *et al*., (1992). Al-Shugeairy *et al.,* (2014).
Gel chambers	Seedling is grown along a 2D plane of agar gel, usually in large petri dishes.	Inexpensive. High through-put. Non-destructive.	Only 2D analysis Difficult to correlate with field.	Shi *et al*., (2013). Bengough *et al*., (2004).

Soil coring	The use of a cylindrical auger to sample the root mass, type and number at various positions and depths in relation to the plant.	3D analysis. Inexpensive. In-field analysis.	Low through-put. Only partial analysis.	Noordwijk *et al*., (1985). Kerbiriou *et al*., (2013).
Computed tomography (CT)	The use of X-rays to view the root system within the pot.	High-resolution 3D imaging. Non-destructive Good correlation with field.	Expensive. Limited pot size. Low through-put. Requires skilled technicians.	Tracy *et al*., (2010). Mooney *et al*., (2011).
X-ray micro-tomography	Silver anode sourced X-ray passes through the sample and differences in intensity are detected on the far side of the object by a detector, producing an image with computer software.	Extremely high-resolution 3D imaging. Relatively inexpensive. Non-destructive.	Very limited pot size. Low-through-put. Requires skilled technicians.	Gregory *et al*., (2003). Hargreaves *et al*., (2009).
Optical Projection Tomography (OPT).	Allows analysis of bioactivity and gene expression on roots grown in agar or other clear gels using microscopy	Relatively inexpensive. Non-destructive.	Low-through-put. Difficult to correlate with field	Lee *et al*., (2006).
Ground Penetrating Radar (GPR)	Passes electromagnetic waves into the soil and measures the reflected signal.	Non-destructive. 3D analysis. In-field analysis.	Very low resolution. Only very dense structures. Low through-put.	Hruska *et al*., (1999). Hirano *et al*., (2009).
Electrical resistivity Imaging (ERI)	Measures the soil resistance to an electrical current o analyse root mass.	Non-destructive. 3D analysis. In-field analysis	Very low resolution. Only very dense structures. Low through-put.	Amato *et al*., (2008).
Trench profile	Digging of a trench close to the plant and then washing the root system clear of soil.	Can be non-destructive. 3D analysis. In-field analysis	Very low through-put. Laborious.	Vepraskas & Hoyt (1988). Neukirchen *et al.,* (1999)

There are many different forms of root analysis platforms available, from simple gel-based 2D assays to high-tech 3D CT MRI and X-ray assays. The high-throughput systems allow the possibility to non-destructively analyse hundreds of genotypes in a very inexpensive platform taking up very little space, however they tend to be 2D assays and can poorly correlate to in-field environments. Assays that correlate better with in-field conditions tend to be low through-put, allowing the analysis of only several genotypes at a single time point and most 3D assays with the exception of CT, MRI and X-ray platforms tend to be destructive. A combination of a high through-put system to analyse a large number of genotypes followed by a low through-put 3D analysis of extremes offers a logical solution.

3 Chapter 3 - 2D high-throughput assay optimization and mapping population selection.

3.1 Abstract.

The study of lettuce seedlings using a high through-put 2D assay has not yet been optimised. The following study identified the need for additional porous sheets to the standard practice of cassette construction at a Hoaglands concentration of 15% is required along with a pre-soaking prior to placement of the seed/seedling in the 2D high-throughput assay. The study also identified greater segregation of the intra-specific parents on the assay than that of the inter-specific parents for the traits primary root length, total lateral root length and total number of laterals that are associated with rapid rooting, indicating that the intra-specific cross is more useful for the study of QTL analysis for the rapid rooting phenotype in the 2D high-throughput assay.

3.2 Introduction.

Lettuce in the UK and Europe is usually sown and raised to the 5-7 true leaf stage in commercial nurseries prior to transplanting to the field. Plants that are transplanted are more uniform, avoid early environmental stresses associated with direct drilling and usually mature earlier than direct sown crops (Leskovar & Cantliffe, 1993). However, when lettuce is transplanted to the field the root system undergoes a major stress through root pruning due to the conjoined blocks, they are raised in and the growing of roots into adjacent blocks. The plant must therefore recover the root area lost in a process known as the recovery phase (Kerbiriou *et al*., 2013). The ability of the plant to restore the root area quickly and establish in the field is beneficial to uniformity and earlier maturity as shoot growth is greatly reduced until the root:shoot ratio is restored (Bar-Tal *et al*., 1994).

Rapid recovery of the root system following transplanting, could be hypothesised to aid rapid field establishment i.e. the quicker the plant can recover any lost root mass and increase root mass in the field soil, the quicker the establishment will be. Therefore, potential traits of interest forming part of a “rapid rooting” phenotype are defined as; increased primary root growth (PRL), increased lateral root growth (TLL) and a higher production of lateral roots, or total number of laterals (TNL). Other traits of interest, that could be beneficial to other in-field requirements following establishment, are root hair length, which has been

linked to uptake efficiency of phosphorus in cow pea (Krasilnikoff *et al*., 2003), root hair density, which would increase the overall root area for nutrient and water acquisition (Bibikova & Kilroy, 2003), and lateral root topology, which could aid in the breeding of cultivated lettuce that have the deeper rooting trait of wild relatives, allowing access to nutrients and water from deeper soil profiles (Johnson *et al*., 2000).

The root system architecture of wild and cultivated Lactuca spp. differ greatly Cultivated lettuce displays a shallow root system inadvertently selected for uniform and rapid shoot growth in cultivated systems (Jackson, 1995). The wild Lacuca spp. develop a deep primary root relying on the acquisition of water from deeper soil zones during surface soil drought making them more tolerant to drought conditions (Werk & Ehleringer 1985; Jackson, 1995; Gallardo *et al*., 1996). It has been suggested altering the root system architecture of cultivated lettuce would provide recovery of deeper soil resources and avoid stress (Johnson et al., 2000).

3.2.1 Assay optimisation.

The 2D high-throughput growth pouch assay used in this study consists of a vertically orientated, A4 germination (or anchor) paper and a polyethylene sheet, which can be easily removed for periodical imaging of the root system. The assay was first employed to identify QTL in a study of wheat seedling root characteristics by Atkinson *et al.,* (2015) and has since been used to identify genetic variation in oilseed rape, including under varying phosphorus supply (*Brassica napus* L.) (Thomas *et al*., 2016a,b; Wang *et al*., 2017b) and to further analyse wheat root architecture related to nitrogen uptake efficiency (Kenobi *et al*., 2017) The assay allows the rapid analysis of root traits in seedlings in a time, cost and labour efficient manner compared with other techniques (Table 2.1). Thomas *et al*., (2016a) estimated that total analysis of each plant in this assay required ~2 min at a cost of <£1 per seedling.

This study aims to identify if the 2D high through-put growth pouch assay can be developed for the adequate growth of lettuce seedlings so that further studies can be undertaken to identify if variation for rapid rooting traits exists within the lettuce mapping populations available for genetic study.

3.2.2 Mapping population selection.

There were two mapping populations available for the identification of QTL and possible genes involved with the rapid establishment of transplanted lettuce. The first was an intra-specific cross, developed at the University of Warwick by Hand *et al*., (2003) for identification of molecular markers for marker assisted selection (MAS) of resistance against the peach-potato aphid (*Myzus persicae*) and downy mildew (*Bremia lactucae*). The parents of the population are the Batavian cultivar Iceberg (syn Batavia Blonde a bord Rouge), bred in France and the crisphead cultivar Saladin (syn Salinas) bred in the USA. The second mapping population has been developed from an inter-specific cross between *L. sativa*, cultivar Salinas with accession LJ03050 of the wild progenitor species, *L. serriola.* The mapping population was first described in a study identifying QTL for rooting traits (Johnson *et al*., 2000).

To identify the most appropriate mapping population to use, the parents must first be analysed, and a difference observed for one or more of the traits of interest (Collard *et al*., 2005). This study also aims to identify if significant genetic variation for the rapid rooting traits, and other root traits of interest, exists in one or both of the pairs of mapping population parents available and hence which population would give the best possible likelihood of identifying QTL associated with the traits.

3.3 Materials and methods.

3.3.1 Plant material.

For the growth pouch optimisation study, which was conducted to identify the optimal conditions within the assay a commercial variety, a variety designated as commercial line C was provided by Syngenta (Fulbourn, Cambridgeshire, UK).

For the identification of the optimal mapping population available the parents of the inter-specific *L. sativa* (cv Salinas) x *L. serriola* developed at the University of California, Davis, and intra-specific *L. sativa* (cv Saladin) x *L. sativa* (cv Iceberg) developed by the University of Warwick were used.

3.3.2 Germination.

Germination paper (SD7640; Anchor Paper Company, St Paul, MN, USA) was placed in petri dishes. The germination papers were pre-soaked with 7 ml of tap water for imbibition of the seed. Once the seeds had been placed in the petri dishes they were put inside a 310 x 340 mm lidded opaque plastic tray and held in a cold store (14-16˚C) with 24 h low irradiance 1.5 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR) lighting. The seeds were left for a minimum of 48 h to reach a pre-determined stage of germination. Following the initial stages of germination when the radicle emerges from the seed and is around 1-5 mm in length root hairs are developed that form an arrowhead-like appearance on the radicle. This stage of germination was easy to identify and use as the optimal point at which to place the seedling on the assay. The seeds were checked every 24 h and this assured that all seedlings were placed on the assay at the same growth stage, thereby removing any variation due to germination time.

3.3.3 Growth pouch optimisation.

Modifications were made to the 2D high through-put growth pouch assay (Atkinson *et al*., 2015; Thomas *et al*., 2016a) (Figure 3.1) through the addition of two sheets of porous paper (TFM Farm and Country Superstore Ltd, Shropshire, UK) altering the position of the seed within the cassette to try and optimise root growth generating four cassette types a-d (Figure 3.2). The four cassette types were tested in three concentrations of Hoagland's solution (Hoagland's No. 2 Basal Salt Mixture, Sigma Aldrich, Dorset UK) were tested at 0%, 15% (0.24 g L^{-1}) and 30% (0.48 g L^{-1}) to identify the most adequate nutrient concentrations required for optimal root growth. The four cassettes types in the three hoaglands concentrations were treated with a further experimental design, where all four cassette types in the three hoaglands concentrations were placed within the tanks to allow diffusion of the nutrient solution up through the cassette or pre-soaked for ~10 s in the solution before being placed in the system.

Following diffusion or pre-soaking of the cassettes the germinated seeds were placed at the top of the growth pouch ensuring the visible radicle was orientated towards the bottom of the cassette.

3.3.4 Seedling growth.

For both experiments, the growth pouches were suspended over drip trays with ~ 50 cm of the lower portion submerged in the hoaglands solution. The growth pouches were supported within an aluminium frame as described by Atkinson *et al*., (2015). Each drip tray had 2 L of tap water containing 15% (0.24 g L^{-1}) Hoagland's solution (Hoagland's No. 2 Basal Salt Mixture, Sigma Aldrich, Dorset UK) added. At a height of 40 cm above the growth pouches, over each tank were six 550 mm strip white light emitting diode (LED) lights (Leyton Lighting, Essex, UK) providing a mean PAR of 90.1 µmol m^{-2} s^{-1}, ranging from 68.5-113.4 µmol m^{-2} s^{-1}. The lights were set to provide a 20 h photoperiod.

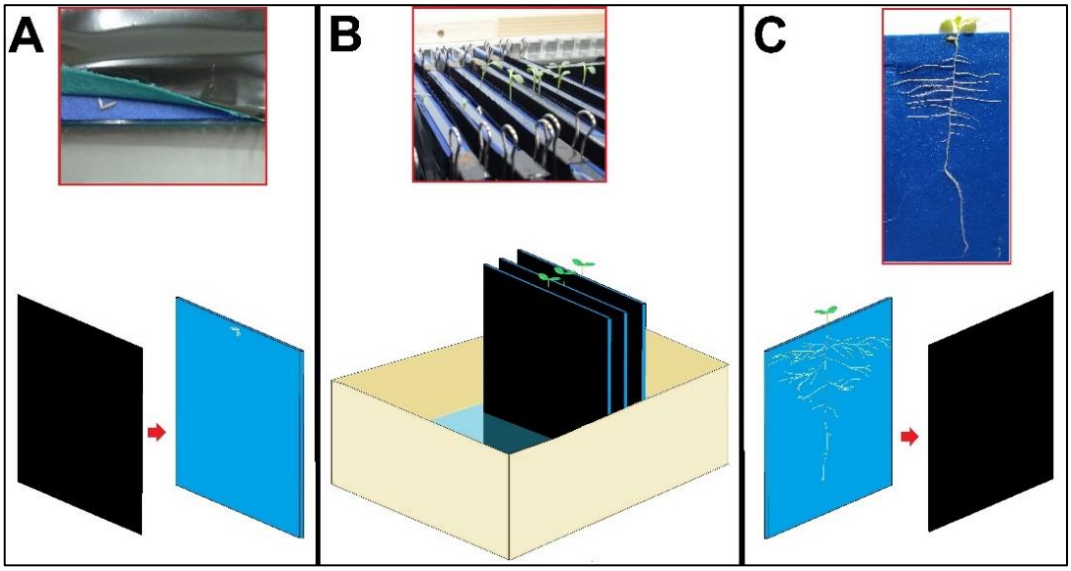

Figure 3.1: schematic representation of the 2D high-throughput system.
A) The seedling was placed in the pouch, next to the blue anchor paper; B) the seedlings were grown for 14 days; C) the pouches were then removed, and the root system exposed for imaging by removal of the black polyethylene sheet.

For the 2D high-throughput growth pouch assay optimisation four replicates of commercial line C was used for each cassette type were used for each treatment. The diffusion and pre-soaked treatment and the Hoagland's concentration treatment. The cassettes for all treatments were allocated to positions in the support frames using a one-way design with no blocking (GenStat 17th edition, VSN International Ltd, Hemel Hempstead, UK).

For the mapping population parents six replicates were grown in cassette type B with 15% Hoagland's solution using the same randomizing method already discussed.

The seedlings of both experiments were grown within one frame unit for a 14-d period with a 20 h photoperiod to encourage as much growth as possible in the 24 d period but allowing a short period without photosynthesis. The temperature and relative humidity (RH) were recorded every 2 hours with a data logger (TinyTag Plus2, Gemini Data Loggers Ltd, Chichester, UK). The mean temperature was 14.1°C and ranged between 13.7°C and 17.6°C. The mean RH was 98.2 % with a minimum of 76.2 % and a maximum of 100%. Following 14 d growth the pouches were removed from the system for imaging.

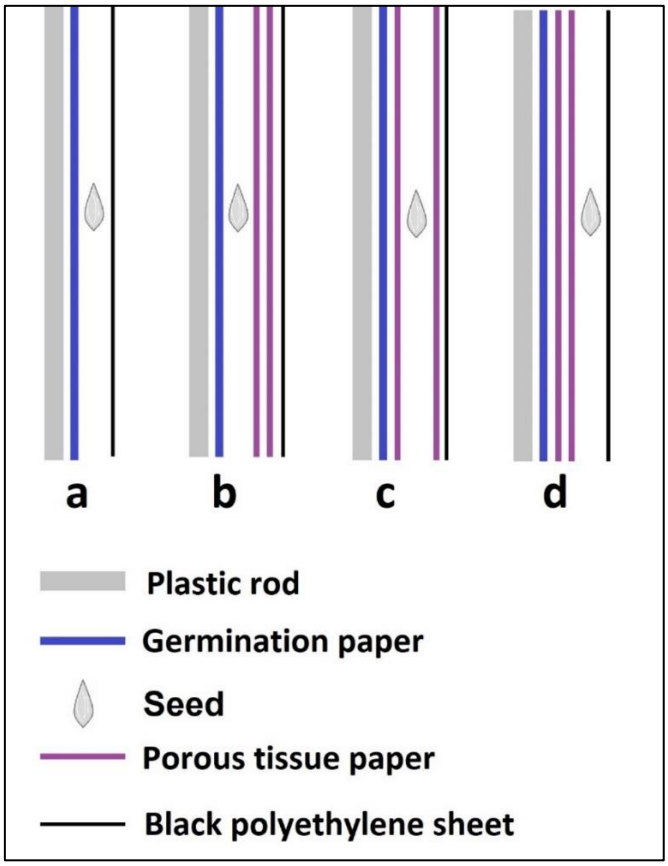

Figure 3.2: An overhead schematic representation of the various iterations attempted.
The cassettes were constructed with the seed placed in different positions. a) Seed was placed next to anchor paper and no porous tissue used (standard cassette); b) two sheets of porous paper were added to the cassette orientated towards the polyethylene sheet; c) the seed was placed between the two additional porous sheets; d) the seed was placed orientated towards the polyethylene sheet.

The cassettes for both experiments were removed from the frame and dismantled to expose the root system. The root system was then imaged with a digital camera (Lumix - DMC-FP2, Panasonic, Berkshire, UK) at fixed distance of 200 mm (Appendix 3.1). The images were analysed using ImageJ (Abràmoff *et al*., 2004; Schneider *et al*., 2012; Thomas *et al*., 2016a). The segmented line selection tool was used to measure individual root lengths. For the growth pouch optimisation experiment, measurements were recorded and

analysed for the pre-defined rapid rooting traits; primary root length (PRL), total lateral root length (TLL) and total number of lateral (TNL) roots. For the mapping population experiment, traits measured were those associated with rapid rooting; PRL, TLL, and TNL and three calculated ratios of the three traits; lateral root length density (LRLD = TLL/PRL), lateral root number density (LRND = TNL/PRL) and the mean lateral root length (MLRL = TLL/TNL) ((LRLD). Additional traits that were measured and analysed for mapping population parents were; mean maximum root hair length (MRHL) [[the longest visible root hair measured], percent root hair coverage (PRHC) [a visual score of the percentage of the root covered in root hair], root hair density (RHD) [a visual score of what percentage of total root was root hair] (Appendix 3.2).

3.4 Results.

3.4.1 Growth pouch optimisation.

The pre-soaked treatment had a significantly longer ($P<0.001$) PRL, which had a mean of 42.5 mm compared to the diffused treatment that had a mean length of 24.0 mm. For the trait TLL the pre-soaked treatment mean of 19.0 mm was significantly ($P<0.001$) greater than the mean of 8.1 mm observed in the standard diffused treatment. The pre-soaked treatment also had a significantly ($P<0.001$) greater number of lateral roots for the trait TNL than the diffused treatment, with a mean of 5.5 lateral roots compared with a mean of 2.8 laterals in the diffused treatment (Figure 3.3).

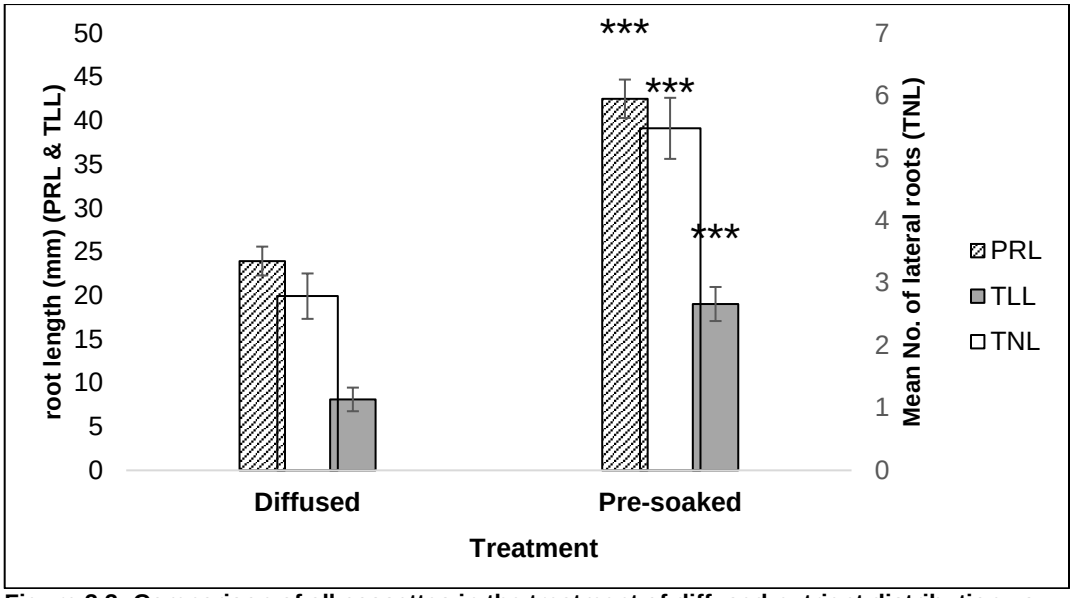

Figure 3.3: Comparison of all cassettes in the treatment of diffused nutrient distribution vs pre-soaking the cassette for ~10s prior to seed placement of commercial line C (n=48). ***=P<0.001.

There was a highly significant difference identified between cassette types in the pre-soaked treatment for PRL (P<0.001), TLL (P<0.001) and TNL (P<0.001). Cassette type D had the highest mean PRL, which was 56.5 mm and was significantly greater (P=0.015) than cassette type B, which had the second highest mean value for PRL. Cassette type C had the highest mean of 25.3 mm for TLL, which was significantly (P<0.001) greater than the mean (4.9 mm) of the cassette type A (standard cassette) but no significant difference was found between cassette types B, C and D. For the trait TNL the highest mean (7.25) was observed in the cassette type D, which was significantly greater (P<0.001) than the mean (2.6) observed for cassette type A (standard cassette), however no significant difference was observed between cassette types B, C and D (Figure 3.4).

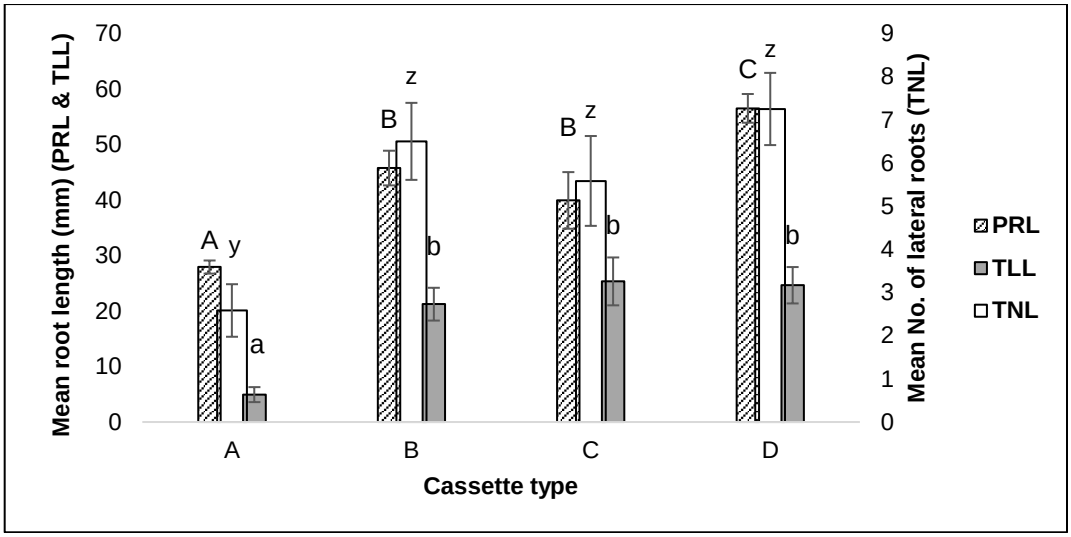

Figure 3.4: Comparison of 14 d old seedlings of commercial line C for the three traits PRL, TLL and TNL for cassette type in the pre-soaked treatment (n=12).
Uppercase letters (A, B and C) indicate the significant differences (P<0.05) between the cassette types for the trait PRL. Lowercase letters (a, b and c) show the significant differences (P<0.05) between the cassette types for the trait TLL. The lowercase letters y and z indicate the significant differences (P<0.05) between the cassette types for the trait TNL.

No significant difference (P=0.555) was found for the trait PRL in the pre-soaked treatment between the different Hoagland's solution concentrations. However, the longest PRL was in the 15% concentration. For the trait TLL in the pre-soaked treatment there was no significant difference (P=0.078) found between Hoagland's solution concentrations with 15% Hoagland's solution having the highest mean TLL of 24.7 mm. There was a significant difference (P=0.035) for the trait TNL between nutrient concentrations for the pre-soaked treatment, with the highest mean (6.8) in the 15% concentration, which was significantly (P=0.010) greater than the mean of 3.8 lateral roots seen in the 0% Hoagland's solution concentration, however no significant difference was observed between the 15% and 30% concentrations (Figure 3.5).

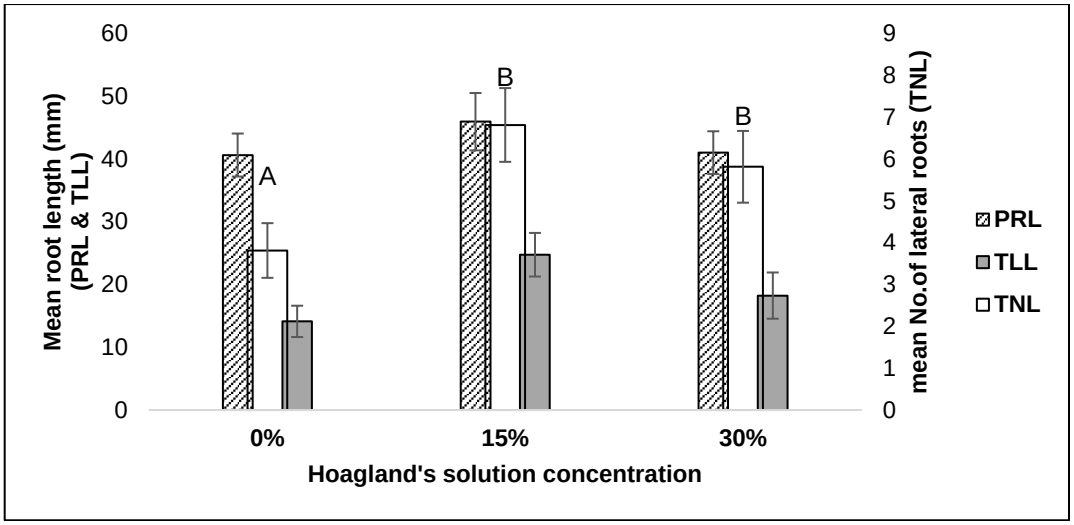

Figure 3.5: Comparison of 14 d old seedlings of the commercial line C in Hoagland's solution concentrations (0%, 15% & 30%) in the pre-soaked treatment (n=16).
The uppercase letters show the significant difference (P<0.05) observed between hoaglands concentrations for the trait TNL.

3.4.2 Mapping population parent segregation.

There was a 58.2 mm difference (P<0.001) in primary root length between the Saladin and Iceberg parents for primary root growth, which was a larger phenotypic difference than the 15.9 mm difference (P=0.004) seen between the cv. Salinas and *L. serriola* parents (Table 3.1).

For the trait TLL the segregation of the intra-specific parents, Saladin and Iceberg, was larger (P=0.008) than that of the inter-specific parents Salinas and *L. serriola* (P=0.244) with a difference of 87.2 mm compared to only 6.5 mm (Table 3.1).

There was a highly significant difference (P=0.003) for the trait TNL in the intra-specific cross parents, Saladin, and Iceberg, with a difference of 9.5 lateral roots. This was greater than the difference of 0.3 lateral roots between the inter-specific parents Salinas and *L. serriola* (P=0.034) (Table 3.1).

There was no significant difference between the Saladin and Iceberg parents of the intraspecific cross for the trait MRHL (P=0.279), however there was a significant difference between the inter-specific cross parents, Salinas and *L. serriola* (P=0.025) (Table 3.1).

No significant differences were identified between the intra-specific cross parents, Saladin, and Iceberg, for any of the three calculated ratios: LRLD (P=0.759), LRND (P=0.16) and MLRL (P=0.192). No significant differences were observed in the inter-specific cross

parents, Salinas, and *L. serriola*, for the three ratios: LRLD (P=0.471), LRND (P=0.706) and MLRL (P=0.068) (Table 3.1).

There was no significant difference (P=0.279) between the intra-specific Saladin and Iceberg parents for MRHL, however there was a significant difference (P=0.025) found between the inter-specific cross parents Salinas and *L. serriola* (Table 3.1).

There was no significant difference (P=0.115) for the trait PRHC in the intra-specific cross parents Saladin and Iceberg. There was, however, a significant difference (P=0.024) observed between the Salinas and *L. serriola* parents of the inter-specific cross (Table 3.1).

There was no significant difference seen between either the parents of the intra-specific (P=0.145) or the inter-specific (P=0.227) cross parents for the trait RHD (Table 3.1).

	Inter-specific parents				**Intra-specific parents**			
Trait	**Salinas**	***L. serriola***	**Difference**	**Sig.**	**Saladin**	**Iceberg**	**Difference**	**Sig.**
PRL	7.77	23.69	15.92	**	32.32	90.5	58.18	***
TLL	2.44	8.97	6.54		45.07	132.27	87.23	**
TNL	1	1.33	0.33	*	2.83	12.33	9.5	**
LRLD	0.35	0.78	0.43		1.49	1.38	0.11	
LRND	0.14	0.1	0.03		0.1	0.13	0.04	
MLRL	1.52	6.13	4.61		17.07	10.77	0.15	
MRHL	0	1.21	1.21	*	0.93	1.07	0.15	***
PRHC	0	43	43	*	19	40.33	21.33	
RHD	0.1	5.7	5.6		2.05	5.05	3	

Table 3.1: Root trait contrasts of the parent lines of the two mapping population parents grown for 14d in the 2D high through-put assay with 15% Hoagland's solution (n=6). *P<0.05, **P<0.01, ***P<0.001.

3.5 Discussion.

3.5.1 Growth pouch optimisation.

The study determined the 2D high-throughput growth pouch assay can be optimised for the adequate growth of lettuce seedlings for the analysis of the root system. Lettuce root growth for the three traits associated with rapid rooting (PRL, TLL & TNL) in the assay could be significantly increased through pre-soaking the cassettes for ~10 s prior to placement of the seed on to the system compared to the original method (Atkinson *et al*., 2015). The study identified the most optimal cassette type for lettuce root growth was cassette type D,

however the placement of the seed on the porous tissue caused issues at the imaging stage as the roots had a tendency to grow intermittently through the porous paper making separation of the root and the porous paper difficult, whereas in the second best system (cassette type B), that had only a lower significant growth of the PRL trait compared to cassette type D, the roots remained on the anchor paper and imaging was relatively simple. The concentration of the Hoagland's solution for the adequate growth of lettuce seedlings was 15%, which was lower than the 25% concentration used in studies of *Brassica napus* (Thomas *et al*., 2016b; Drizou *et al*., 2017; Wang *et al*., 2017b).

The 2D high-throughput growth pouch assay will enable the analysis of lettuce seedling rooting traits and other studies have been able to correlate the findings in the assay to field grown crops. The study of *B. napus* seedling root traits by Thomas *et al.*, (2016a), was able to link specific root traits to nutrient capture and ultimately seed yield in field-grown *B. napus*. This is promising for the study of lettuce transplant establishment, as the transplant is closer to the development stage of seedlings than fully mature seed setting plants. In the original study utilizing the assay, wheat rooting traits of wheat seedlings for nitrogen uptake in the assay did not strongly correlate to nitrogen uptake in the field, however one possible cause for this was that no adventitious roots were observed for the seedlings in the assay, but make up a large area of the root system in mature plants and account for a large proportion of nutrient and water capture in wheat (Atkinson *et al*., 2015). This issue may not be a problem correlating lettuce seedling root traits in the assay with transplanted lettuce performance as only the initial root mass that produces field establishment would be measured, although studies would need to be undertaken to analyse if this is the case.

3.5.2 Mapping population selection.

The study identified phenotypic variation for rooting traits in a 2D high-throughput growth pouch assay in both mapping populations. There was significant segregation between the parents of both mapping populations for the trait PRL, although the greater difference was between the intra-specific cross parents, Saladin and Iceberg. For the trait TLL the only significant difference was seen in the intra-specific cross parents, which was also the case for the trait TNL. The results indicate that the optimal mapping population to use for identification of QTL linked to the "rapid rooting" (greater growth of PRL, TLL and TNL) phenotype would be the intra-specific Saladin X Iceberg recombinant inbred line (RIL)

mapping population, as the parental lines of the population need to phenotypically and therefore genetically different for the trait of interest (Collard *et al*., 2005).

For the rooting trait MRHL, which could prove to be an important factor in nutrient and water capture, there was a significant difference identified between the inter-specific parents Salinas and *L. serriola*, but not between the parents of the intra-specific cross parent lines. For the trait RHD no significant segregation was observed between the parents of the intra-specific cross, but there was a significant difference seen between the parents of the inter-specific cross. The observations also suggest that any future studies that analyse root hair length/density in lettuce should consider inter-specific crosses for maximum genetic variation between the parental lines.

3.6 Conclusion.

The study has been able to identify the optimal cassette type for the study of lettuce seedlings in the 2D high-throughput assay for the study of lettuce seedlings. The optimal set-up of the cassette requires the addition of two porous tissue sheets to the cassette orientated towards the polyethylene sheet side of the cassette with the seed in contact with the anchor paper. The cassette requires pre-soaking in the optimal 15% Hoagland's solution. The study also identified greater phenotypic variation for the rapid rooting traits in the intra-specific cross parents compared to the inter-specific parents, indicating this population should be used for the study of the rapid rooting trait in the 2D assay, while further traits such as those associated with root hairs would benefit from the use for the interspecific mapping population.

4 Chapter 4 - Phenotypic variation identified in a 2D high-throughput phenotyping assay for root traits in seedlings of a lettuce diversity fixed foundation set.

4.1 Abstract.

Lettuce in western Europe is transplanted and crops need to establish quickly in the field to optimise growth, uniformity, and mild initial drought tolerance. The following study used a 2D high through-put assay to screen 14 day old seedlings of a lettuce diversity fixed foundation set (DFFS) consisting of 96 accessions of wild and cultivated lines and identified genotypic variation for key rooting traits that could aid establishment and could be important for future breeding programmes. The DFFS accession (CGN04628) known as Kakichishia White had the greatest growth for the traits defined to constitute a “rapid rooting” phenotype. The study also identified genetic variation within the DFFS and found that wild relative lines have a deeper rooting potential of lateral roots than the cultivated varieties and tend to have shorter root hairs covering a larger area of the root surface than the cultivated lines, which tend to have longer, but fewer root hairs.

4.2 Introduction.

Root development in young seedlings can affect the production of an optimum root system for the remainder of a plant’s lifetime. It is suggested that early rapid root growth and branching provides an advantage for efficient soil water use and plant establishment (Nicola, 1998). The distribution of the root system within in the soil can determine the plant’s potential to access irregularly distributed resources throughout the soil profile (Lynch, 1995). Root system architecture can differ greatly across species and between species genotypes (Lynch, 1995) and although root systems display extensive phenotypic plasticity to environmental cues, some of the variation is also under genetic control (Fitter & Strickland, 1991).

Root development in lettuce (*Lactuca sativa*) takes place rapidly under ideal conditions, with lateral roots appearing on the initial 2.5 – 3.5 cm of the primary root after just six days following seed germination (Weaver & Bruner, 1927). Within Western Europe, lettuce is commonly grown as transplants in compact peat blocks in commercial nurseries prior to planting to fields at the 5-7 true leaf stage, which provides several benefits to the

farmer including prevention of thermodormancy and associated cost of seed priming, faster germination, a more competitive crop against early weed infestation and importantly, a more uniform crop (Maltais, Gosselin, Tremblay & van Winden, 2008). Crop uniformity is considered an essential trait in crops such as lettuce, which still require manual harvesting on a single pass through the crop. However, transplantation is a time of high stress for such crops with a higher susceptibility to drought and nutrient stresses prior to the growth of roots out of the transplant block and establishment of a root system in the surrounding soil (Figure 2.1).

Crops with a high-water content, such as lettuce (95%) are very sensitive to drought conditions, , furthermore cultivated lettuce have relatively shallow roots with the majority of the root system architecture functioning within the top 20 cm of the soil (Johnson *et al*., 2000). Therefore, rapid root elongation could be advantageous to their establishment as surface soil layers are extremely vulnerable to drying out (Sharp *et al*., 1988). Drought conditions are expected to be amplified by climate change, increasing the establishment time for seedlings and increasing both the intensity of drought and rate of drying (Trenberth *et al*., 2014). Droughts, exacerbated by global warming, have been forecast to reduce global crop yield by approximately 1.5% per decade (Lobell & Gourdji, 2012).

The root system architecture of the wild relative (*Lactuca serriola*) differs to that of cultivated lettuce. The wild relative is tolerant to drought (Werk & Ehleringer 1985), develops a long taproot, relies on water from deep soil zones during surface soil drought, and displays a lower level of developmental plasticity in its roots than the cultivated species *L. sativa* (Jackson, 1995; Gallardo *et al*., 1996). The differences in root architecture and root growth patterns between wild and cultivated lettuce suggest that inadvertent selection has occurred for root characteristics in *L. sativa* that result in rapid growth and shoot uniformity under cultivation (Jackson, 1995) but due to the shallow rooting phenotype, high losses of nutrients through leaching below the root zone. Breeding programmes that alter the root system architecture of commercial lettuce cultivars would provide recovery of deeper soil resources and avoid stress (Johnson *et al*., 2000).

The lettuce Diversity Fixed Foundation Set (DFFS) employed in this study was developed by the Vegetable Genetic Improvement Network (VeGIN) and consists of 96 accessions (*L. sativa* = 79 accessions, *L. serriola*=12 accessions, *L. saligna*=3 accessions and *L. virosa*= 2 accessions). The DFFS was developed to maximise genetic variation in a smaller, more manageable number of accessions than wider collections of lettuce diversity in

various Genebanks (Walley *et al*., 2012). The accessions were selected from the UK Vegetable Genebank, Wellesbourne, UK (19 accessions) and the International *Lactuca* collection at Centre for Genetic Resources Netherlands (CGN) (77 accessions). The DFFS has been previously used to quantify nitrate content (Burns *et al*., 2011), post-harvest discolouration (Atkinson *et al*., 2013a), and lettuce aphid resistance (Walley *et al*., 2017). The DFFS contains the parents of two mapping populations. The first is from an inter-specific cross between *L. sativa* cv. Salinas and the wild relative *Lactuca serriola* (Johnson *et al*., 2000). The second is an intra-specific cross between the crisphead *L. sativa* cv Saladin (syn Salinas) bred in the US and the Batavian *L. sativa* cv Iceberg, bred in France (Atkinson *et al.,* 2013b).

In this study, a hydroponic system was used to study the DFFS and three additional commercially available cultivars to determine: 1) if there is significant phenotypic variation at the seedling stage for root system architecture, root hair morphology and a rapid rooting phenotype that currently does not exist in current commercial varieties, and 2) if elite lines can be identified for these traits that could, through breeding programmes, be introgressed into commercial varieties to reduce the impact of drought stress in transplanted field crops.

4.3 Materials and methods.

4.3.1 Plant material.

The study included three commercially available varieties from Syngenta (Fulbourn, Cambridgeshire, UK) described in the study as lines A, B and C and utilised a lettuce DFFS, developed by VEGIN, consisting of 96 lines with accessions from the cultivated *L. sativa* (n=79), and the wild relatives *L. serriola* (n=12), *L. saligna* (n=3) and *L. virosa* (n=2) (Burns, *et al*., 2011). Within the *L. sativa* accessions from the morphological types Batavian (n=4), Cos (n=17), Butterhead (n=25), Crisp (n=11), Cutting (n=10), Latin (n=7), Stem (n=2), Leaf (n=1), oilseed (n=1) and Stalk (n=1) are represented.

4.3.2 Seed germination.

Blue germination paper (SD7640; Anchor Paper Company, St Paul, MN, USA) was cut and placed into petri dishes and individually numbered sections marked out with a pen. To the petri dishes 7 ml of tap water was then added to imbibe the seeds. The seeds were allotted to a section of the germination paper before being placed in a 310 x 340 mm lidded

opaque plastic tray. The plastic tray was then located in a cold store under constant 1.5 µmol m^{-2} s^{-1} of photosynthetically active radiation (PAR). The seed were left for a minimum of 48 h to reach a pre-determined stage of germination (when the radicle had reached 1- 5 mm in length and initial root hairs were visible). Use of the pre-determined growth stage before placing the seedlings into the assay removed any variation in growth caused by differing germination times.

4.3.3 Seedling growth assay.

A high through-put vertical growth pouch assay was used (Atkinson, *et al.,* 2015; Thomas *et al*., 2016a, b; Wang *et al*., 2017b; Xie *et al*., 2017). The method described by Thomas *et al*., (2016a) was modified for the growth of lettuce, which included the addition of two sheets of porous tissue paper, arranged as described for cassette type B in chapter 3 (TFM Farm and Country Superstore Ltd, Shropshire, UK), to increase water availability to the seedlings. The growth pouches were pre-soaked horizontally for 10 s in tap water with a concentration of 15% (0.24 g L^{-1}) Hoagland's solution (Hoagland's No. 2 Basal Salt Mixture, Sigma Aldrich, Dorset UK) before being affixed above drip trays that contained 2 L of tap water with a concentration of 15% (0.24 g L^{-1}) Hoagland's solution (Hoagland's No. 2 Basal Salt Mixture, Sigma Aldrich, Dorset UK). At 40cm above each tank were six 550 mm strip white light emitting diode (LED) lights (Leyton Lighting, Essex, UK) providing a mean PAR of 90.1 µmol m^{-2} s^{-1}, ranging from 68.5 – 113.4 µmol m^{-2} s^{-1}.

At the pre-determined germination stage (see above), six replicate ½ growth pouches (each growth pouch divided vertically) of each accession were allocated to positions in the two support frames using a one-way randomised design with no blocking (GenStat 17th edition, VSN International Ltd, Hemel Hempstead, UK). The seedlings were placed around 5 mm from the top edge and around 50 cm from the relative vertical edge on the germination paper with the radicle in a downward orientation. All seedlings were grown in a 2.2 m wide x 3.3 m long x 3.0 m high controlled environment (CE) room for 14 days from the point they were put in the system with a 20 h photoperiod. The temperature and relative humidity (RH) were recorded every 2 hours with a data logger (TinyTag Plus2, Gemini Data Loggers Ltd, Chichester, UK). The mean temperature was 13.4°C and ranged between 13.1°C and 17.9°C. The mean RH was 98.7% with a minimum of 78.6% and a maximum of 100%. After 14 days, the pouches were removed from the system and images collected.

4.3.4 3D sand assay.

To translate findings from the pouch assay to a 3D environment, sixteen lines were selected to represent eight extreme phenotypes (the eight highest and eight lowest for the three traits; primary root length (PRL), total lateral length (TLL) and total number of laterals (TNL)) and were germinated as previously described before being placed in 125 mL transparent polypropylene pots (General stores LTD, Enfield, London, UK) that had four 2 mm holes drilled 3 mm from the base of the pot. The pots were pre-filled with horticultural grade sharp sand, with an average maximum grain size of 3 mm (Vitax Ltd, Leicestershire, UK).

The pots containing six replicates of each line were then placed in 570 mm long x 390 mm wide x 50 mm high plastic trays (Garland Products Ltd, Kingswinford, West Midlands, UK). The pot location within the trays was randomised using Genstat 17th edition (VSN International Ltd, Hemel Hempstead, UK). To each tray, 6 L of 15% concentration Hoagland's solution made with tap water was added and replenished with 1 L of tap water after 4 d. The seedlings were grown for 10 d in a CE room. The sand was then washed from the seedling roots and images collected.

Photosynthetically active radiation (PAR) was 191 µmol m-2 s-1 (190 SB quantum sensor; LI-COR Inc., Lincoln, NE, USA) at plant height produced by 400 W white fluorescent lamps (HIT 400w/u/Euro/4K, Venture Lighting, Rickmansworth, UK). Relative humidity throughout the trial in the CE room ranged from 31.7 – 100% with an average of 69.2 %. Temperature ranged from 12.6 – 27.5˚C with an average of 17.5˚C. The sand was then washed from the seedling roots and images collected.

4.3.5 Image analysis.

The growth pouches were deconstructed following removal from the frame and the root system exposed. The root system for each plant was then imaged using a digital camera (Lumix - DMC-FP2, Panasonic, Berkshire, UK) with a fixed distance of 200 mm (Appendix 4.1 & 4.3). The images were then analysed using *ImageJ* (Abràmoff, *et al*., 2004; Schneider, *et al*., 2012) and measurements for primary root length (PRL), total lateral length (TLL), number of laterals (TNL), maximum root hair length (MRHL) [the longest visible root hair measured] were made using the segmented line selection tool. The traits root hair coverage (PRHC) [a visual percentage score given for how much of the root surface was

covered in root hair],root hair density (RHD) [a visual percentage score given for how much of the total visible root was root hair], lateral root number topology (LRNT) [calculated from the mean percentage of number of laterals at a given percentage position on the primary root] and lateral root length density topology (LRLDT) [calculated from the mean percentage of total lateral root length at a given percentage position on the primary root] were recorded and analysed. For the trait of mean lateral root spacing (MLRS), any seedlings with fewer than two lateral roots were discarded from the analysis. For the sand pot assay, traits focused on were those previously defined in chapter 3 as involved with the “rapid rooting” phenotype; primary root length, total number of laterals and total lateral length. As these traits have been highlighted as probably the most important for in-field establishment following transplanting, analysing if these traits correlate with sand grown plants is essential for further studies on transplant root growth (Appendix 4.2 & 4.3).

4.3.6 Data analysis.

Accessions that had a greater root growth and development had greater variance of the residuals for the traits PRL, LRL, TNL, resulting in data that had non-constant variances, which were not normally distributed. The raw data for the traits PRL and TLL were therefore transformed by square rooting the data. For the trait TNL some of the accessions had a low number of lateral roots or no lateral roots whatsoever, therefore, to normalise the data an increase of 0.1 lateral roots was added to all data sets and data were then transformed to their natural logarithms.

The data sets were then analysed using restricted maximum likelihood (REML) variance component analysis which accounted for the spatial variation, such as light level or edge effect that may have occurred within the frames. The resultant predicted means for all lines were then analysed for significant differences. All statistical analysis of the DFFS data was done using GenStat 17th edition.

4.4 Results.

4.4.1 Primary root length (PRL).

The primary root length for the 99 lines tested ranged from 2.9 mm to 133.7 mm in 14 d old seedlings (Figure 4.1). The accession with the greatest primary root growth was CGN04628, a Cos variety named Kakichisha White. Accession CGN04628 had a 3.3-fold

larger PRL than commercial line A ($P<0.001$), 4.0-fold larger than commercial line B ($P<0.001$), and 2.5-fold larger than commercial line C ($P<0.001$). There was a significant difference in PRL between cultivated *L. sativa* and wild relatives of the DFFS ($P<0.001$). The cultivated species *L. sativa* had the greatest primary root growth with a mean of 63.0 mm compared to the mean of 24.9 mm for the wild relatives (Figure 4.2).

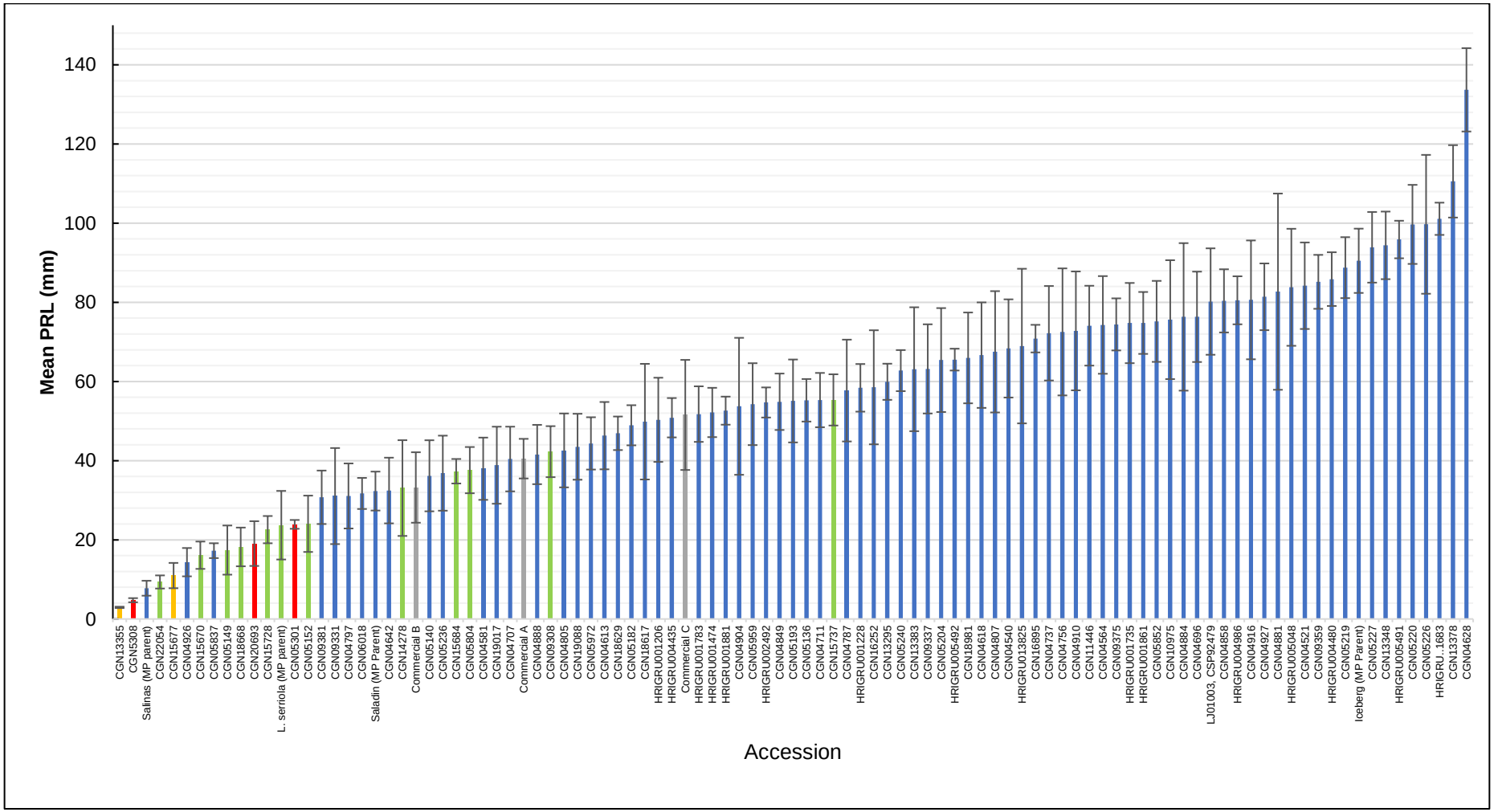

Figure 4.1: Primary root length (PRL, mm) of the DFFS and commercial lines A, B and C after 14 d in the growth pouch assay (n=6).
Lines shown belong to the species *L. sativa* (blue bars), *L. serriola* (green bars), *L. virosa* (orange bars), *L saligna* (red bars) and commercial lines A, B and C (grey bars). Error bars are =/- one SEM.

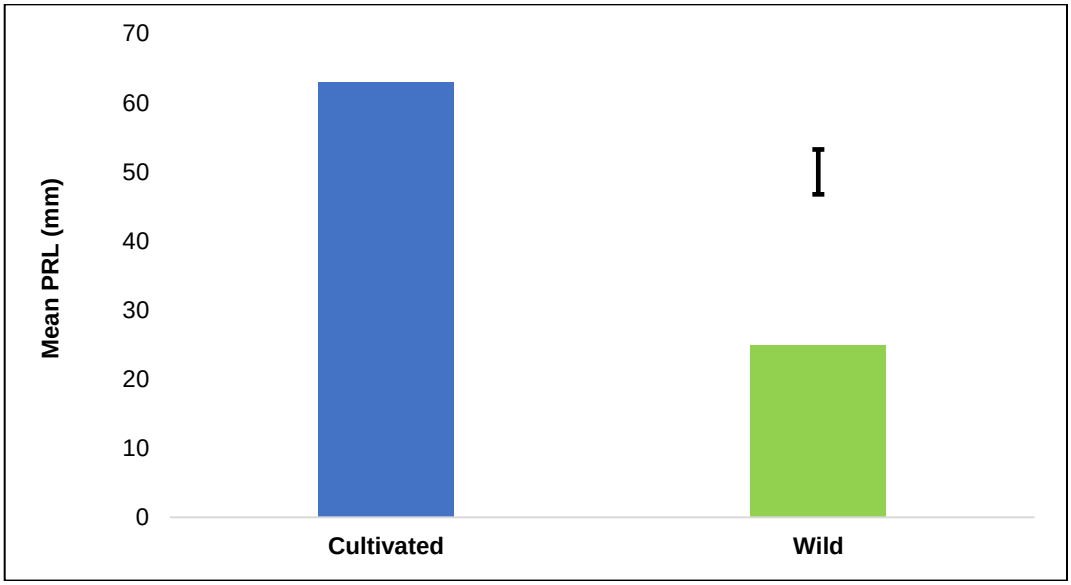

Figure 4.2: Primary root length of the lettuce DFFS accumulative cultivated (n=474) and wild species (n=102) after 14 d growth in the growth pouch assay. Error bar is LSD (P<0.05).

Sixteen accessions, representing the eight highest and eight lowest extremes of PRL were then grown in the sand pot assay. PRL for these lines had a positive correlation (R^2=0.43) between the pouch and sand assays. The variation was greater in the growth pouch assay and the accession HRIGRU005491, named Lilian, swapped from a rapid rooting extreme in the growth pouch assay to a slow extreme in the sand assay (Figure 4.3).

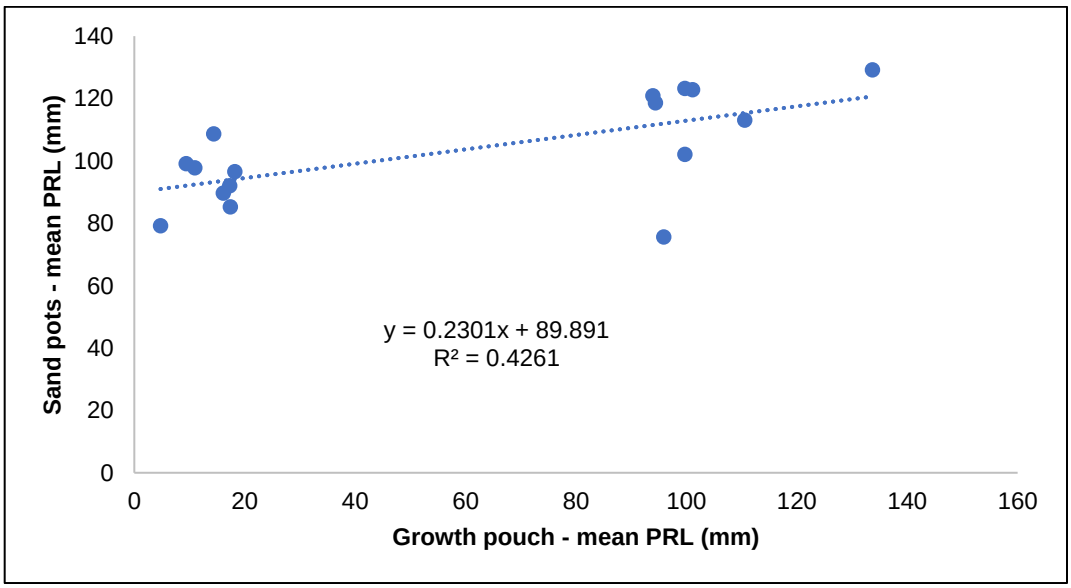

Figure 4.3: Comparison of extreme lines of DFFS for primary root length (PRL) (n=6) following 14 d in the growth pouch assay (x-axis) compared to 10 d growth in 3D sand pot assay (Y-axis).

4.4.2 Total lateral root length (TLL).

The DFFS and commercial lines A, B and C differed significantly for total lateral root length (TLL) ($P<0.001$), with values ranging from 0.0 mm to 267.8 mm (Figure 4.4). The line with the highest value for the trait TLL was found to be accession CGN04628, previously found to have the highest PRL. For the TLL trait, accession CGN04628 was 5.2-fold larger than commercial line A ($P<0.001$), 2.9-fold larger than commercial cultivar B, and 3.7-fold greater than commercial line C ($P<0.001$).

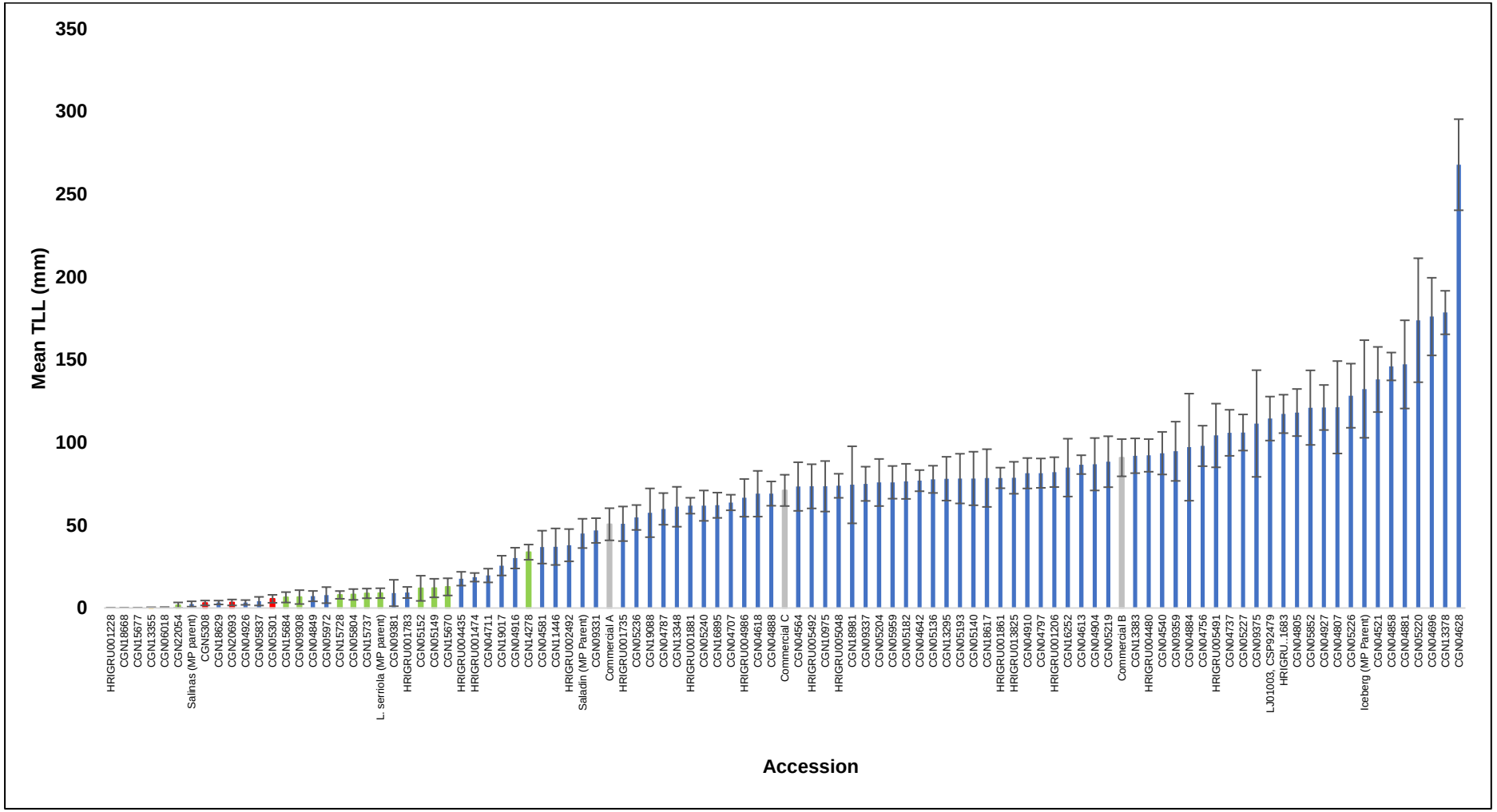

Figure 4.4: Total lateral root length of the lettuce DFFS and commercial lines A, B and C after 14 d on the growth pouch assay (n=6).
Accessions are shown from *L. sativa* (blue bars), *L. serriola* (green bars), *L. virosa* (orange bars), *L. saligna* (red bars) and commercial lines A, B and C (grey bars). Error bars = SEM.

The cultivated *L. sativa* (76.38 mm) had a significantly higher (P<0.001) TLL than the accumulative mean of the wild relatives (8.49 mm) (Figure 4.5).

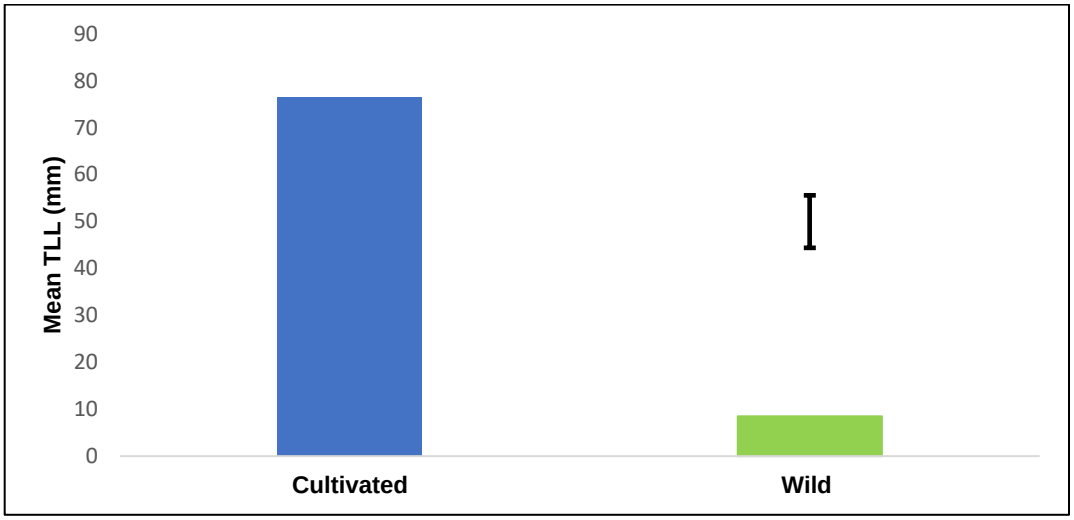

Figure 4.5: Total lateral root length of grouped, cultivated (n=474) and wild relatives (n=102) of the lettuce DFFS seedlings after 14 d growth in the growth pouch assay. Error bar is LSD (P<0.05).

Sixteen extreme accessions (the eight highest and eight lowest) that had the highest segregation in the growth pouch assay, for the total lateral length trait had a positive correlation (R^2=0.43) following 10 d growth in the sand pot assay. The accession HRIGRU001228 had no visible lateral root growth in the growth pouch assay but had the highest mean TLL in the sand pots (530.46 mm) (Figure 4.6).

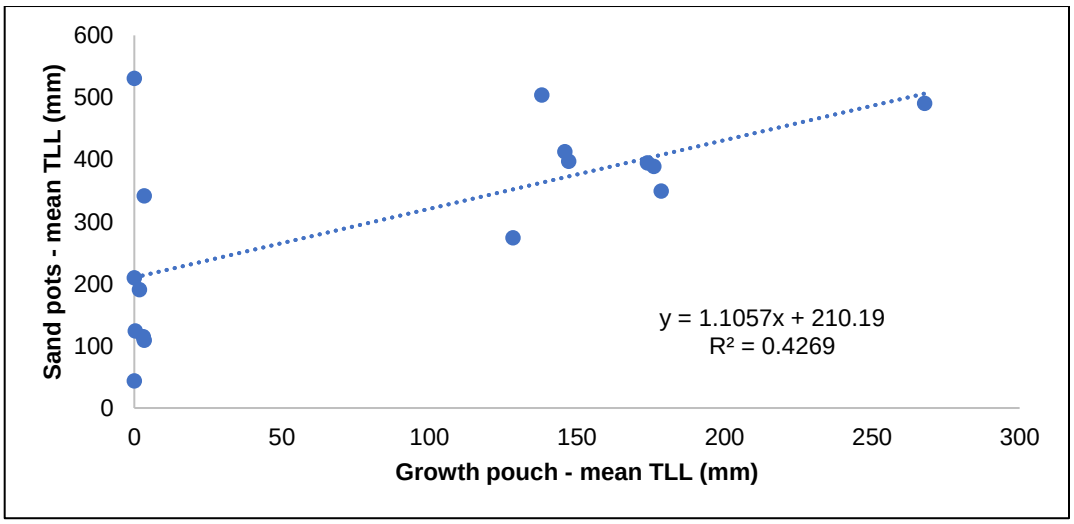

Figure 4.6: Comparison for the trait total lateral length following 14 d growth in the 2D high throughput growth pouch assay (x-axis) compared to 10 d growth in a 3D sand pot assay (y-axis) (n=6).

4.4.3 Total number of lateral roots (TNL).

The lettuce DFFS and lines A, B and C showed significant (P,0.001) variation for the trait total number of lateral roots (TNL), ranging between no laterals produced to a mean total of over 25 lateral roots in the aforementioned accession CGN04628 (Figure 4.7). The accession CGN04628 produced 6.6-fold more lateral roots than commercial line A ($P<0.001$), 5.1-fold more than commercial line B ($P<0.001$), and 5.1-fold more than commercial cultivar C ($P<0.001$).

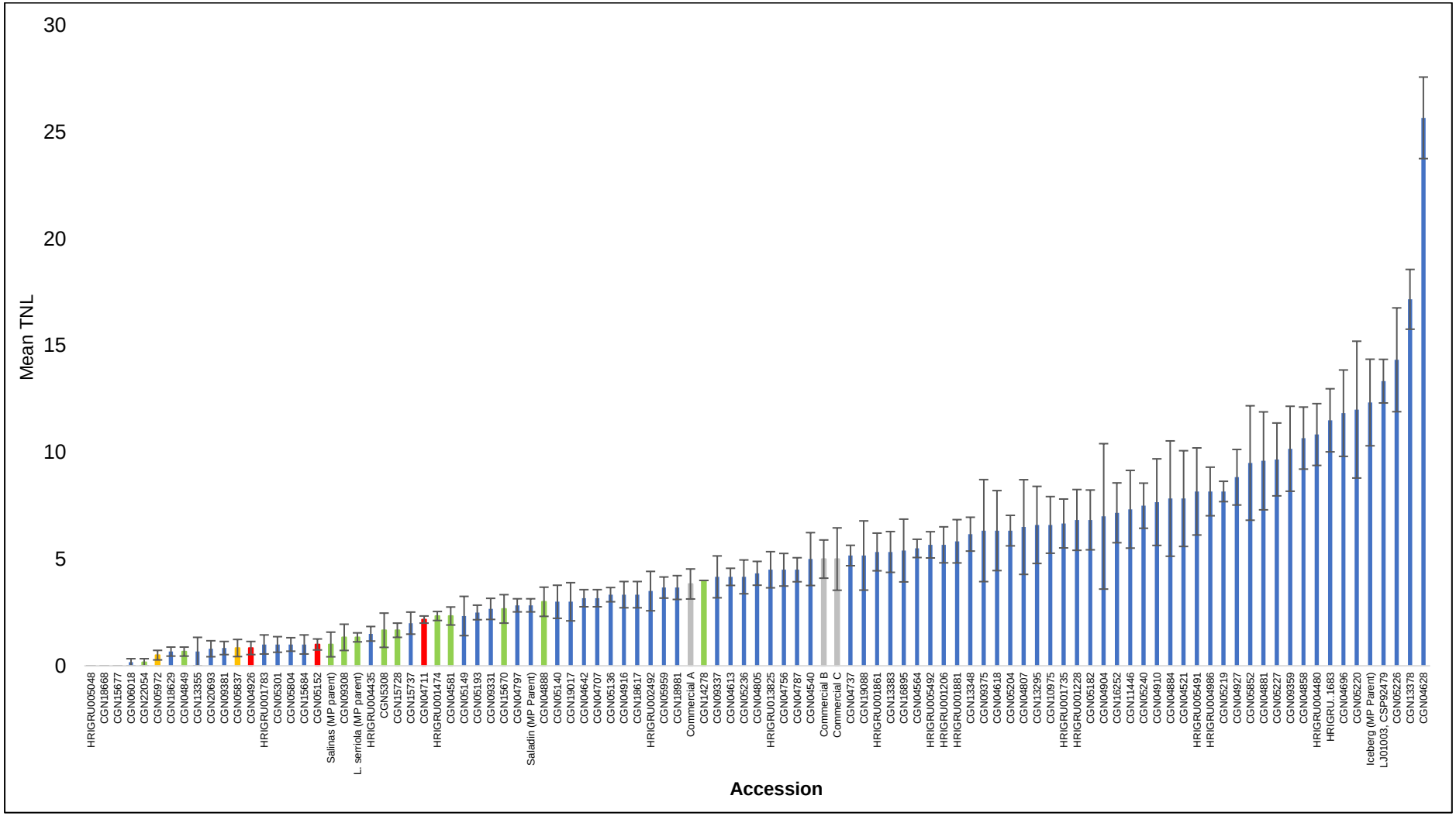

Figure 4.7: Total number of lateral roots of the lettuce DFFS and commercial lines A, B and C after 14 d on the growth pouch assay (n=6).
Accessions are shown from *L. sativa* (blue bars), *L. serriola* (green bars), *L. virosa* (orange bars), *L. saligna* (red bars) and lines A, B and C (grey bars). Error bars = SEM.

The cultivated species *L. sativa* had a mean total lateral root number of 5.83, which was significantly greater (P<0.001) than the mean of the wild DFFS species, which had an accumulative mean total number of lateral roots of 1.41 (Figure 4.8).

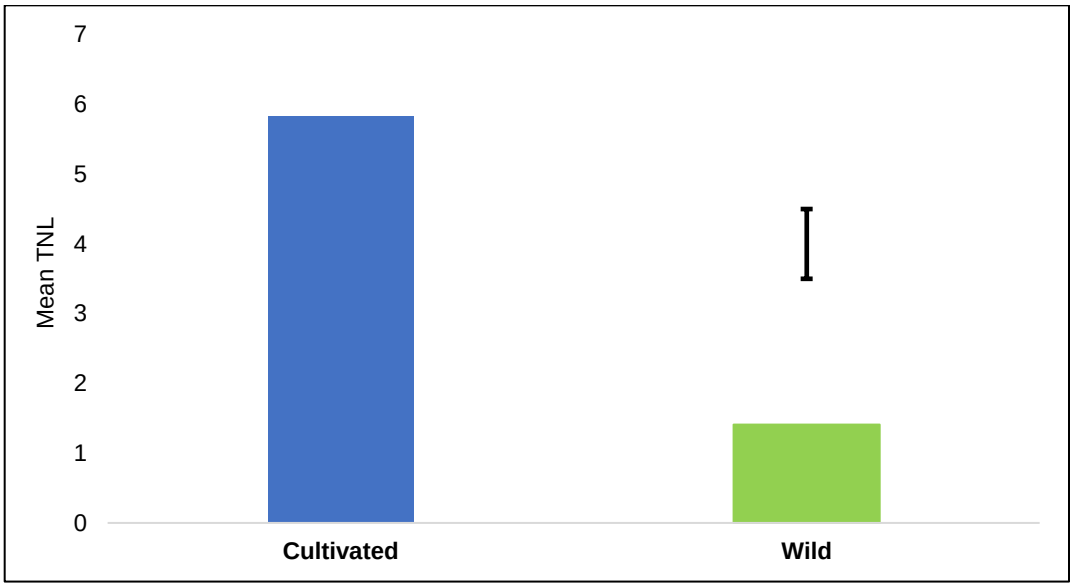

Figure 4.8: Total number of lateral roots of lettuce DFFS seedlings after 14 d growth in the growth pouch assay grouped by cultivated (n=474) and wild relatives (n=102). Error bars are LSD (P<0.05).

There was a weak positive correlation (R^2=0.25) observed for extreme lines (the eight highest and eight lowest) from the growth pouch assay when compared to the sand pot assay for TNL. The accession HRIGRU001228, again had no lateral roots visible in the growth pouch assay but the second highest in the sand assay with a mean of 23.25 lateral roots (Figure 4.9).

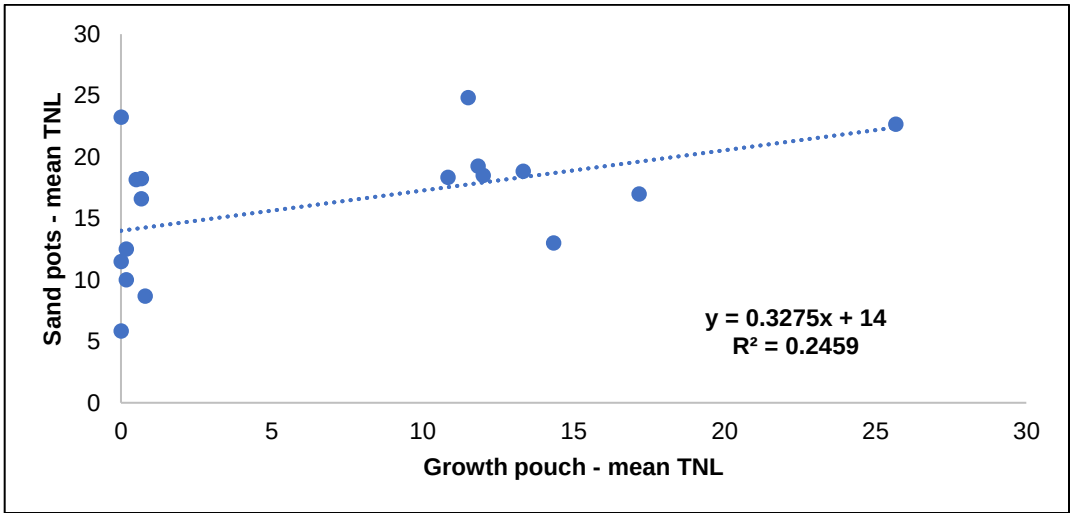

Figure 4.9: comparison between seedlings of extreme lines of the trait total number of lateral roots, grown in the growth pouch assay (x-axis) and in the sand pot assay (y-axis) in extremes of the DFFS (n=6).

4.4.4 Maximum root hair length (MRHL).

There was a large difference observed across all lines for maximum root hair length (MRHL) in the DFFS and commercial cultivars grown on the pouch assay (Figure 4.10). The line with the greatest root hair length was the accession CGN04849, which was a *L. sativa* cutting variety, named Simpson. The accession CGN04849 had a mean MRHL 1.9-fold longer than commercial line A($P<0.001$), 2.3-fold longer than commercial line B ($P<0.001$), and 1.7-fold longer than commercial line C ($P=0.003$). The accession CGN04849 had a mean root hair length of 1.9 mm. Accession CGN5308, which is a wild *L. saligna* line, had no observable root hairs.

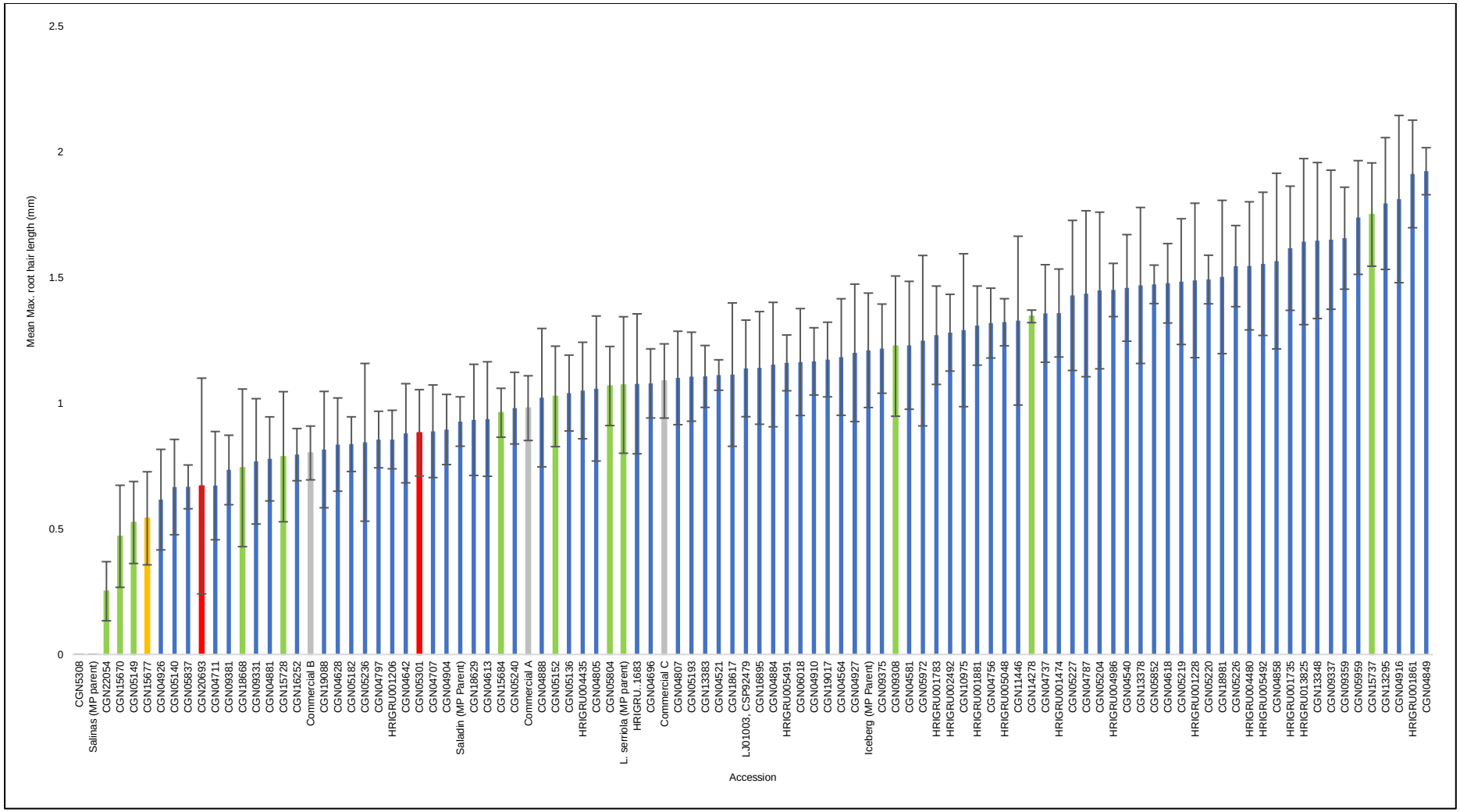

Figure 4.10: Maximum root hair length of the lettuce DFFS and lines A, B and C after 14 d on the growth pouch assay (n=6).

Accessions are shown from *L. sativa* (blue bars), *L. serriola* (green bars), *L. virosa* (orange bars), *L. saligna* (red bars) and lines A, B and C (grey bars). Error bars are SEM.

There was a significant difference (P<0.001) observed between the accumulative mean MRHL of the cultivated *L. sativa* (1.2 mm) and that of the wild species (Figure 4.11).

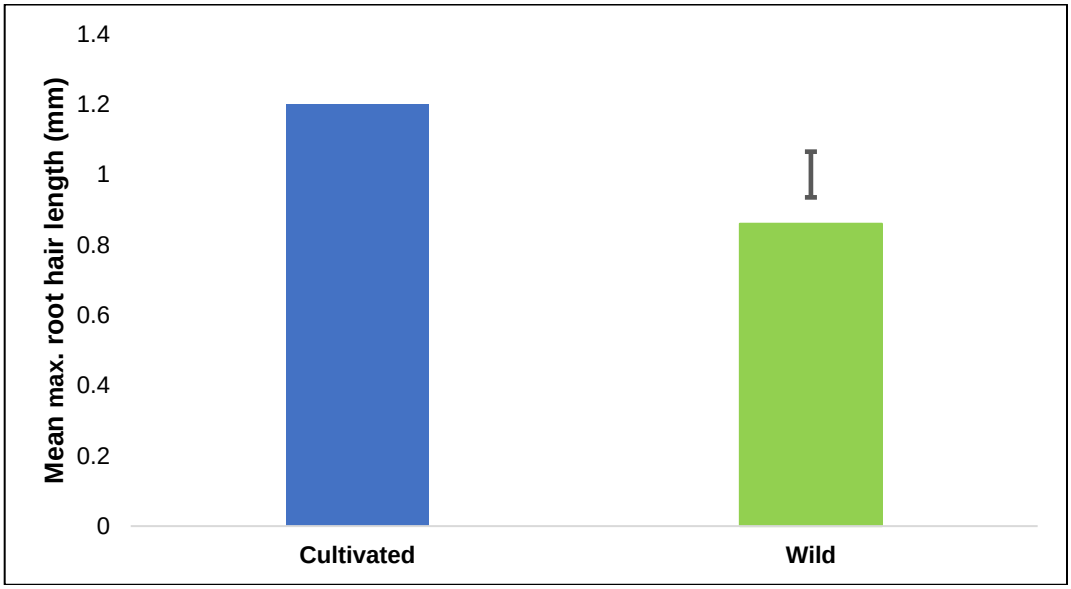

Figure 4.11: maximum root hair lengths of 14d old seedlings of the DFFS grouped by cultivated (n=474) and wild relatives (n=102) from the growth pouch assay. Error bar is LSD (P<0.05).

4.4.5 Percent root hair coverage (PRHC).

The lettuce DFFS and commercial lines had a significant difference (P<0.001) across all lines for PRHC (Figure 4.12). The highest coverage was observed in a *L. serriola* wild relative accession, CGN14278, which had 70% of the total visible root area covered compared to the lower mean scores of between 20.8 % and 42.5 % for the three commercial lines.

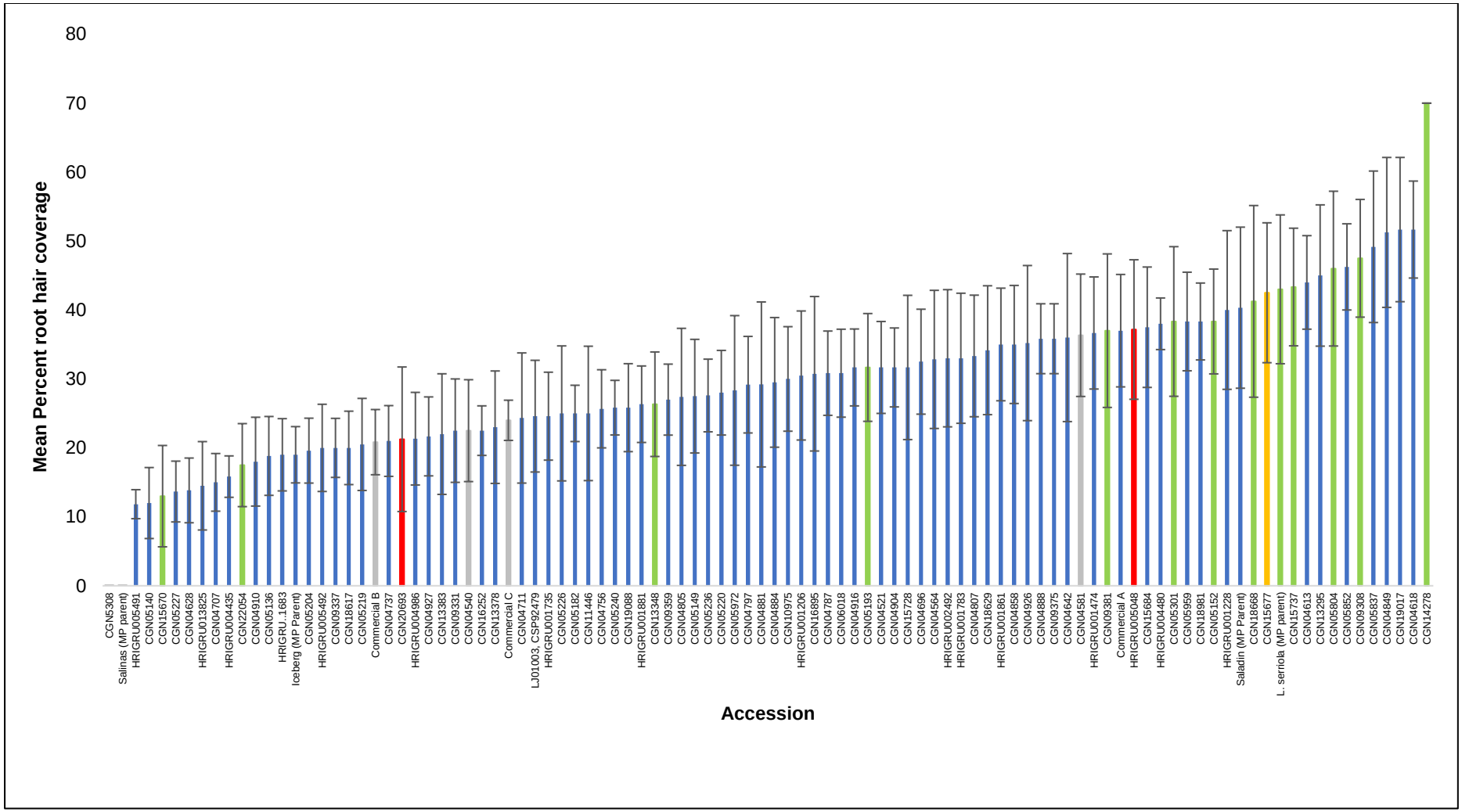

Figure 4.12: Percent root hair coverage (PRHC) of seedlings grown in the growth pouch assay (n=6).
Accessions are shown from *L. sativa* (blue bars), *L. serriola* (green bars), *L. virosa* (orange bars), *L. saligna* (red bars) and lines A, B and C (grey bars). Error bars are SEM.

There was a significant difference (P=0.011) between the accumulative mean (28.65%) of the cultivated *L. sativa* species and that of the wild relatives (34.49%) for PRHC (Figure 4.13). The species *L. virosa* had the highest mean for the trait PRHC with a mean of 42.5%. The second highest was *L. serriola* with a mean of 37.7%. The cultivated species *L. sativa* and the wild species *L. saligna* had a significantly lower mean PHRC (28.9% and 19.7%, respectively).

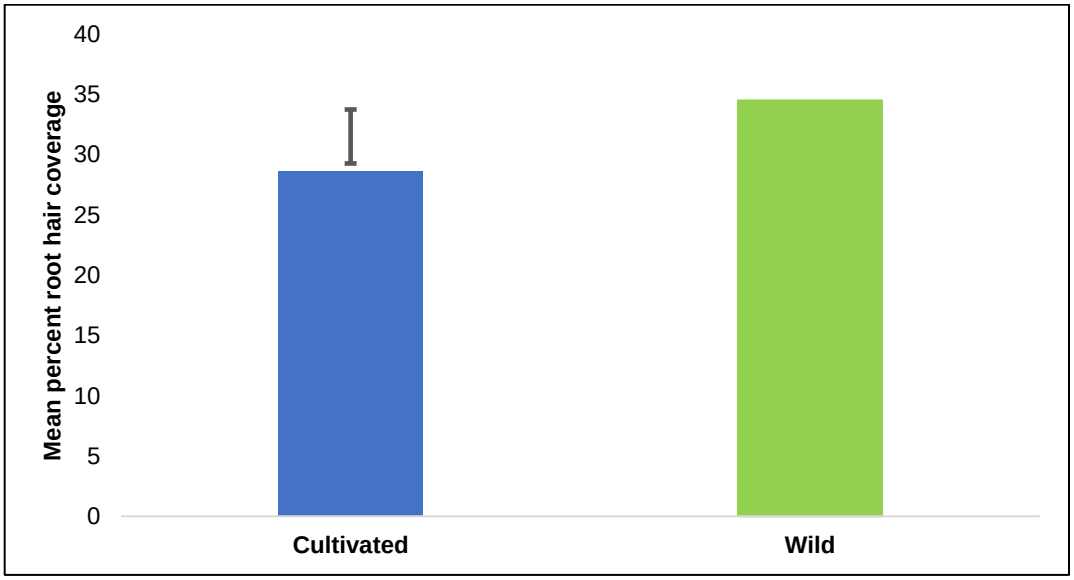

Figure 4.13: Lettuce DFFS 14 d old seedlings grouped by cultivated (n=474) and wild relatives (n=102) for the trait percent root hair coverage (PRHC), in the growth pouch assay. Error bar is LSD (P<0.05).

4.4.6 Root hair density (RHD).

The lettuce DFFS and commercial lines segregated with a significant difference (P=0.001) for the trait root hair density (RHD) in the growth pouch assay

The line with the largest amount of RHD was the accession CGN14278, a wild relative *L. serriola* line which has been previously mentioned to have the greatest PRHC. The accession CGN14278 had a mean RHD of 12.5%, which was greater than the three commercial lines, which ranged from 2.3% - 5.3%. (Figure 4.14).

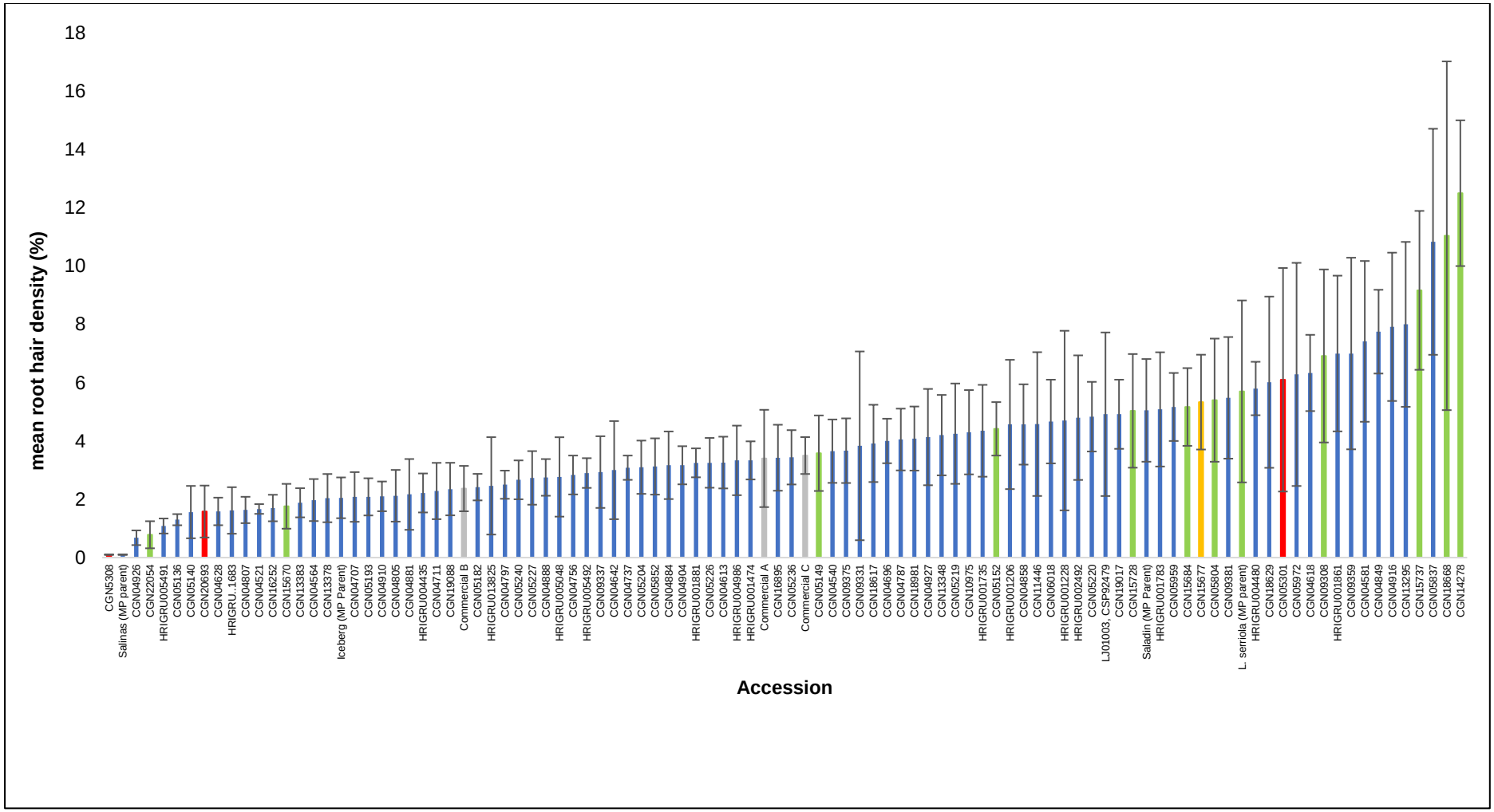

Figure 4.14: Growth pouch assay of 14 d old seedlings of the DFFS for the trait root hair density (n=6).
Accessions are shown from *L. sativa* (blue bars), *L. serriola* (green bars), *L. virosa* (orange bars), *L. saligna* (red bars) and lines A, B and C (grey bars). Error bars are SEM.

The accumulative mean of the wild relatives (5.13%) was significantly greater (P=0.002) than that of the cultivated *L. sativa* (3.65%) for the trait RHC (Figure 4.15).

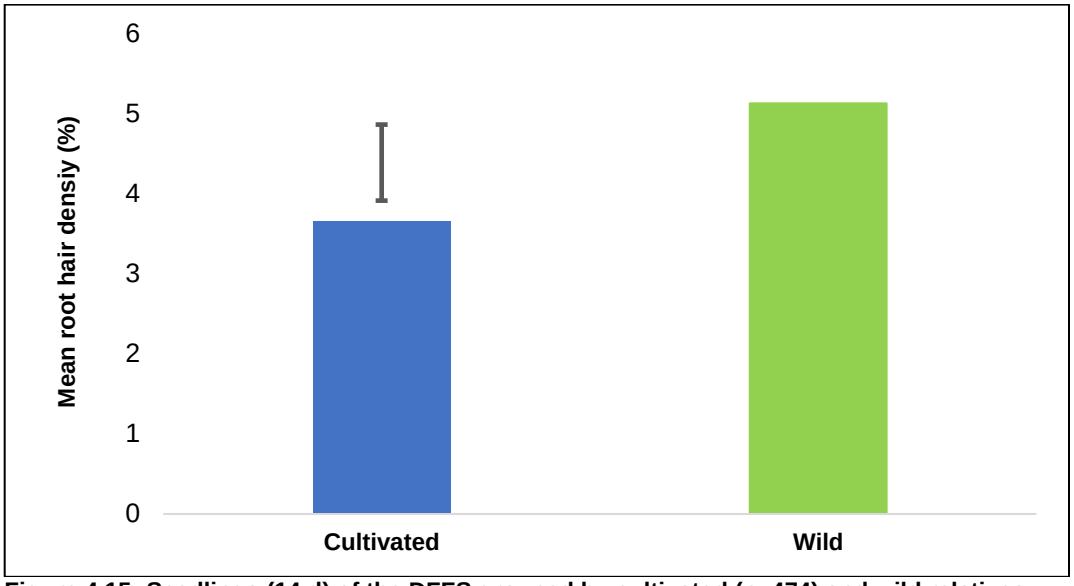

Figure 4.15: Seedlings (14 d) of the DFFS grouped by cultivated (n=474) and wild relatives (n=102) for the trait RHD. Error bar is LSD (P<0.05).

4.4.7 Mean lateral root spacing (MLRS).

There was a significant difference (P<0.001) identified across all lines of the DFFS for the trait mean lateral root spacing (MLRS) (Figure 4.16). The accession with the greatest mean distance between lateral roots was a wild relative, *L. serriola* line, CGN05804, which had a mean spacing of 16.1 mm between lateral roots, which was greater than the three commercial lines that ranged between a mean lateral spacing of 1.6 mm – 4.8 mm.

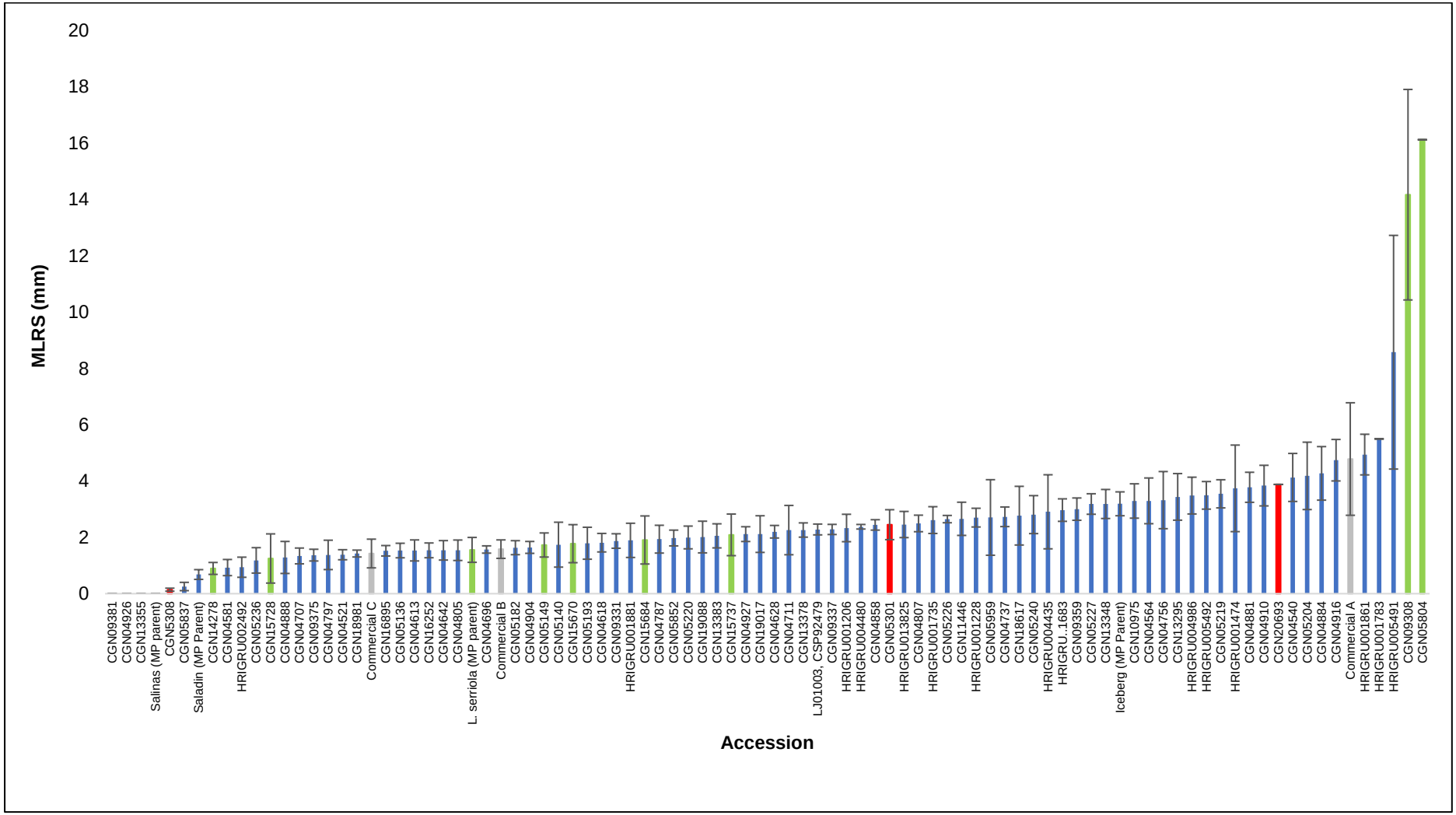

Figure 4.16: Phenotypic variation for mean lateral root spacing of 14 d seedlings of the lettuce DFFS in the growth pouch assay (n=6).
Accessions are shown from *L. sativa* (blue bars), *L. serriola* (green bars), *L. virosa* (orange bars), *L. saligna* (red bars) and lines A, B and C (grey bars). Error bars = SEM.

4.4.8 Lateral root number density topology (LRNDT).

The study observed a significant difference (P=0.026) only at the 20-30% primary root position in a comparison of cultivated and wild accessions (Figure 4.17).

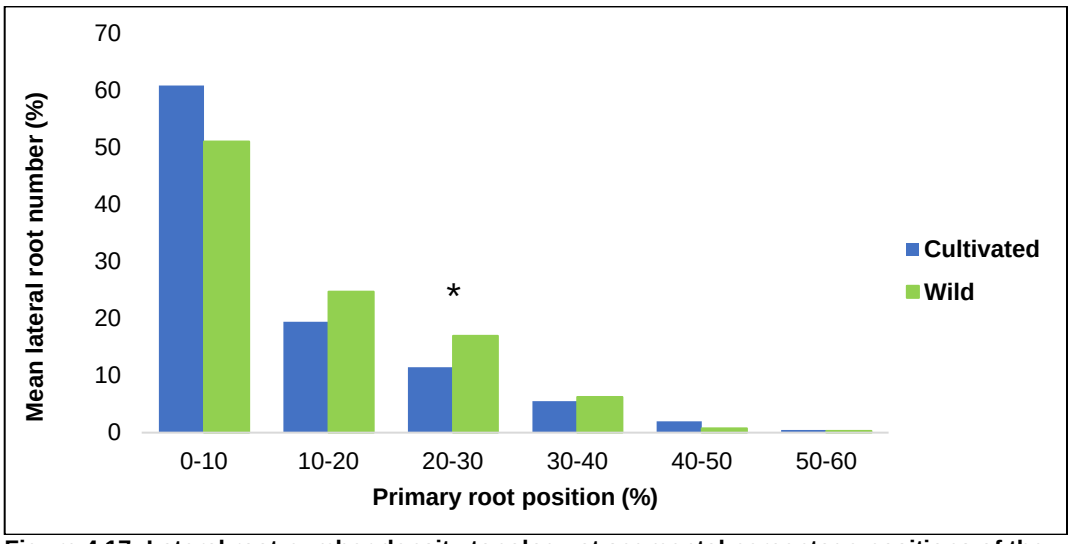

Figure 4.17: Lateral root number density topology at segmental percentage positions of the primary root for 14 d seedlings of the lettuce DFFS (n=6): L. sativa lines (blue bars; n=474); wild relatives (green bars; n=102). *P<0.05.

4.4.9 Lateral root length density topology (LRLDT).

When comparing cultivated lines against wild species, significant difference for LRLDT was found at primary root positions 0-10% (P=0.015) and at 20-30% (P=0.015) (Figure 4.18).

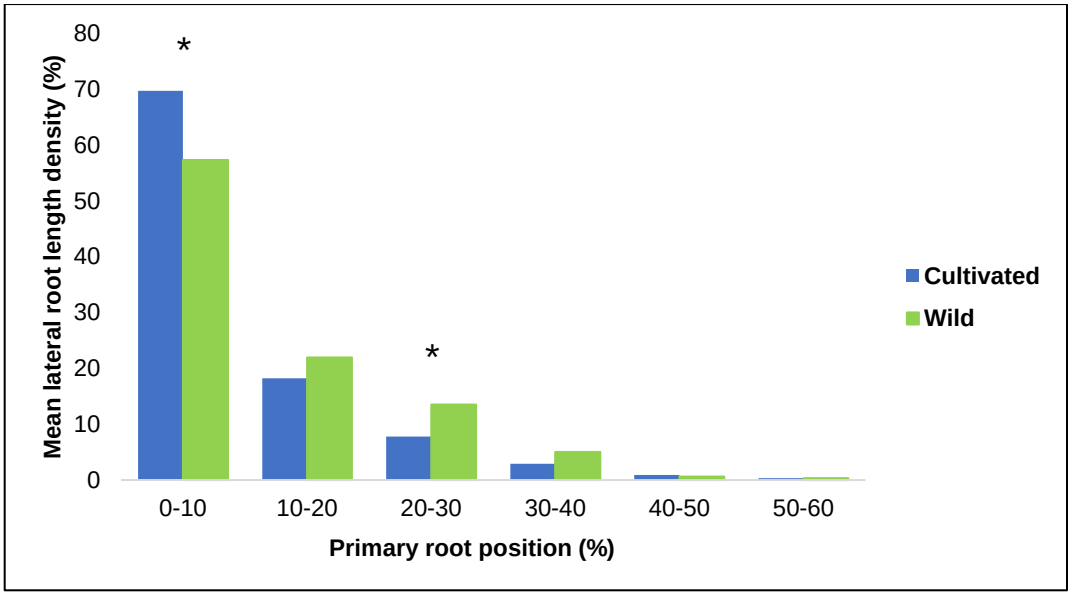

Figure 4.18: Lateral root length density topology at segmental percentage positions of the primary root for 14 d seedlings of the lettuce DFFS (n=6): L. sativa lines (blue bars; n=474); wild relatives (green bars; n=102). *P<0.05.

4.5 Discussion.

This study has identified genetic variation within the lettuce DFFS for a rapid rooting phenotype in seedlings at the 14-day old stage. In particular, one line was observed to have a rapid rooting phenotype i.e. having a combination of a longer primary root, a greater total lateral root length and a higher total number of lateral roots. The Cos variety of the cultivated species, *L. sativa*, accession CGN04628, known as Kakichisha White, had the greatest root growth for the traits PRL, TLL & TNL. This accession had at least a 2.5-fold increase in primary root length, 2.9-fold increase for total lateral root length, and a 5-fold increase for lateral root number in 14-day old seedlings compared to any of the commercial varieties tested in this study. These results would indicate that there is potential within lettuce breeding programmes to improve root growth rate and hence soil exploration, which could reduce the amount of fertiliser inputs required. The phenotype could also lead to a reduction in establishment time and susceptibility to environmental stresses, such as drought following transplanting.

The introgression of such traits into commercially available cultivars should prove to be relatively straight forward, as the rapid rooting traits are within an *L. sativa* line and traits associated with rapid rooting have already been successfully genetically mapped through quantitative trait loci (QTL) analysis of a mapping population (discussed in chapter 5), although other alleles and genes could be involved with the trait in Kakichisha White as the root growth was much higher in this accession than the Iceberg parent. The greater means

of the *L. sativa* species over the wild relatives for overall root growth traits; PRL, TLL & TNL could be an indication of how and where the species have evolved. Wild relatives occur widely in arid regions and so accessions will be under selection pressure to adapt to low water conditions where water and nutrient availability may not support the rapid growth observed in the cultivated varieties (Johnson, *et al*., 2000). Adaptation to arid climates may also explain why wild relatives tend to root deeper in the soil profile and have a higher distribution of root hairs as seen in this study. The higher density of root hairs could also support the slower root growth of the wild relatives seen in the study, as the root hairs would increase the root area for nutrient and water capture in poor conditions. The positive correlation between the high-throughput pouch assay and the extreme lines grown in the sand pot assay for the traits PRL, TLL, & TNL indicate the results from the two-dimensional pouch assay may be transferable to 3-dimensional root growth, however evidence of phenotypic variation for differing environmental cues was observed..

The study identified genetic variation within the lettuce DFFS for the trait MRHL. The accession that had the longest root hairs observed was CGN04849, a cutting variety within the *L. sativa* species, known as Simpson. The mean maximum root hairs of CGN04849 was at least 1.7-fold greater than the commercial varieties used in the study. The phenotype for increased root hair length could be integrated into a commercial variety through intra-specific crossing of CGN04849 and a commercially important variety within a breeding programme. Introducing this phenotype to commercial lines could improve yields through improved nutrient acquisition in reduced input farming schemes. This could be especially true for phosphate uptake efficiency in phosphorus deficient soils where increase root hair length has already been observed to increase grain yield in barley (Gahoonia & Nielsen, 1997; Gahoonia & Nielsen, 2004) and identified as being involved with phosphorus uptake efficiency in cowpea (*Vigna unguiculate*) (Krasilnikoff, *et al*., (2003). The trait for increased root hair length has also been genetically mapped through QTL analysis in maize (*Zea mays* L.) (Zhu, *et al*., 2005) and common bean (*Phaseolus vulgaris* L.) (Yan, *et al*., 2004).

Although the *L. sativa* accessions were observed to have a greater MRHL than the accessions of wild relatives, for percentage root hair coverage and root hair density it was the wild species, and more specifically the wild *L. serriola* accession CGN14278 that had the greatest root hair coverage and root hair density. This may be due to a higher number of trichoblasts formed during cell differentiation in the transitional zone. Root hairs only form in the trichoblast cells and the higher the number of trichoblasts cells within the root epidermis the higher potential for root hair formation is (Cho & Cosgrove, 2002) .What is unknown is if

a higher number of shorter root hairs, with higher overall root hair density is better than a lower number of longer root hairs for nutrient and water acquisition. Further work would be required to test which is the more beneficial phenotype under field conditions.

In this study, where there was adequate nutrient availability to the plants, the high root hair density and increased lateral spacing at higher spatial positioning down the primary root suggests that the wild relatives accessions in the DFFS are adapted to low nutrient environments where the root system will need to scavenge available nutrients from the soil profile and are less plastic developmentally to different conditions as suggested by Jackson (1995). The wild relative accessions in this study had greater lateral root spacing than the cultivated *L. sativa* accessions, with the *L. serriola* accession CGN05804 having the greatest lateral root spacing phenotype. Wild relatives also differed in lateral root positioning with *L. sativa* producing a greater number of lateral roots in the top 10% of primary root position than the wild relatives, which produced a greater number of lateral roots in the 20-30% position than the cultivated *L. sativa* accessions. These results appear to confirm the findings of Jackson (1995), who reported similar results in much older pot grown plants, indicating root architecture can be modelled in 14 d old seedlings in a 2D assay in lettuce. The introgression of a deeper lateral root phenotype into commercial lettuce cultivars could prove to be a successful approach to allow the reduction of the input of nitrogen as fertilizers and alleviate environmental pollution risks for lettuce producers.

There are two explanations for the initiation of lateral root development from the pericycle cells. The first is the initiation of roots through root bending, with lateral root initiation taking place unilaterally at places of bending along the root axis. The second is where lateral roots are initiated through a regulated oscillatory pattern along the main root axis (Kircher & schopfer, 2015).

Lateral root initiation through root bending is caused by the process of the root bending itself. To cause the root to bend auxin is translocated to site of elongation. Pericycle cells at this location, on the outside of the bend, also elongate resulting in an increase of auxin levels in these cells and the abundance of the AUX1 transporter within one or more pericycle cells in the region. The increase in auxin and AUX1 correlates with the reduction of PIN-FORMED (PIN) proteins. PIN proteins are plant-specific transmembrane proteins that mediate the directional transport of auxin (Křeček, *et al*., 2009). The reduction of the expression of the PINs in the cells and therefore the pericycle cell is pushed towards cell division and the formation of founder cells (Laskowski, *et al*., 2008).

Lateral root initiation may also occur through the regulatory oscillating expression pattern within the basal meristem, which lies before the elongation zone, which is also an auxin mediated expression (Nakamura, *et al*., 2003). As the roots of the seedlings in this study did not undergo any restrictions that could cause root bending the oscillatory explanation may be hypothesised as the mechanism causing the difference in lateral root spacing.

Root systems of the wild relatives had greater lateral root spacing along the primary root axis and had greater numbers of lateral roots further down the primary root position. This might be explained by differential expression of the oscillation zone genes. In the cultivated *L. sativa* species, the genes associated with oscillation may undergo rapid oscillatory expression in the very early stages of primary root growth before ceasing, whereas oscillating expression may be over a much longer period with longer pauses within the wild relatives. Further RNA transcriptomic analysis of *L. sativa* accessions and accession CGN05804 would need to be undertaken to test this.

The breeding of commercial lettuce has prioritised the development of cultivars that have greater shoot growth and have neglected rooting traits. The study has shown that within the lettuce DFFS there are lines that could be introduced to breeding programmes that could introduce traits of benefit to potentially cope with changing climate conditions. Firstly, the rapid rooting phenotype (greater primary root growth and total lateral root length along with a higher number of total lateral roots) could be beneficial to lettuce transplants in reducing establishment time and having a greater root area to access nutrients and water in mild drought conditions, following transplanting (Fitter & Stickland, 1991). The study has identified that wild relatives tend to have a higher percentage of their lateral roots deeper in the soil profile compared to *L. sativa* and current commercial varieties, which was also observed in the study by Johnson, *et al.,* (2000).

The greater root hair length (MRHL) observed in the *L. sativa* species could also be an indirect result from commercial breeding programmes. Although *L. sativa* and commercial lines tend to have long root hairs, they were observed to have fewer of them and they did not cover as much of the root surface as seen in wild relatives. This phenotype could also be a response to intensive high input farming, where the nutrient and water availability is within the upper soil profile and the commercial varieties have been inadvertently bred to have longer less frequent root hairs within this region, whereas the wild relatives benefit from

shorter, much more dense root hairs over the whole root profile capturing water and nutrients throughout the soil profile. Further work is needed to understand this adaptation.

4.6 Conclusion.

Within the lettuce DFFS phenotypic variation was observed at seedling stage for the three traits defined to be involved with rapid rooting; PRL, TLL and TNL indicating commercial lines could be improved to aid establishment within the field. The study was able to observe moderate correlations between the 2D growth pouch assay and seedlings grown in sand, which indicates the 2D assay can be used to infer elite root trait lines for the improvement of transplants at seedling stage. The study identified genetic variation for traits involved with root hair morphology, such as MRHL, PRHC, RHD, which could be key traits for the improvement of water and nutrient capture in mild drought and poor soil conditions. The study was also able to correlate the findings of Jackson, (1995) and Johnson *et al.*, (2000) within the 2D high through-put growth pouch assay for root system architecture in that wild relatives tend to have deeper rooting lateral roots compared with those of cultivated varieties. Overall elite lines have been identified that could be utilised in breeding programmes to introgress these traits into commercial varieties to reduce the impact of drought stress or those associated with reduced inputs in transplanted field crops.

5 Chapter 5 - Quantitative trait loci (QTL) linked with root growth in lettuce (*Lactuca sativa*) seedlings.

5.1 Abstract.

In-field variation of transplanted lettuce (*Lactuca sativa* L.) due to variable soil and environmental conditions is one of the major restrictions in the optimization of production and yield. Marker assisted breeding for lettuce varieties with a rapid rooting phenotype has the potential to improve the performance of lettuce transplants. This study aimed to identify traits linked with increased primary root length (PRL),total lateral root length (TLL) and total number of lateral roots (TNL) in 14 d *L. sativa* seedlings from an intra-specific cross (Saladin x Iceberg). In total 16 significant quantitative trait loci (QTL) were associated with increased root growth traits that would allow direct introgression of the traits. Six of the QTL were associated with increased primary root growth, accounting for 60.2 % of the genetic variation for the trait. Three QTL were associated with lateral root growth (38.6 % of genetic variation); two QTL were associated with lateral root length density (27.6 % of genetic variation) and three with root number density (33.4 % of genetic variation) and two QTL were associated with mean lateral root length (21.1 % of genetic variation). The QTL were located across 9 different linkage groups (LGs) representing loci on 7 of the 9 *L. sativa* chromosomes. A combination of restriction fragment length polymorphism (RFLPs) and Kompetitive allele specific PCR (KASPs) markers linked to these rooting traits were identified, which could allow breeders to select for a rapid establishment phenotype.

5.2 Introduction.

In Europe and North America, lettuce (*Lactuca sativa* L.) seedlings are typically grown during the early stages of production in glasshouses prior to transplanting out into the field. This removes issues associated with direct drilled seed such as, germination, crop uniformity and avoidance of early weed infestation, while optimizing growth and yield (Sharma *et al*., 2005; Maltais *et al*., 2008). Transplant establishment requires the regeneration of new roots and resumption of shoot growth in the field following transplanting (Orzolek 1991). Transplanted crops differ morphologically from direct drilled crops with loss of the primary root from the mechanical separation of the peat blocks prior to transplanting, which results in the development of a larger number of lateral roots (NeSmith & Duvall 1998).

Each lettuce plant within a crop needs to achieve similar establishment to give as uniform a crop as possible for the optimization of production. Lettuce is still manually harvested, and growers will only carry out 'once-over' harvest therefore crop uniformity is essential for maximising profit. Transplant establishment can be negatively impacted by many factors within a field. For example, the variability of soil parameters, such as pH can reduce nutrient availability and root growth (Orzolez 1991). Compaction and poorly tilled soil result in poor root penetration (Grassbaugh & Bennett 1998). Soil moisture can be too high or low for adequate root development (Grassbaugh & Bennett 1998). Transplant shock, which describes the sudden transient stresses at transplanting (Kerbiriou *et al*., 2013), such as temperature change can also impact establishment. Better establishment would improve crop uniformity by minimising the variation between plants caused by abiotic stress at the time of transplanting through the rapid establishment of young plants and the associated access to nutrient and water (Johnson *et al*., 2000).

As for most crops, lettuce breeding has to date been focused on yield, leaf/head traits and pest and disease resistance with little or no direct attention given to the root system. A root breeding strategy in lettuce would be to identify quantitative trait loci (QTL) linked to beneficial root growth traits and introduce these into crop varieties through marker assisted selection breeding programmes to develop lettuce cultivars capable of rapid establishment under variable soil conditions. The introduction of root trait QTL has been previously shown to be successful in upland rice (*Oryza sativa*), where root traits for longer and broader roots were introduced into a new variety, resulting in improved yields (Steele *et al*., 2006; Steele *et al*., 2013). Identifying genetic resources that allow lettuce cultivars to achieve uniform establishment will be of great importance as future more 'sustainable' crop production will most likely be carried out under conditions of lower fertilizer and water use (Zhu *et al*., 2011) and increased fertilizer prices as nutrients such as phosphorus diminish (Le Marié *et al.,* 2014).

Previously, QTL based on segregating root traits have been identified in two studies on lettuce. Both studies used an inter-specific cross between cv. Salinas and the wild relative *Lactuca serriola* (Johnson *et al*., 2000; Wei *et al*., 2014). The first study analysed drought tolerance through deep soil water exploitation and identified QTL involved with root growth and biomass (Johnson *et al.,* 2000). The second study analysed salt tolerance in seedlings through changes to root system architecture (Wei *et al.*, 2014). Both studies demonstrated that a number of *Lactuca* species root traits are under genetic control in

seedling assays. However, it is not known whether these traits are related to a “rapid rooting” phenotype with a combination of increased primary and lateral root growth and lateral root number. The study reported here utilised a high-throughput growth pouch assay to analyse root growth traits in an intra-specific cross mapping population with the aim of identifying QTL associated with an increased root growth phenotype in 14 d old seedlings that may then be used for marker assisted breeding for the improvement of lettuce transplant establishment.

5.3 Materials and methods.

5.3.1 Plant material.

A mapping population was previously produced from an intra-specific cross between the crisphead *L. sativa* cv Saladin (syn Salinas) bred in the US and the Batavian *L. sativa* cv Iceberg, bred in France (Atkinson *et al.,* 2013b). The mapping population used in this study for QTL analysis consists of 125 F_8 recombinant inbred lines (RILs) that were selected as the most genetically informative subset from 254 F_5 genotyped individuals (Atkinson *et al.,* 2013b).

5.3.2 Seed germination.

Germination paper (SD7640; Anchor Paper Company, St Paul, MN, USA) was placed in petri dishes with 10 numbered sections marked out with a pen (Figure 5.1a). The germination papers were pre-soaked with 7 ml of tap water for imbibition of the seed. Once the seeds had been placed on the sections they were placed in a 310 x 340 mm lidded opaque plastic tray and held in a cold store (14-16˚C) with 24 h low irradiance lighting (1.5 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR)). The seeds were left for up to 48 h to reach a pre-determined stage of germination, which was defined as the presence of a radicle 1- 5 mm long and initial root hairs that formed an arrowhead-like appearance (Figure 5.1b). This assured all seedlings were placed on any given assay at the same growth stage, removing any variation due to germination time.

5.3.3 High through-put growth pouch assay.

A high through-put growth pouch assay (Atkinson *et al.,* 2015; Thomas *et al*., 2016a,b) was constructed as described by Atkinson *et al*., (2015) but modified for use with

lettuce by the inclusion of two sheets of porous tissue paper (TFM Farm and Country Superstore Ltd, Shropshire, UK), which increased water availability to the seedlings. Germinated seeds were placed at the top of the growth pouch with the radicle orientated towards the bottom of the paper (Figure 5.1c), with 2 seeds on each side of the pouch at approximately 15 cm spacing (Figure 5.1d). The growth pouches were suspended over drip trays supported within an aluminium frame as described by Atkinson *et al*., (2015). Each drip tray had 2 L of tap water containing 15% (0.24 g L^{-1}) Hoagland's solution (Hoagland's No. 2 Basal Salt Mixture, Sigma Aldrich, Dorset UK) added. Above each tank were six 550 mm strip white light emitting diode (LED) lights (Leyton Lighting, Essex, UK) providing a mean PAR of 90.1 µmol m^{-2} s^{-1}, ranging from 68.5-113.4 µmol m^{-2} s^{-1}.

5.3.4 Seedling growth.

Following germination six replicate growth pouches (each growth pouch =2 seedling positions) of each genotype were allocated to positions in the support frames using a one-way design with no blocking (GenStat 17th edition, VSN International Ltd, Hemel Hempstead, UK). Each of the seedlings were grown across two frames for a 14-d period with a 20 h photoperiod. The temperature and relative humidity (RH) were recorded every 2 hours with a data logger (TinyTag Plus2, Gemini Data Loggers Ltd, Chichester, UK). The mean temperature was 13.8°C and ranged between 13.6°C and 18°C. The mean RH was 99.2 % with a minimum of 78.7 % and a maximum of 100%. Following 14 d growth the pouches were removed from the system for imaging.

5.3.5 Image analysis.

Following 14 d growth of each individual seedling the growth pouches were removed from the frame and dismantled to expose the root system. The root system was then imaged with a digital camera (Lumix - DMC-FP2, Panasonic, Berkshire, UK) at fixed distance of 200 mm (Appendix 5.1). The images were analysed using ImageJ (Abràmoff *et al*., 2004; Schneider *et al*., 2012) and measurements for primary root length, total lateral length and number of laterals were recorded and analysed.

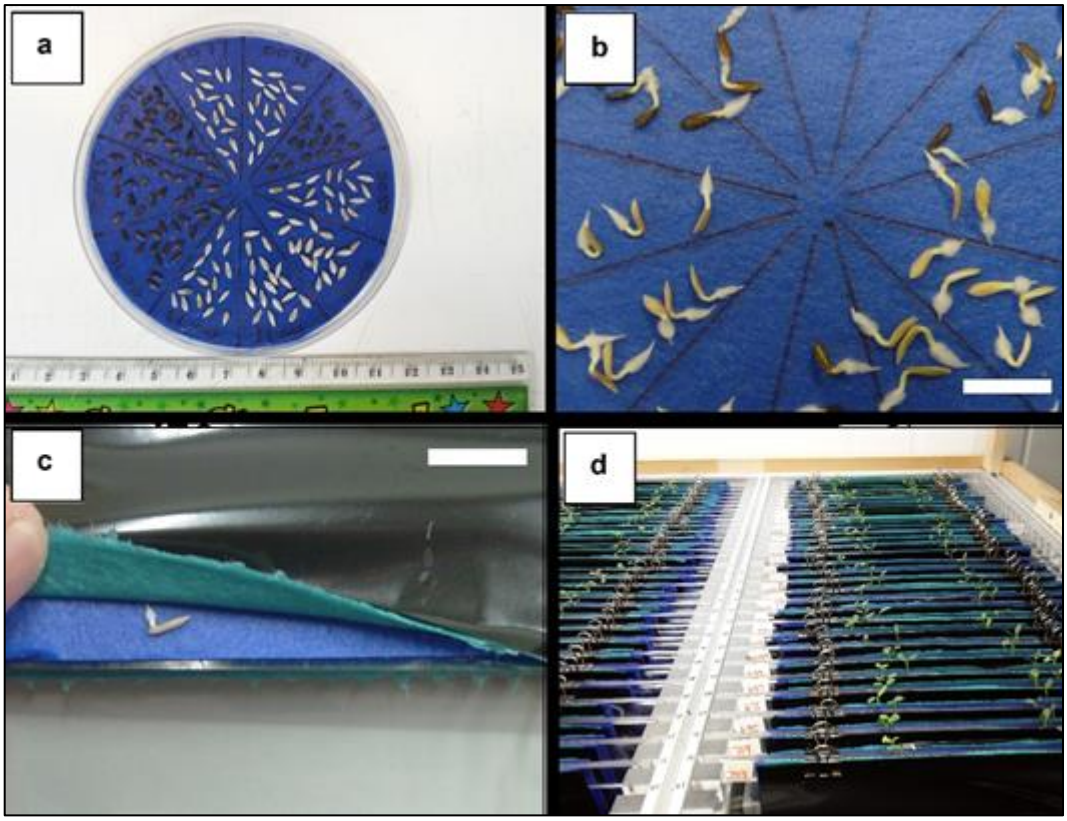

Figure 5.1: Seed germination, position, and growth in the pouch assay.
The imbibed seed on the germination paper in a petri dish (a). The predetermined stage at which the germinated seedlings were placed in the growth pouches, scale bar =1 cm; (b). A germinated seed of the parent Iceberg at the position placed in the growth pouch with the radicle orientated towards the bottom of the paper, scale bar = 1 cm. (c). Seedlings 10 d from the date they were placed in the growth pouch (d).

5.3.6 Data analysis.

An increase in root growth resulted in an increase in the variance of the residuals indicating that the data had non-constant variance and was not normally distributed. The raw data for the RILs and the parental lines were therefore transformed to square root and the mean calculated to normalise the distribution of the data for statistical analysis.

The transformed data were analysed using restricted maximum likelihood (REML) variance component analysis which accounted for variation, such as light level or edge effect that occurred within the frames. The resultant predicted means for all lines were then analysed to determine significant differences between genotypes. From the three measured phenotypes; primary root length (PRL), total lateral root length (TLL) and total number of lateral roots (TNL) three further ratios were produced, which were lateral root length density ($LRLD = TLL/PRL$), lateral root number density ($LRND = TNL/PRL$) and the mean lateral root length ($MLRL = TLL/TNL$) (Appendix 5.2). Broad sense heritability (H^2) for each trait was calculated from the variance component analysis ($VG/(VE + VR)$ where VG is the

genotypic variance, VE the sum of the component variance and VR is the residual variance). All statistical analysis of the mapping population data was done using GenStat 17th edition (VSN international Ltd, Hemel Hempstead, UK).

5.3.7 QTL analysis.

A combination of restriction fragment length polymorphism (RFLPs) and Kompetitive allele specific PCR (KASPs) markers were used to genotype both the parents and the RIL population. The linkage maps were constructed using JoinMap4 (Kyazma B.V, Wageningen, The Netherlands). Following REML transformation of the data the predicted mean values for all traits were analysed using MapQTL6 (Kyazma B.V, Wageningen, The Netherlands). Initially the data were analysed using interval mapping to identify putative QTL (Zeng 1994) before further analysis was done using multiple QTL model (MQM) mapping, adding significant cofactor markers to eliminate genetic variation (background noise) caused by QTL located elsewhere on the genome (Jansen & Stam 1994). The statistical logarithm of odds (LOD) score was calculated for a genome wide and chromosome wide significance of P<0.05 ($1 - \alpha c = \sqrt[n]{(1 - \alpha g)}$, where αc is the chromosomal significance threshold, αg is the genome wide significance threshold and n is the number of chromosomes) (van Ooijen 1999) (Appendix 5.3).

5.4 Results.

Some individual seedlings did not emerge, had severely inhibited primary root growth or browning of the root tissue. These seedlings were not included in the data analysis (99 seedlings from a total of 726). In total 42 lines had one data point missing, 19 lines had 2 data points missing, 5 lines had 3 data points missing and 1 RIL (RIL 36) had 4 data points missing.

There was very high significant variation (P<0.001) across all lines of the mapping population, including the parents, for all six root traits; primary root length (+/- 7.6, SEM=0.041, Figure 5.2a); total lateral root length (+/- 6.3 SEM=0.116, Figure 5.2b); total number of lateral roots (+/- 3.0 SEM=0.029); total lateral root, length/primary root length (+/- 0.8 SEM 0.013 Figure 5.2c); number of laterals/primary root length (+/- 0.4 SEM=0.003) and mean lateral root length (+/- 2.1 SEM=0.025).

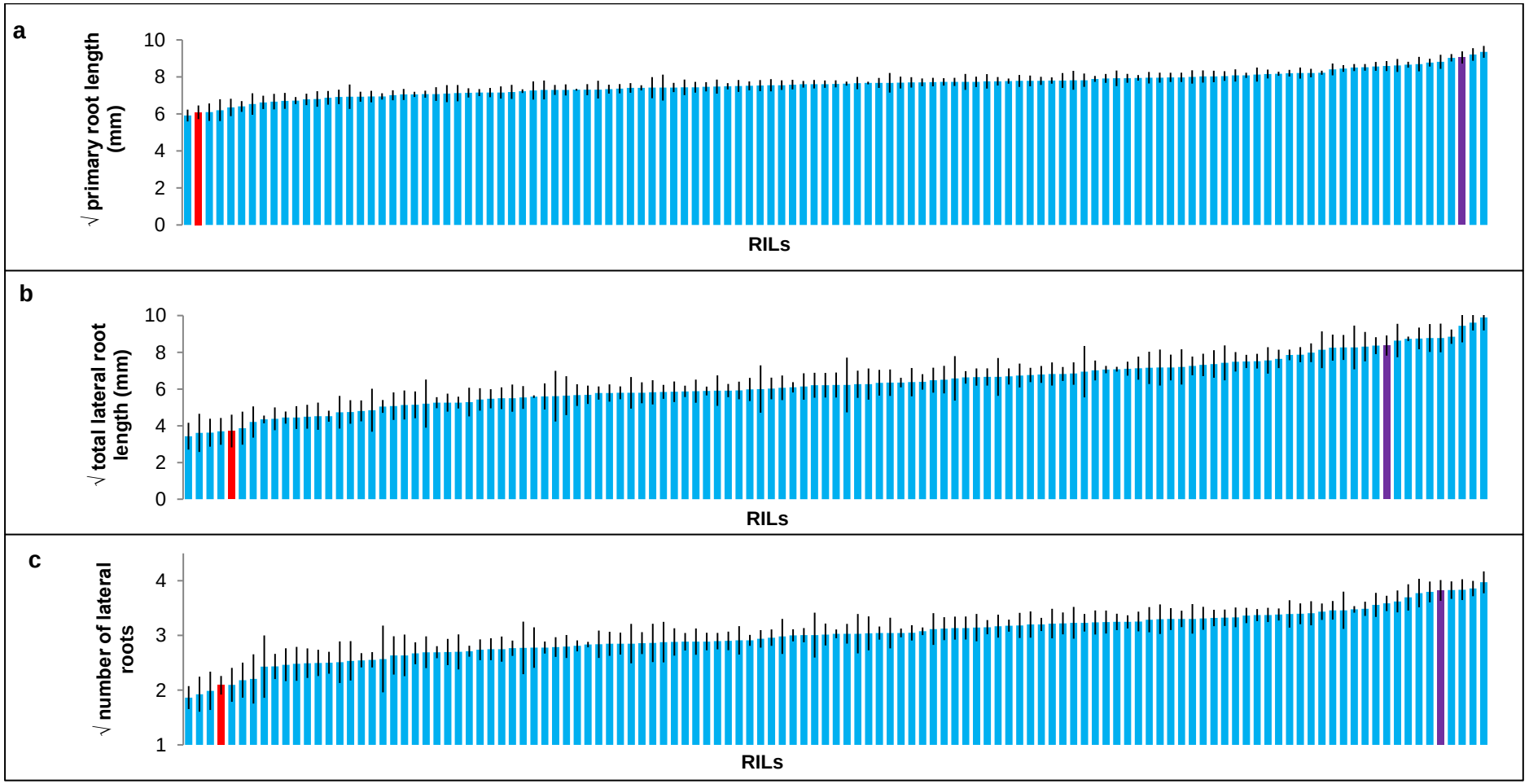

Figure 5.2: Segregation of 14 d seedlings of the 125 RILs of the Saladin X Iceberg mapping population and the parents for the measured traits (n=6).
(a) Primary root length, (b) total lateral root length and (c) total number of lateral roots. Red bars are the Saladin parent. Purple bars are the Iceberg parent and blue bars are the RILs; Error bars are SEM.

The chromosomal wide and genome wide significance at P<0.05 was 0.994. This value when interpolated into the table by van Ooijen (1999) corresponding to the average chromosomal map length of 116 cM gave a LOD score of 3.1 for statistically significant QTL (P<0.05) for the size and type of population used. The permutation test using the MapQTL software gave a LOD score of 3.2 and using this more conservative value a total of 16 statistical QTL were identified (Table 5.1; Figure 5.3).

Six significant QTL were found for primary root length on linkage group (LG) 2c, 4b, 5b, 7b, 8 and 9, which were labelled PRL-01 through to PRL-06 and 60.2 % of the genotypic variance can be explained by these QTL. Variance components analysis showed that the primary root length trait had a H^2 score of 0.37. For total lateral root length three statistical QTL, labelled TLL-01 through to TLL-03 were identified on LG 3, 5b and 9b and 38.6 % of the phenotypic variance was explained by these QTL. The H^2 score was 0.35 for the total lateral root length trait. No statistical QTL were discovered for total number of lateral roots. The H^2 score for total number of lateral roots was 0.28 (Table 5.1; Figure 5.3).

The first of the three ratios, LRLD had two statistical QTL on LG 4 and 9b and were labelled LRLD-01 and LRLD-02. The H^2 for the LRLD trait was 0.29. These two QTL explained 27.6 % of the phenotypic variance for this trait. Three statistical QTL were found for LRND. These QTL were on LG 7b, 8b and 9, explaining 33.4 % of the phenotypic variation for the trait and were labelled LRND-01, LRND-02 & LRND-03. The H^2 for the ratio LRND was 0.24. For MLRL two statistical QTL were identified on LG 8 and 9b and these QTL were labelled MLRL-01 and MLRL-02. A total of 21.1 % of the phenotypic variance of the MLRL trait can be explained by these two QTL and the H^2 score for MLRL was 0.24 (Table 5.1; Figure 5.3).

Table 5.1: Statistical QTL (P<0.05) for root traits and their genetic positions in 14 d old seedlings of the Saladin x Iceberg mapping population.

QTL (P<0.05)	LOD score	Linkage Group	Position (cM)	Associated markers	Allelic contribution	% phenotype explained
PRL-01	5.82	7b	33.5 – 35.5	7_LS1_750 ;39	Iceberg	17.1
PRL-02	3.84	9a	10.0 - 10.5	AQYG-OP3 9_LS1_319 ;53	Iceberg	11.1
PRL-03	3.38	5b	0.0 – 1.0	E35M47_191i E45M60_160i	Saladin	8.4
PRL-04	3.27	8a	24.4 – 27.4	ARRK-OP4 AKDB-OP4 BVTF-OP4 E35M61_280s	Iceberg	7.8
PRL-05	3.26	4b	14.9 – 15.9	4_LS1_324 ;23 AVZB-OP4	Iceberg	7.5
PRL-06	3.22	2c	35.3 - 35.3	2_LS1_664 ;11	Iceberg	8.3
TLL-01	8.83	9b	12.8 – 12.8	9_LS1_694 ;52	Saladin	23.8
TLL-02	3.24	5b	0.0 – 1.0	E35M47_191i E45M60_160i	Saladin	7.2
TLL-03	3.2	3a	13.7 - 13.7	AVSI-OP3 3_LS1_14 ;15	Saladin	7.6
LRLD-01	6.78	9b	8.6 – 12.8	9_LS1_392 ;52	Saladin	19.0
LRLD-02	3.33	4a	5.1 - 5.1	BSCC-OP3-1	Saladin	8.6
LRND-01	4.13	8b	10.0 – 12.0	E45M59_265i AKQB-OP4	Saladin	10.4
LRND-02	4.02	7b	31.7 – 31.7	E35M47_244i	Iceberg	8.7
LRND-03	3.97	9a	10.5 – 11.2	9_LS1_470 ;53 9_LS1_496 ;53	Saladin	14.3
MLRL-01	4.38	9b	7.4 – 7.4	BEMX-OP4	Saladin	10.8
MLRL-02	4.18	8a	1.0 – 3.1	8_LS1_591 ;48 8_LS1_58 ;49 8_LS1_229 ;49	Saladin	10.3

Trait abbreviations are PRL (primary root length), TLL (total lateral root length), LRLD (lateral root length density), LRND (lateral root number density) and MLRL (mean lateral root length)

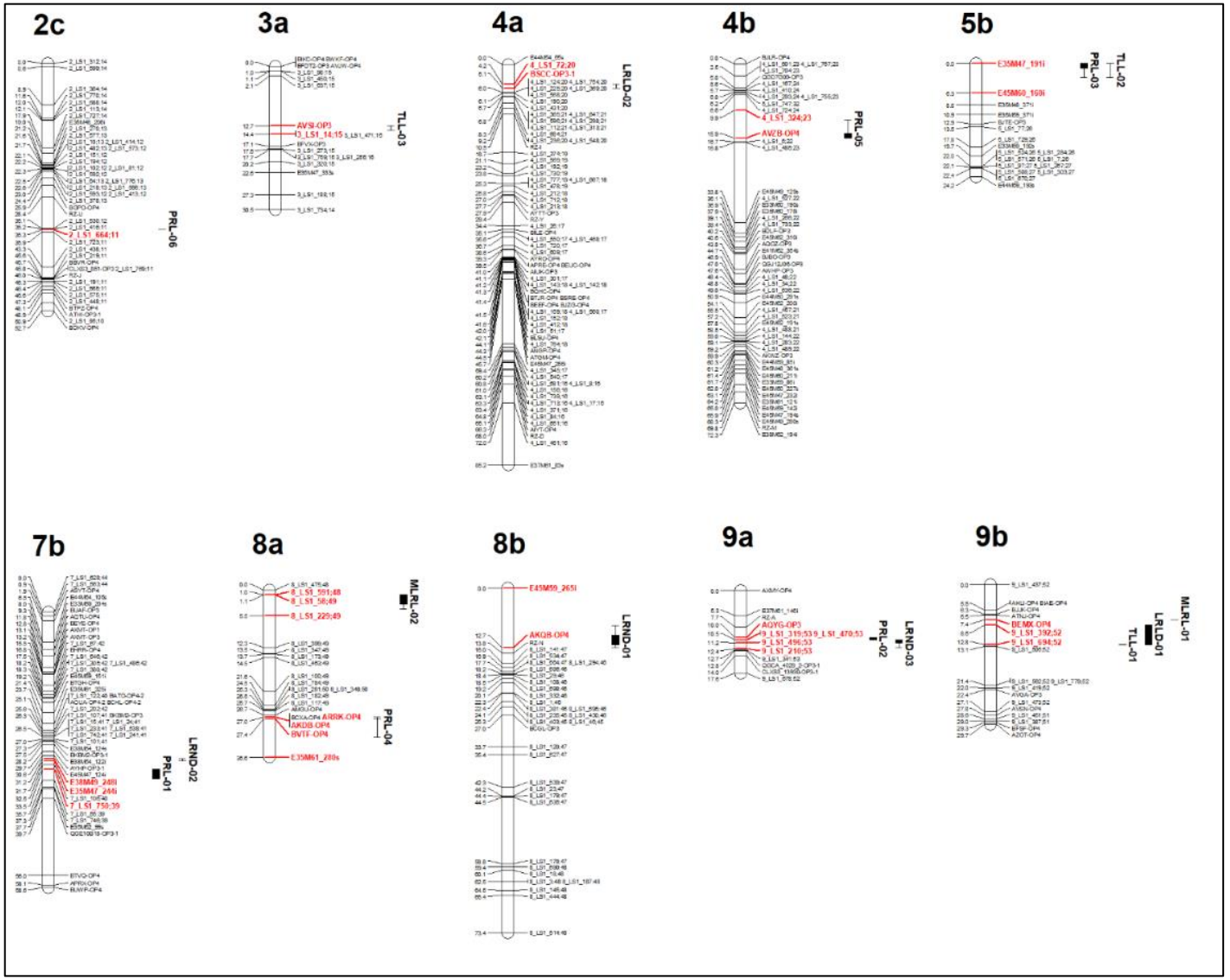

Figure 5.3: QTL positions and associated markers for root traits on the linkage groups of the Saladin x Iceberg mapping population.
Statistical (black bars) QTL positions in centimorgans (cM) on the Saladin x Iceberg linkage map. The solid blocks are the 1-LOD threshold (LOD score of 3.2), the outer intervals are the 2-LOD threshold. The markers in bold red are those associated with the significant LOD of the QTLs. Abbreviations of traits are PRL (primary root length), TLL (total lateral root length), TNL (total number of lateral roots), LRLD (lateral root length density), LRND (lateral root number density) and MLRL (mean lateral root length).

5.5 Discussion.

The study identified 16 statistical QTL associated with early stage rapid root development in an intra-specific *L. sativa* mapping population. The markers associated with these traits could be used for marker assisted selection in breeding programmes in the future should an increase in root growth prove to be associated with better establishment in field grown lettuce transplants.

The study has identified genetic potential, within the intra-specific cross in a 2D assay that could be utilised within a breeding programme. Further studies would need to be undertaken however, to better understand what interaction the environment has on these traits in field conditions. The 2D high-throughput assay used in this study allows the rapid analysis of root traits in seedlings in a time, cost and labour efficient manner compared with other techniques, such as computed tomography (CT) 3D analysis (Mooney *et al*., 2012). Over 762 germinated seedlings were sown in <6 h, covering an area <1.5 m^2 at a cost of <£0.50 per seedling. This technique offers greater efficiency than sand or soil pot grown root analysis, which increases area use, labour, and time costs dramatically as the roots need to be washed and separated before imaging/measuring can be accomplished.

In directly seeded crops the ability to produce a longer tap root early may be advantageous. Greater primary root length observed in 14 d old seedlings using the pouch system has been positively correlated with root emergence, faster establishment and increased seed yield in field grown *Brassica napus* (Thomas *et al*., 2016b). Increased primary root length in seedlings potentially allows root access to deeper water resources (Johnson *et al.*, 2000). Cultivated lettuce was described by Jackson (1995) as having a short tap root compared to its wild progenitor *L. serriola.* This study has observed significant difference within a *L. sativa* intra-specific cross for primary root length that may allow the ability to explore deeper soil layers and allow faster establishment and emergence in field grown lettuce. Six QTL were identified for increased primary root length of which one was on LG 5b (PRL-03) while the others were located on LG 7b (PRL-01), 9 (PRL-02), 8 (PRL-04), 4b (PRL-05) and 2c (PRL-06). Further work would be needed to identify if the RIL lines with a greater primary root length trait in 14 d seedlings emerge and establish faster and develop a longer, deeper tap root at maturity in the field.

In transplanted lettuce where mechanical pruning of the primary root often occurs (Kerbiriou *et al*., 2013), recovery of the root:shoot ratio may be governed by the plants ability to rapidly replace lost root mass through lateral root growth. Establishment is also dependent on the crops ability to regenerate lateral roots during establishment (Orzolek 1991) allowing early capture of the resources available to further optimise shoot growth. Longer total root length of wheat seedlings in a growth pouch assay has been associated with increased yield and shoot biomass in the field (Xie *et al*., 2017). Five statistical QTL were found that were linked with total lateral root length. Two QTL were located along LG 9b (TLL-01 and LRLD-01) overlapping the same region and probably represent a single locus. The further three QTL were located on LG 5b (TLL-02), 3 (TLL-03) and 4 (LRLD-02).

Decapitation of the root tip from primary lateral roots in lettuce seedlings has been shown to slow and even cease the emergence of any further secondary or tertiary lateral roots along the length of the decapitated root (Biddington & Dearman 1984). The pruning of the lateral roots often occurs as a consequence of the mechanical separation of adjacent peat blocks in the process of transplanting lettuce. Hence, breeding for cultivars that can regenerate greater numbers of primary lateral roots more efficiently may be a desirable trait that helps plants establish more rapidly. There were three individual statistical QTL linked to the total number of lateral roots. The QTL were for the lateral root number density trait and were located on LG 8b, (LRND-01), 7b (LRND-02) and 9 (LRND-03). LRND-01 and LRND-03 were contributed by the Saladin parent, while LRND-02 was contributed by the Iceberg parent.

The ability of a lettuce transplant to produce fewer longer lateral roots (greater MLRL) may be advantageous. Fitter *et al.,* (1991) suggested exploitation efficiency (amount of soil exploited per carbon unit cost of root) may be beneficial to crops. If lettuce transplants were able to produce fewer longer lateral roots with less branching following transplanting, then the plant would be able to utilise the resources captured mainly on shoot growth. There were two statistical QTL identified for MLRL in this study that may be beneficial to exploitation efficiency in lettuce transplants. The first was located on LG 9b (MLRL-01) and the second was on LG 8 (MLRL-02).

The region on LG 9b between 8.6 and 12.8 cM where QTL for the traits total lateral root length and lateral root length density traits (3 QTL), but not total number of lateral roots, lateral root number density and mean lateral root length suggests that this region is genetically involved with increased individual lateral root length or decreased

branching/topology, which would indicate this region could be exploited to increase the root exploration potential (Fitter & Stickland 1991) in lettuce transplants.

Only one of the six statistical QTL identified in this paper for primary root length (i.e. tap root length) was located on LG 2-(LG 2c), where QTL for the trait were identified by Johnson *et al*., (2000), however, the study cannot identify if the loci are the same. One of the QTL in this study located to the region towards the end of linkage group 2c (35.3 cM) which is similar to where Johnson *et al.,* (2000) had mapped a QTL associated with tap root length contributed by the wild parent. A further QTL identified in this study (TLL-03) mapped to LG 3 (13.7 cM), which is in asimilar area to the QTL identified by Johnson *et al*., (2000) associated with number of lateral roots.

The two QTL identified on the LG 5 group (5b), PRL-03 and TLL-02 locate to the same LG as a QTL linked to lateral root length and lateral root number observed by Wei *et al*., (2014). Johnson *et al*., (2000) also located a QTL on LG 5 that was linked to lateral root number in the lower soil profile contributed by the wild relative *L. serriola*. This region is therefore strongly linked to lateral root emergence and growth in both cultivated and wild parents. The QTL PRL-02, TLL-01, LRLD-01, LRND-03 and MLRL-01 located on the same LG (LG 9 and 9b) to the QTL identified by Wei *et al.,* (2014) linked to general root growth. Our study identifies this region as being linked with all the root growth traits; primary root growth, lateral root growth and lateral root emergence.

Of the population, RIL 87 and RIL 114 had the highest and lowest scores respectively for the three measured traits primary root length, total lateral root length and total number of lateral roots indicating that these lines would be the best candidates to use in a gene expression study to identify the genes underlying the QTL and others that are involved with increased root growth rate traits. Increased root growth traits could reduce the period of the recovery phase, caused by transplant shock (van Iersel 1998), by quickly restoring the root:shoot ratio and therefore increasing crop uniformity by reducing transplant establishment time. However, certain negative possibilities could occur. In a rapid rooting line, the increase in growth could mean more lateral roots are pruned leading to an enhanced transplant shock, meaning no benefit to establishment would apply. These concerns would need further studies to be resolved.

No QTL were identified for the trait TNL even though the parents significantly differed for the trait. One simple explanation for this could be if the total genetic variation for this trait was not captured in the mapping population.

5.6 Conclusion.

A rapid rooting phenotype may be beneficial to the establishment of lettuce transplants in commercial field production. Such a phenotype could reduce transplant shock and alleviate reduction in shoot growth due to mild abiotic stresses that occur in the field. The use of a high throughput rooting growth pouch assay revealed significant genetic variation in a Saladin X Iceberg cross RIL population to identify QTL linked to the traits associated with a rapid rooting phenotype in 14 d old seedlings. A total of 16 statistical QTL were identified. The statistical QTL were located across 9 different LGs representing loci on 7 of the 9 *L. sativa* chromosomes. DNA markers linked to these rooting traits were identified, which could allow breeders to select for a rapid establishment phenotype.

The linked markers could also be directly applied in lettuce breeding programmes and may be of more direct utility compared to markers from inter-specific crosses, which would be difficult to cross and will undoubtably contain unwanted genetic material from the wild *L. serriola* parent (Atkinson *et al*., 2013b).

6 Chapter 6 – Combination of QTL mapping and Transcriptomics for the identification of candidate genes responsible for the variation in root growth traits in lettuce seedlings.

6.1 Abstract.

Lettuce transplant establishment is an important factor in crop uniformity and maturity. The study used QTL mapping in combination with targeted RNA-seq transcriptomic analysis to identify nine candidate genes involved with various pathways of root growth that were located under five of the previously reported QTL for a “rapid rooting” trait identified in lettuce seedlings. Two genes, a *WAT1* related gene and *IRX9* gene, with known functions in cell expansion in *A. thaliana*. A further three genes were identified that play a role in cell proliferation -an *ALP1*-like gene, the *ACA8* calcium ATPase and the *PUX4*-like gene. The *NCED4* gene and *RCF3*-like genes involved with regulating ABA synthesis and ABI gene silencing were identified, which may promote root growth through the down-regulation of ABA synthesis and downstream genes associated with root growth suppression. A gene known as *PMT2*, which plays a role in cell wall synthesis was identified and a novel gene previously reported to play a key role in pollen tube development was identified to be expressed for the first time in roots.

6.2 Introduction.

The uniform establishment of lettuce transplants could increase yield uniformity and hasten crop development to full head maturity in an environment where there is growing pressure to reduce inputs such as water use and fertilizer use. A further constraint already seen in lettuce transplanting is the unavoidable mechanical pruning of the root system during block seperation. The problem in lettuce is that when the primary lateral roots are pruned, secondary lateral growth stops from these laterals (Biddington & Dearman, 1983), therefore in-field establishment is likely to be dependent on how quickly undamaged laterals can grow and emerge from intact roots from inside the transplant block in to the soil. A “rapid rooting trait” which is defined here in this thesis as increased primary (PRL) and lateral root growth (TLL) along with an increased lateral root emergence (TNL) would be expected to reduce

establishment time and recovery phase, restoring the root:shoot ratio more quickly and therefore improve crop uniformity and yield.

Quantitative trait loci have been previously identified for root growth traits in *Lactuca. sativa* seedlings using a high-through-put phenotyping assay for the rooting traits associated with PRL, TLL and TNL, along with three calculated ratios of these traits; lateral root length density (LRLD = TLL/PRL), lateral root number density (LRND = TNL/PRL) and the mean lateral root length (MLRL = TLL/TNL). For the trait PRL a total of six of the QTLs accounted for 60.2 % of the genetic variation (PRL-01-PRL-06). Three QTLs, TLL-01, TLL-02 & TLL-03, were associated with lateral root growth accounting for 38.6 % of genetic variation; two QTLs, LRLD-01 & LRLD-02, were associated with lateral root length density accounting for 27.6 % of genetic variation, three QTLs, LRND-01, LRND-02 & LRND-03, accounted for 33.4 % of genetic variation in lateral root number density and two QTLs, MLRL-01 & MLRL-02, were associated with mean lateral root length accounting for 21.1 % of genetic variation in the trait. The QTLs were located across 9 different linkage groups (LGs) representing loci on 7 of the 9 *L. sativa* chromosomes.

There are several described pathways involved with root growth in *A. thaliana* each regulating the expression of many genes that may be linked to an increased root growth rate such as that defined here as a “rapid rooting” trait in lettuce. These include genes involved with auxin signal responses, which among other growth promotions activates lateral root formation and position (Xiangdong & Harberd, 2003). Other pathways include the cytokinin signalling response pathway associated with root epidermal cell differentiation in to atrichoblasts or the trichoblasts that allow root hair development (Lee & Schiefelbein, 1999; Cho & Cosgrove, 2002). The genes associated with the gibberellin signal response pathway, including the *DELLA* proteins regulate auxin responses (Weston *et al*., 2008). There are also the genes involved with abscisic acid signalling responses during stress (Shkolnik-Inbar & Bar-Zvi, 2010). Together the hormone response pathways regulate the expression of genes that control cell division (Mar Castellano *et al*., 2001; Gutierrez, 2009; Gallois *et al*., 2013) and other physiological responses such as polar cell elongation (Verbelen *et al.,* 2006), which must be synchronized with the expression of genes involved with cell wall expansion (Vissenberg *et al.,* 2000), and cell wall synthesis (Cosgrove *et al.,* 2002).

Previous studies have combined QTL mapping and transcriptome profiling to successfully identify candidate genes for rice grain aroma and salt tolerance in rice (Pandit, *et al*., 2010; Pachauri, *et al*., 2014). The technique was used to identify root-knot nematode

resistance genes in cowpea (Santos *et al*., 2018) and rice (Petitot *et al*., 2017). Combining QTL analysis with a transcriptomic approach was also useful in the identification of Fusarium head blight resistance, and seed dormancy in wheat (Schweiger et *al*., 2013; Barrero *et al*., 2015). Of particular relevance are the studies by Zheng *et al*., (2003 & 2006) where genetic variation for the differential growth of seminal and lateral roots was analysed and combined with the targeted approach of QTL mapping and transcriptomics in a method known as cDNA-AFLP analysis to identify four genes involved with cell wall expansion in rice exposed to flooding and upland conditions. These studies highlight the possibility that the combined technique of QTL mapping and transcriptomics can be used to determine genes associated with genotypic variation in root growth traits.

This study aimed to identify candidate genes that are responsible for the QTL-based genetic variation in root growth observed in extreme phenotype seedlings of the *L. sativa* Saladin x Iceberg mapping population. Rather than determining the entire differentially expressed genes of the root transcriptome, the QTL are used to target the expression studies. As far as the authors are aware no differential gene expression has been previously carried out in *Lactuca sativa* comparing seedlings of lines with known differences for root growth rates.

The aim of this study was to identify differentially expressed genes that are directly or indirectly involved with root growth rate in lettuce seedlings and that are located under the QTL regions identified in chapter 5.

6.3 Material and methods.

6.3.1 Plant material.

The study used the *L. sativa* parents and four F_8 recombinant inbred lines (RILS) , which were selected from chapter 5, that displayed rapid root growth (Iceberg parent and RILs 87 and 35) or slow root growth (Saladin parent and RILs 41 and 111) across all three rooting traits associated with the “rapid rooting” trait i.e. primary root length (PRL), total lateral length (TLL) and total number of lateral roots (TNL), from the intra-specific cross between the crisphead L. sativa cv Saladin (syn Salinas) bred in the US and the Batavian L. sativa cv Iceberg, bred in France (Atkinson *et al.,* 2013b).

6.3.2 Plant growth.

The seeds were germinated as described in chapter 5. The germinated seedlings (n=12) were placed into 568 mL transparent polypropylene pots (General stores LTD, Enfield, London, UK) that had six 2 mm holes drilled 3 mm from the base of the pot. The pots were pre-filled with horticultural grade sharp sand, with an average maximum grain size of 3 mm (Vitax LTD, Leicestershire, UK). The germinated seedlings were sown in a randomized design with no blocking (GenStat 17th edition, VSN International Ltd, Hemel Hempstead, UK), The pots had been placed in 570 mm long x 390 mm wide x 50 mm high plastic trays (Garland Products LTD, Kingswinford, West Midlands, UK) containing 8 L of Hoagland's solution. Each tray had a total of 24 pots.in a 6-row, 4-column orientation. The seedlings were grown for 10 days following germination in a glasshouse at the University of Nottingham Sutton Bonington campus (52°49′59″ N, 1°14′50″ W). The glasshouse temperature was set at 20˚C days and 15˚C nights with a mean temperature of 17.3 and mean RH of 62.6% recorded. The photoperiod for the glasshouse was set at 16h/8h photoperiod.

6.3.3 RNA extraction and sequencing.

Following 10 days growth six seedlings were selected that were representative of the average root growth of each line i.e not the largest and not the smallest. The seedlings were removed from the sand by gently washing the sand from the plant root system, before the root was cut below the hypocotyl and immediately snap frozen in liquid nitrogen. The RNA was extracted from the roots of the seedlings following the QIAGEN RNeasy Mini kit protocol (Qiagen, Manchester, UK). The RNA quality was tested with an Agilent 2100 bioanalyzer at the University of Warwick and the RNA integrity number (RIN) ranged between 8.3 and 10.0 for the samples (Appendix 6.1). The samples were sent to the Earlham Institute (Norwich Research Park, Norwich, UK), where 18 stranded RNA libraries were constructed and sequenced on an Illumina HiSeq_4000 platform with 150bp paired end read metric. The parent lines were run on at a higher depth of coverage (mean coverage of 43.6 million reads) than the RIL lines (mean coverage of 22.3 million reads). The mean insert size of the parent lines were 385.7 bp, while the mean insert size of the RILs was 385.3 bp.

6.3.4 Bioinformatics.

All the bioinformatics were undertaken using the online platform Galaxy.org (Afgan *et al.,* 2018). The raw read quality was checked using the FASTQC tool (Andrew, 2014) (Appendix 6.2) before being aligned against the *L. sativa* genome sequence produced by Reyes-chin-Wo *et al*., (2017) using the HISAT2 (Kim *et al.,* 2015) alignment tool (Appendix 6.3). The aligned reads were then initially assembled using the StringTie tool and a reference annotation developed using the StringTieMerge tool (Appendix 6.4) before finally undertaking quantification with StringTie (Pertea *et al.,* 2015) (Appendix 6.5). The rapid rooting lines were pooled and contrasted against the pooled slow rooting lines and differential expression analysis was performed using EdgeR (Robinson *et al.,* 2009). The Gene ID numbers were then linked to the sequences with the Extract Genomic DNA tool (Appendix 6.6).

Differentially expressed (DE) genes were identified using a threshold of $\log^2$ fold change (FC) >2 and false discovery rate (FDR) <0.05. Differentially expressed genes were analysed to identify those that were situated within the QTL regions reported in chapter 5 using the flanking Kompetitive allele specific PCR (KASP) marker sequences. The KASP marker sequences were blasted against the *L. sativa* genome [GCF_002870075.1] (Reyes-chin-Wo *et al.*, 2017) on the website of the National Center for Biotechnology Information (NCBI) – Basic Local Alignment Search Tool (BLAST) to identify the genomic positions of the derived QTL. Those positions were then compared to the positions of the differentially expressed genes. Once the differentially expressed genes within the QTL regions were identified (Appendix 6.7) a literature search of genes and BLAST analysis of conserved domains was undertaken to confer gene function.

6.3.5 EdgeR analysis.

The EdgeR differential expression analysis software (Robinson *et al.,* 2009) was used with a $\log^2$ fold change >2 and a false discovery rate (FDR) of <0.05 (Benjamini & Hochberg, 1995) (Appendix 6.8).

6.4 Results/Discussion.

The analysis identified 171 genes that were up regulated in the rapid rooting lines compared to the slower rooting lines. Of these a total of 18 genes were located within QTL regions identified previously (Table 6.1). A total of 176 genes were identified as being down-

regulated by log2 fold change >-2 in the rapid rooting lines compared to the slow rooting lines and a total of 11 genes were under the QTLs identified previously (Table 6.2).

Table 6.1: Up-regulated genes associated with rapid rooting in the Iceberg parent and the two RILs 87 and 35 compared to the Saladin parent and RILs 41 and 111. (n=3). ***=P<0.001, **=P<0.01, *=P<0.05.

NCBI BLAST ID	logFC (EdgeR)	FDR (Edge R)	Assoc. QTL	gene/conserved domain
LOC111920980	9.2	**	MLRL-02	LTR Retrotransposon
LOC111892412	8.9	**	PRL-03/TLL-02	SEC16A homolog
LOC111915198	8.1	***	LRND-01	beta-1,4-xylosyltransferase IRX9
LOC111880186	7.6	***	LRND-02	TIM23-2-like
LOC111913637	7.5	***	LRND-02	calcium-transporting ATPase 7 (ACA7)
LOC111919034	7.3	***	PRL-03/TLL-02	calcium-transporting ATPase 8 (ACA8)
LOC111915190	7.2	***	LRND-01	cysteine protease inhibitor cystatin-1-like
LOC111876313	7.1	**	LRND-01	Alpha/beta hydrolase protein
LOC111919621	7.0	**	TLL-03	RCF3-like
LOC111887546	6.8	***	LRND-02	DNA-J domain novel protein
LOC111907680	5.7	***	LRND-01	PB1_UCP2 domain & STK domain protein
LOC111921315	5.4	*	LRND-03	MBD domain & Zinc finger domain protein
LOC111893337	4.5	*	LRND-02	Ethanolamine phosphotransferase protein
MK522161	4.4	**	MLRL-01	leucoanthocyanidin dioxygenase
LOC111890110	4.0	***	LRND-01	Phospho-methyltransferase 2 (PMT2)
LOC111879804	3.2	***	LRND-02	Resistance Methylated Gene 1 (RMG1)
LOC111898129	2.8	***	MLRL-02	WAT1-related protein
LOC111879553	2.6	***	PRL-01	No conserved domains identified

logFC represents the log fold change in expression identified by EdgeR. The FDR represents the fold difference of significance in expression. Gene/conserved domain is the identified gene or conserved domain identified through BLAST. (*** P=<0.001, ** P=<0.01, * P=<0.05).

Table 6.2: Down-regulated genes associated with rapid rooting in the parent Iceberg cultivar and the two RILs 87 and 35 compared to the Saladin parent and RILs 41 and 111. (n=3). ***=P<0.001, **=P<0.01, *=P<0.05.

NCBI BLAST ID	logFC (EdgeR)	FDR (Edge R)	Assoc. QTL	gene/conserved domain
LOC111910593	-6.0	**	LRND-01	NPH3 domain protein
LOC111879799	-6.3	***	LRND-02	Disease resistance protein RML1A-like
KC676791	-6.7	***	LRND-01	nine-cis-epoxycarotenoid dioxygenase 4 (NCED4)
LOC111912029	-7.5	***	LRND-01	UBX domain-containing protein 4-like (PUX4-like)
LOC111883800	-7.9	***	LRLD-02	palmitoyl-acyl carrier protein thioesterase
LOC111881507	-8.5	*	MLRL-02	ALP1-like
LOC111881454	-8.8	*	PRL-03/TLL-02	Cell wall invertase 1-like (CWINV1-like)
LOC111896810	-8.9	*	LRND-01	RICESLEEPER 3-like
LOC111879569	-9.5	***	PRL-01	no conserved domains identified
LOC111882257	-9.9	***	LRND-02	ankyrin repeat-containing ITN1-like protein
LOC111880061	-11.2	***	LRND-01	CDC6 homolog B-like

logFC represents the log fold change in expression identified by EdgeR. The FDR represents the fold difference of significance in expression. Gene/conserved domain is the identified gene or conserved domain identified through BLAST. (*** P=<0.001, ** P=<0.01, * P=<0.05).

6.4.2 Cell expansion genes.

The study has identified genes associated with cell expansion under QTL for mean lateral root length (MLRL-02) and lateral root number density (LRND-01). The gene *WAT1-like*, located under the QTL MLRL-02 was up-regulated by a mean of 2.8-fold (P<0.001) in the rapid rooting lines (Table 6.1). This protein is within the *WAT1* family of genes which have been shown to be involved with integrated auxin signalling, secondary cell wall formation in *A. thaliana* interfascicular fibres (Ranocha *et al*., 2010) and light-independent cell expansion (Denancé *et al*., 2010). The differential expression of *WAT1* therefore could be enabling the rapid rooting lines to expand the cells within the root more quickly. *WAT1-like* expression is associated with mean lateral root length indicating the protein is contributing to an increased lateral root length compared to total number of lateral roots. The shoot growth rate of the *wat1* mutant in *A. thaliana* was not observed to be reduced until around 4 weeks after germination (Ranocha *et al*., 2010). However, the study did not undertake any observations of the root system or establish whether there was any reduction in root growth that could lead to the reduction of shoot growth further along maturation, which would need to be investigated.

Interestingly, another protein that has been directly shown to be an important contributing factor to cell wall synthesis and therefore linked with cell expansion is the β-1,4-xylosyltransferase, IRX9, which is an essential enzyme in the production of xylan in the plant secondary cell wall transferring UDP-Xyl to xylosyloligomers (Ren *et al*., 2014). This protein was up-regulated by a mean of 8.1-fold (P<0.001) in the rapid rooting lines (Table 6.1) and associated with a higher number of lateral roots compared with overall lateral root length (LRND) as it was located under the QTL LRND-01. The absence of *IRX*9 expression in *A. thaliana* leads to the collapse of xylem vessels in the shoots, shortened xylan chain length and dwarfed plant growth (Lee *et al.,* 2010). Therefore, increased expression of *IRX9* as seen in this study could be linked with increased synthesis of cell walls during elongation required for rapid rooting. Surprisingly, the gene was linked to LRND i.e increased number of lateral roots (cell proliferation) rather than cell elongation, which would be more closely linked with total laterl root length.

6.4.3 Cell proliferation genes.

The study identified genes associated with cell proliferation under QTL for lateral root number density (LRND-01), primary root length (PRL-03), total lateral root length (TLL-02, TLL-03) and mean lateral root length (MLRL-02).

The -7.5 fold (P<0.001) down-regulated expression of the *PUX4-like* gene (Table 6.2) in the rapid rooting lines could lead to increased root cell proliferation and cell elongation in these lines as PUX4 has been observed to interact with CDC48 and lead to ubiquitin-based degradation of CDC48 in *A. thaliana* (Rancour *et al*., 2004). CDC48 has been shown to be involved with the up-regulation of cell division and cell expansion (Feiler *et al.,* 1995) therefore lower expression of the *PUX4* gene could allow higher accumulation of CDC48*,* which in turn, could lead to increased root cell division and expansion. The *PUX4* gene was located under the QTL for the trait LRND (LRND-01).

Calcium plays a key role in plant growth and root development and morphogenesis through the mediation of cellular signalling (Xiao Pan *et al.,* 2018). Extracellular signals cause an elicit change in cellular calcium levels, which leads to signal transduction pathway responses (Tuteja & Mahajan, 2007). The study identified two calcium ATPase membrane pumps, ACA proteins where the expression was up-regulated in the rapid rooting lines compared to the slow rooting lines. The first was the CALCIUM ATPase 8 (ACA8), which was up-regulated by 7.3-fold (P<0.001) and was located under the QTLs for the trait primary root length (PRL-03) and total lateral root length (TLL-02) (Table 6.1). ACA8 has been identified in *A. thaliana* to play a key role in sucrose signalling (Smeekens *et al*., 2009) and the down regulation of expression of the cell cycle regulators, such as CYCLIN DEPENDENT KINASE B (CDKB) CYCLIN B (CYCB) and CYCD that supress cell proliferation in roots. ACA8 controls cell proliferation in a sucrose dependent manner, aca8 mutant seedlings have stunted root growth and have a reduced root apical meristem (Zhang *et al*., 2014). The differential expression of ACA8 in the study could be a direct result of larger root apical meristems in the root systems of the rapid rooting lines.

The second calcium ATPase identified was ACA7 which was up-regulated by a mean 7.5-fold (P<0.001) in the rapid rooting lines. This has been identified in *A.thaliana* to play a key role in pollen development (Lucca & León, 2012) including pollen tube cell expansion (Bossi *et al*., 2019) (Table 6.1). The expression of ACA7 has not been reported previously in the root system and here we present a novel possible function that involves ACA7 in the

expansion of cells of the root system. The study is unable to determine in which root cells ACA7 is expressed. One hypothesis is that ACA7 could be involved with lateral root initiation as it located under the LRND-01 QTL and could indicate ACA7 is involved in root development in a similar role to that in pollen tube development that has already been reported. ACA7 is upregulated during mitosis in pollen grain development and *aca7* mutants displayed high levels of pollen fatality (Bossi *et al*., 2019). ACA7 could be expressed in founder cells during the initiation phase of lateral root development and aid anticlinical divisions. Further studies would be required to test this theory.

6.4.4 ABA synthesis and gene silencing.

The gene REGULATOR OF CBF GENE EXPRESSION 3-like (RCF3-like) was identified to be up-regulated by a mean of 7.0-fold (P=0.005) in the rapid rooting lines compared to those of the slow rooting lines (Table 6.1). The protein RCF3 is a regulator of miRNA biogenesis in plants through the interaction with CPL1 and the phosphorylation of HYL1. *A. thaliana* rcf3 mutants display hyperphosphorylation of HYL1, lower cellular levels of miRNA and a reduced phosphorylation of the DICER like-1 protein involved with miRNA silencing (Karlsson *et al*., 2015). The increased expression of RCF3-like in the rapid rooting lines could be causing an indirect effect on root growth by firstly, increasing the ability of the CPL1 protein to interact with HYL1, leading to a reduced expression of RD29A, which is an ABA mediated transcription factor that would lead to a reduction in root growth. A study by Shu *et al*., (2016) identified that overexpression of ABI4 caused by ABA lead to increased expression of RD29A. ABI4 has been linked with inhibition of lateral root development by the reduction of polar auxin transport by inhibiting the expression of PIN-FORMED (PIN) proteins (Shkolnik-Inbar & Bar-Zvi, 2010) and this would explain why the RCF-3 like gene is located under the QTL for total lateral root length (TLL-03).

The increased expression of ABI4 has been identified to inhibit lateral root development in *A. thaliana* (Shkolnik-Inbar & Bar-Zvi, 2010). Interestingly this study identified a mean -8.5-fold (P=0.050) reduced expression of the gene ANTAGONIST OF LIKE HETEROCHROMATIN PROTEIN 1-like (ALP1-like) (Table 6.2). The protein ALP1 has been observed to interrupt the gene silencing activity by the POLYCOMB REPRESSIVE COMPLEX 2 (PRC2) (Liang *et al*., 2015), which inhibits the expression of ABI4 through the histone methylation of the gene (Mu *et al*., 2017). One explanation is that the downregulation of the ALP1-like protein in the rapid rooting lines leads to increased activity of PCR2 followed by a reduced expression of ABI4 and increased lateral root development. This hypothesis is

strengthened by the ALP1-like gene being linked to the mean lateral root length trait located under the QTL MLRL-02.

One gene associated with ABA synthesis was located under the QTL for lateral root number density (LRND-01). The expression of the gene encoding 9-cis-EPOXYCAROTENOIDDIOXYGENASE 4 (NCED4) was down-regulated by -6.7-fold ($P<0.001$) in the rapid rooting lines compared to the slow rooting lines (Table 6.2). NCED4 is a key regulatory enzyme in the promotion of biosynthesis of ABA, which has already been stated to reduce root growth (Leung *et al*., 1997). A study which used CRISPR/Cas9 knockout of the NCED4 gene in lettuce identified reduced thermoinhibition of germination with no detrimental effect on shoot growth (Bertier *et al.,* 2018). Unfortunately, root growth was not included in the study although a study by Huo *et al*., (2013) has previously identified NCED4 gene expression in lettuce roots.

6.4.5 Cell membrane synthesis genes.

One gene identified was associated with cell membrane synthesis and was located under a QTL for lateral root number density (LRND-01). The gene was for the enzyme PHOSPHO-METHYLTRANSFERASE 2 (PMT2), which had a 4.0-fold ($P<0.001$) higher expression in the rapid rooting lines compared with the slower rooting lines (Table 6.1). The PMT enzymes are key enzymes in the biosynthesis of the phospholipid, phosphatidylcholine and PMT2 has been previously observed to be expressed greatest in developmental process and highest in the roots. The pmt2 pmt1 double knockout in *A. thaliana* had reduced root growth (Liu *et al*., 2019). What is not known is if lettuce has the PMT1 gene and if the redundancy is similar in the species.

6.4.6 Other genes of interest.

A gene of interest that was down-regulated by -8.9-fold ($P=0.023$) in the rapid rooting lines under the LRND-01 QTL was the *RICESLEEPER 3-like* gene (Table 6.2). The *RICESLEEPER* genes encode retro-transposons that have lost their original function and are involved with developmental processes as mutant knockouts of *RICESLEEPER1* and *RICESLEEPER2* have been shown to have abnormal development phenotypes (Knip *et al*., 2012), however *RICESLEEPER3* or *3-like* functionality has yet to be defined and its effect on root development is yet to be determined. This study has shown that *RICESLEEPER3-like*

gene is expressed in the root system in lettuce and has probable links to root development, although further studies would be needed to elicit its function.

The gene coded LOC111920980 contains the gag2, gag3, RVE and RVT2 conserved domains, which are associated with the *copia*-like LTR retrotransposons. Many genes within plant genomes contain fragments of *copia*-like retrotransposons in flanking regions and they provide regulatory sequences and facilitate gene duplication (White *et al*., 1994). LTR transposons have been found to influence the expression of genes that are found up-stream and down-stream of them. Notably, the *copia*-like LTR transposon (LOC111992098) up-regulated by 9.2-fold (P=0.009) in this study is found down-stream of the up-regulated WAT1 related protein under the QTL MLRL-02, however the distance is over 402 Kbp and therefore their interaction is highly unlikely.

There were two differentially down-regulated genes in the rapid rooting lines that responded unexpectedly. The first is the *CDC6* homolog B-like gene, which was down-regulated by a mean of -11.2-fold (P<0.001) (Table 6.2). CDC6 has been identified to play a key role in the promotion of cell division in *A. thaliana* (Mar Castellano *et al*., 2001), and would be expected to be up regulated in rapid rooting lines, especially in increased lateral root number. CDC6 has been identified as being involved with endoreplication in dark grown hypocotyls interrupting normal cell division (Mar Castellano *et al*., 2001), which could explain why CDC homolog B-like is tightly regulated in the root cells. The second is the CWINV1-like gene, which was found to be down-regulated by a mean of -8.8-fold (P=0.039) in the rapid rooting lines compared to the slow rooting lines (Table 6.2). A knock-out mutation in the CWINV1 gene in *Daucus carota* (carrot) has been shown to have retarded root development (Tang *et al*., 1999), which is due to cell-wall invertase playing a key role in the hydrolysis of sucrose in to fructose and glucose subunits shown in *A. thaliana* (Verhaest *et al*., 2006).

The study has found several differentially expressed genes that are associated with the cell expansion, cell division and ABA synthesis pathways under the QTL identified in chapter 5. These genes are novel findings in lettuce in a comparison study of lines with differential growth rates of primary and lateral roots and lateral root initiation. The genes identified indicate identifying genetic markers to improve lettuce establishment through increased root growth within breeding programmes will be difficult without further analysis through fine mapping to target DNA markers associated to these genes and even then, understanding the genes which have the largest phenotypic effect would need to be selected for first through field observations of lines that carry the genes.

Of the 30 differentially expressed genes under QTLs, the greatest number were under the QTL LRND-01, which was 2 cM is size, although this is genetic distance and the physical size of the QTL is unknown.. A total of 11 differentially expressed genes were identified under LRND-01 and seven of the genes identified were associated with pathways involved with root development in *A. thaliana*. Of the three genes identified under the QTL MLRL-02 two were associated with root development. Using the DNA markers associated with MLRL-02 in a marker assisted breeding programme could increase the expression of the WAT1 related gene during lettuce seedling growth enabling the lateral roots to grow from the block faster and reduce establishment time.

There are, however, some limitations to interpreting the results of this study. QTL are known to be affected by environmental factors, and QTL with low heritability (H^2) can be difficult to identify candidate genes for (Norton *et al.,* 2008). The candidate genes identified in this study were located under traits with broad sense H^2 scores ranging between 0.24 for the traits lateral root number density (LRND) and mean lateral root length (MLRL) to 0.37 observed for the trait primary root length (PRL).The QTL for lateral root number density (LRND-01) was reported as explaining 19% of the phenotypic variation for the trait, which would likely be only partially explained by the candidate genes identified within this QTL. Similar can be said of the other candidate genes identified under the QTLs; MLRL-02, PRL-03/TLL-02 and TLL-03. In addition, the plants from which the gene expression was assessed were grown in sand-filled pots, whereas the QTL were identified in a 2D high through-put assay. Ideally, stable rooting trait QTL need to be identified in varying environments before combining with a targeted transcriptomic approach. Alternatively, to fully capture the genetics of differential root growth across the “rapid rooting” phenotype the whole transcriptome could also be analysed utilising an untargeted approach. Nevertheless, the study reported here has demonstrated that a targeted approach can be successfully applied to rooting traits in *L. sativa*.

6.5 Conclusion.

The study using QTL mapping in combination with targeted transcriptomic analysis identified a total of nine candidate genes under five QTL associated with the traits primary root length, total lateral root length, lateral root number density and mean lateral root length. Two genes, a WAT1 related gene and IRX9 gene, were identified and had known functions in cell expansion. A further three genes were identified that play a role in cell proliferation -an

ALP1-like gene, the ACA8 calcium ATPase and the PUX4-like gene. The NCED4 gene and RCF3-like genes involved with regulating ABA synthesis and ABI gene silencing were identified. A gene known as PMT2, which plays a role in cell wall synthesis was identified and a novel gene previously reported to play a key role in pollen tube development was identified to be expressed for the first time in roots. These genes identified could explain a proportion of the phenotypic variation for the rapid rooting trait. Further studies in lines segregating for the individual genes are needed to analyse the effect of the candidate genes on the rapid rooting trait.

7 Chapter 7 - A rapid root growth phenotype is maintained in lettuce (*Lactuca sativa*) following root pruning and exhibits genotypic variation.

7.1 Abstract.

Lettuce plants with rapid rooting traits could be able to quickly access water and nutrients to support growth following transplanting into the field. This trait may also lead to increased root damage by mechanical handling during transplanting, leading to a recovery phase where plant growth is reduced. In addition, the trait may not be maintained following root pruning, reducing the utility of seedling phenotyping. In a controlled environment study ten recombinant inbred lines (RILs) from an intra-specific lettuce (*Lactuca sativa*) population (Saladin X Iceberg), known to vary in root growth rate at a seedling stage, and three current commercial varieties were grown in sand and nutrient solution following root pruning at the transplant stage to determine if a rapid rooting phenotype was maintained following root damage associated with transplanting processes. Image analysis identified two lines (RILs 35 and 87) that have consistently high root growth and have a reduced duration of post pruning recovery phase.

7.2 Introduction.

Many vegetable crops, including lettuce, are grown as transplants in glasshouses prior to planting out into the field. This provides several benefits for producers, including faster germination, better crop establishment, prevention of weed infestation, crop uniformity, growth and subsequently yield (Sharma *et al*., 2005: Maltais *et al*., 2008).

Transplants invariably sustain root damage at the time of transplanting (van Iersel, 1998). Lettuce blocks are conjoined, with the roots allowed to grow in to adjacent blocks. Separation of the blocks causes root pruning of the individual plants (Figure 7.1), leading to a stress termed in this paper as root pruned transplant shock (RPTS). RPTS can occur when transpiration is larger than the uptake of water resulting in a sudden plant water deficit. The severity of RPTS can depend on several attributes of the plant, including a tolerance to root disturbance, water and nutrient uptake efficiency and the ability of the plant to replace lost root area quickly (Leskovar, 1998) in addition to environmental conditions that can exacerbate transplant shock, such as soil type and field conditons. The ability of the plant to

restore the root area quickly is beneficial to earlier marketable yield as shoot growth is greatly reduced until the root:shoot ratio is restored (Bar-Tal *et al*., 1994), which is a process known as the recovery phase (Kerbiriou *et al*., 2013).

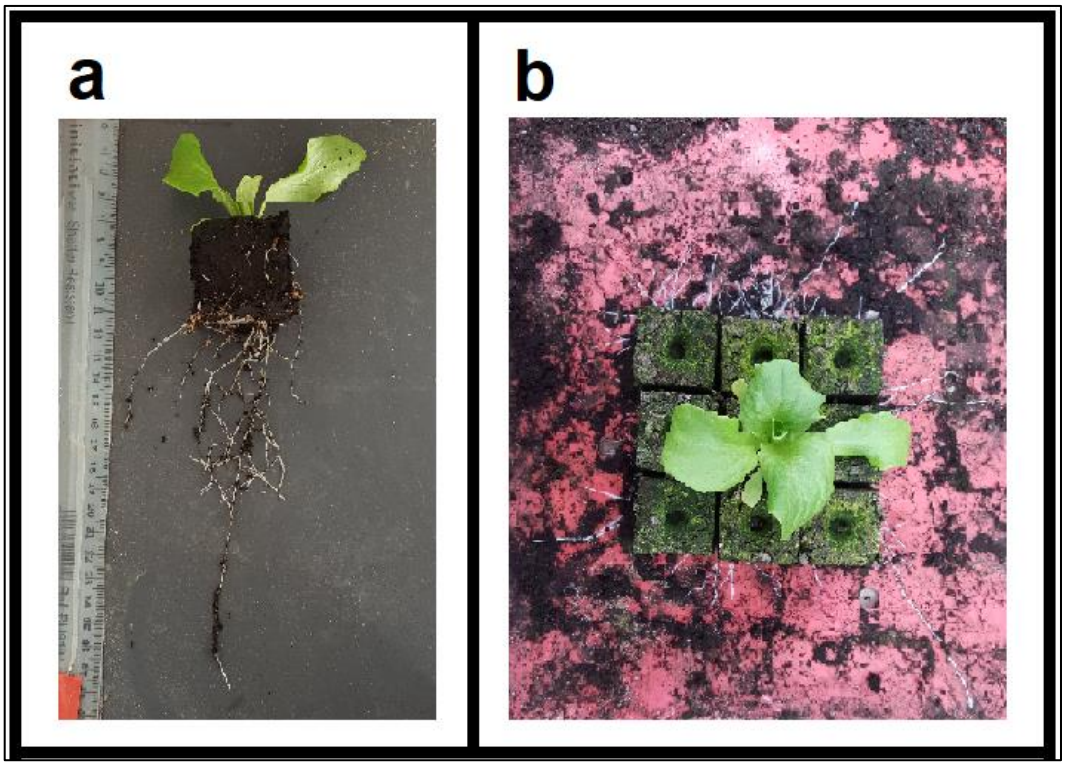

Figure 7.1: Root growth of a lettuce plant in a peat block.
The primary root growth in a 19d seedling (Webbs Wallace) exceeds 190 mm in length (a). After 24 d the roots of a lettuce transplant have reached blocks two places away from the originating block (b).

In Western Europe commercially grown lettuce are grown to around the 4-6 leaf stage in 64 cm^3 peat blocks. As peat is a non-renewable resource and its use is critical to wetland ecosystems there is pressure on producers to reduce its use in transplant production (Mininni & Santamaria, 2012). One option to achieve this is to produce transplants in smaller substrate volumes, which would also alleviate costs of transportation and greenhouse space. However, the volume of substrate used to produce transplants influences subsequent growth (Sharma *et al*., 2005). Larger module volume in lettuce transplants has been shown to reduce the time taken to achieve marketable head weight and improved stand establishment (Gianquinto, 1991; Nicola & Cantliffe, 1996). Smaller transplants may reduce variability in lettuce head weight compared to older transplants (Wurr & Fellows, 1986), which could remove the variability seen in transplant size in commercial lettuce known to cause significant variation in final yield (Harwood *et al*., 2010). However younger lettuce plants may be more susceptible to transplant shock caused by environmental change (Kerbiriou *et al*., 2013).

It may be possible to breed lettuce plants that regenerate roots rapidly following root pruning at the transplant stage. Genetic variation in root traits has been observed to increase nutrient capture in *Brassica napus* (Thomas *et al*., 2016b) and breeding for beneficial root traits, such as longer root structure, has been demonstrated to improve field performance in rice (Steele *et al*., 2013). However, lettuce root production rates have been reported by Biddington & Dearman (1984) to vary over time following pruning, which could indicate that the phenotype could be lost following RPTS.

A rapid rooting phenotype in lettuce may maintain rapid root growth following root pruning and, if so, could also reduce the impact of RPTS on lettuce transplants by reduction of the recovery phase. In this controlled environment study, we have compared how ten lines of a Saladin X Iceberg RIL population previously selected for extremities of overall root growth from the growth pouch assay used in chapter 5, including primary root growth, lateral root emergence and lateral root length, compared to three current commercial varieties. We compared the root and shoot growth of lettuce plants at the 3-7 true leaf stage, which were propagated in commercial blocks and were either root pruned or not at the point of transplanting to sand medium and tested the following hypotheses: a) The rapid rooting trait is under genotypic control; b) The rapid rooting trait in seedlings is maintained in transplants following root pruning; and c) The rapid rooting phenotype is not associated with reduced shoot growth following root pruning.

7.3 Materials and methods.

7.3.1 Plant material.

The study used ten F_8 recombinant inbred lines (RILs) from a Saladin X Iceberg mapping population of lettuce developed at the University of Warwick, UK (Atkinson *et al*., 2013b), previously identified as having either a high or low rapid rooting phenotype identified in the growth pouch assay in chapter 5, and three commercially grown cultivars (Syngenta Seeds B.V, Enkhuizen, NL) identified here as commercial A-C.

7.3.2 Growth conditions.

300 preformed peat blocks were supplied by a commercial plant raiser (Farringtons Ltd, Preston, UK) in two trays. The blocks measure 4x4x4cm (64 cm^3 volume) and each tray (600x400 cm) had 150 blocks in a 15 x 10 layout. To each tray 10 guard plants (Webb's Wonderful; Johnsons-seeds, Newmarket, Suffolk, UK) were sown in the first and last

columns. All lines were sown randomly to all the remaining blocks of the tray ensuring each line appeared once on both the top and bottom row. (Figure 7.2) Location of lines was randomised using Genstat (17th edition) VSN International LTD, Hemel Hempstead, UK).

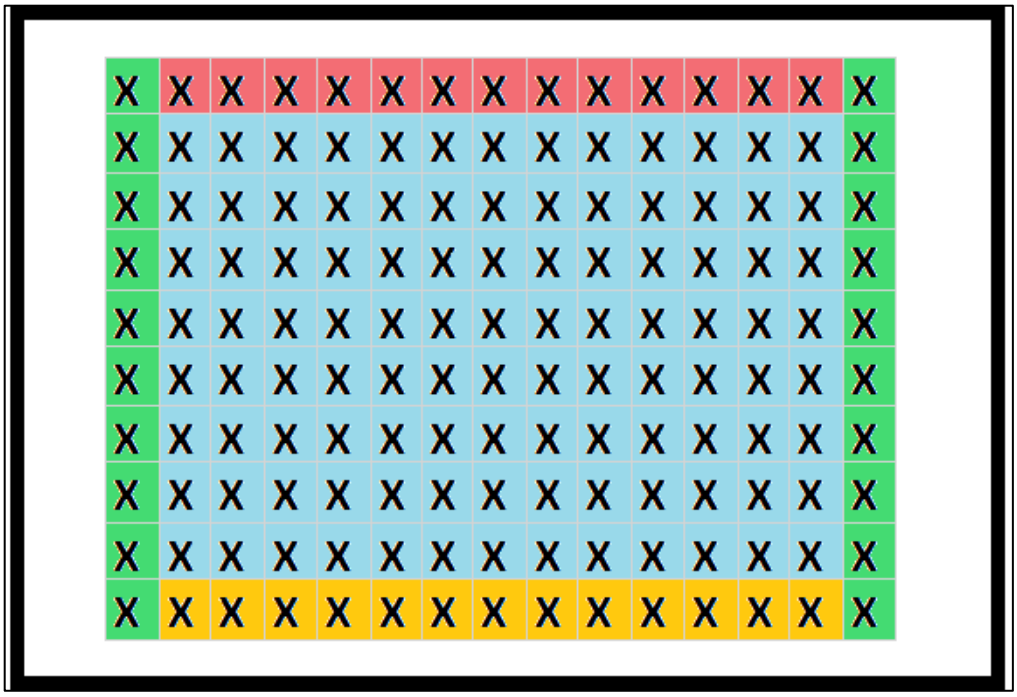

Figure 7.2: Diagram of the peat block layout.
The green blocks are the guard plants (Webbs Wallace) placed in column 1 and 15 of the tray. The red blocks are the first row where each line appeared once. The orange blocks are the last row where each line appeared once. In the blue blocks, lines were randomly appointed a position.

Each peat block was separated at the base and then individually wrapped around four sides and base with plastic clingfilm (Tesco plc, Welwyn Garden City, UK) to prevent outgrowth of roots and intermingling of the roots with those of adjacent blocks. Transplants were grown in a 2.2 m wide x 3.3 m long x 3.0 m high controlled environment (CE) room with a 20 h photoperiod. Photosynthetically active radiation (PAR) was 191 µmol m^{-2} s^{-1} (190 SB quantum sensor; LI-COR Inc., Lincoln, NE, USA) at plant height produced by 400 W white fluorescent lamps (HIT 400w/u/Euro/4K, Venture Lighting, Rickmansworth, UK). Relative humidity throughout the trial in the CE room ranged from 31.7 – 100 % with an average of 69.2 %. Temperature ranged from 12.6 – 27.5˚C with an average of 17.5˚C. As the genotypes were known to differ in growth rate the plants were grown until all plants of a pre-selected cultivar (commercial C) had reached a growth stage of 5 true leaves, with lines ranging from a minimum of 3 to a maximum of 7 true leaves.

The plants were removed from the tray and the plastic film removed. The plant shoot area was imaged as described below. The transplants were then either transplanted with the

roots unpruned (n=10) or roots pruned before transplanting (n=10). In commercial transplanting the amount of root pruned from the separation of the blocks is uncontrolled and usually occurs on block faces adjoined to other blocks and any root growth along the bottom of the tray orientated under other blocks (Figure 7.1). The root pruning treatment in this study was imposed by scraping a knife over all the faces of the peat blocks to remove all visible roots, to ensure all root growth out of the block was post transplanted growth..

Each plant was transplanted into a 568 mL transparent polypropylene pot (General stores Ltd, Enfield, London, UK) that had six 2 mm holes drilled 3 mm from the base of the pot. The pots were pre-filled with horticultural grade sharp sand, with a maximum grain size of 3 mm (Vitax Ltd, Leicestershire, UK). Each peat block was placed so that the top of the block was level with the surface of the sand. The pots were then placed in 570 mm long x 390 mm wide x 50 mm high plastic trays (Garland Products Ltd, Kingswinford, West Midlands, UK). Each tray had a total of 24 pots arranged with guard pots in all but one location in the first and last column. The pot locations within the trays was randomised using Genstat. To each tray, 8 L of 15% concentration Hoagland's solution (Hoagland's No. 2 basal salt mixture, Sigma Aldrich, Dorset, UK) made with tap water was added and replenished with 1 L of tap water after 4 days. The transplants were grown for 7 days in the sand. The transplants were then gently removed from the sand, the roots washed and imaged.

7.3.3 Imaging.

At the end of the propagation stage (d0), each plant was imaged non-destructively, ensuring each leaf tip was visible on the image to enable an estimation of plant size. The leaf area was derived from the leaf length using standard curves developed from the post-transplanting shoot area data. Standard curves were developed, for initial leaves developed pre-heading and later leaves that were forming the initiation of the heading, for each cultivar. At the end of the transplanting stage (d7), the leaves were removed from the stem and laid flat and imaged. The younger heading leaves that were curled and would not lay flat were folded along the mid rib. The roots that were visible on the 5 faces of the peat block at d0 of the pruned treatment, following removal of the plastic film and d7 of both treatments that had been submerged in the sand were imaged by rotating the block around all four sides before imaging the base (Appendix 7.1).

All images were taken at a set distance of 200 mm using a Samsung Galaxy S6 Edge SM-G925F smartphone (Samsung Electronics (UK), Chertsey, Surrey, UK) over a black background. The images were analysed with ImageJ (Abràmoff *et al.*, 2004; Schneider *et al*., 2012) for leaf length, using the segmented line selection tool to measure individual leaf lengths and the polygon selection tool to trace around each leaf to measure leaf area. Leaves that would not lie flat were folded along the mid rib had their measured area doubled.

Each root image pre and post pruning, was cropped, and the background removed using the freehand selections tool and the fill tool set to black. The peat block and all roots that did not originate from that face were removed in the same way. The image was then made binary with the background set as black before the quantity of white in the image was calculated. The measurements for all 5 faces of the peat block were then summed (Figure 7.3).

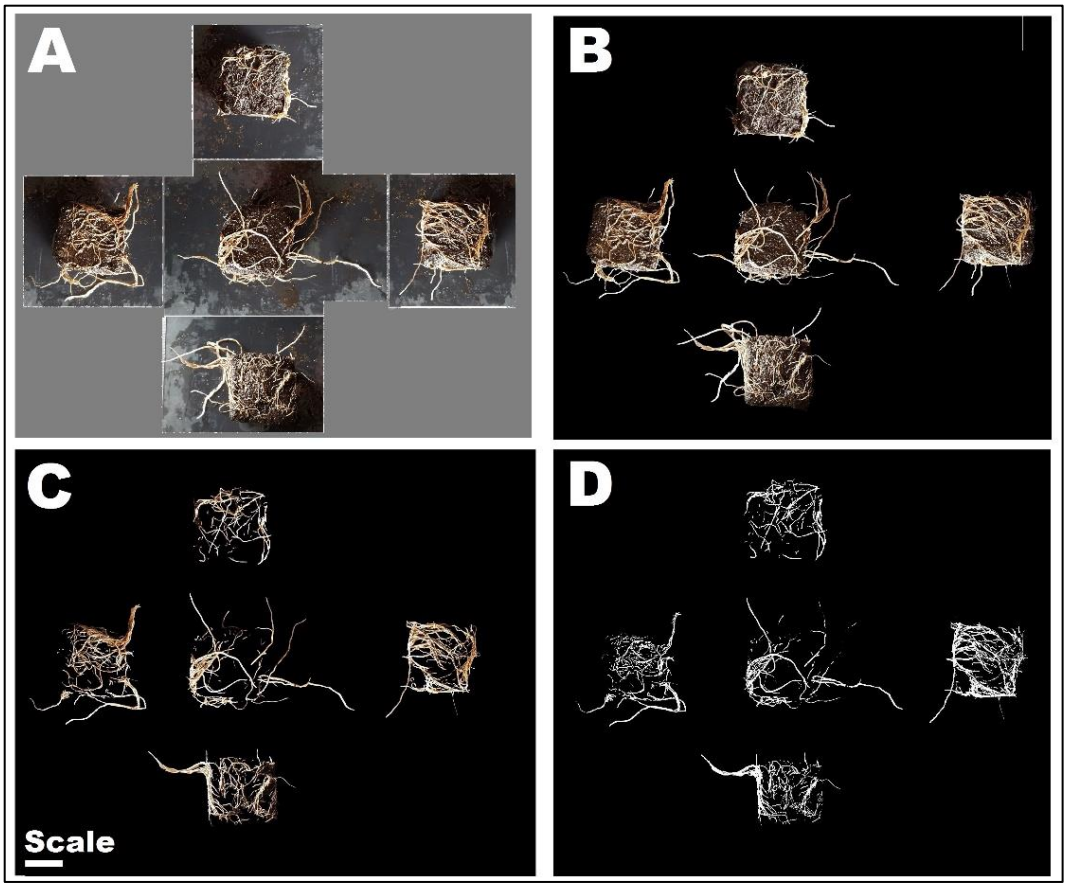

Figure 7.3: Root area analysis with ImageJ.
The five faces of the peat block were imaged (A) and the background removed (B), before then removing the peat block and any roots that did not originate from that face (C). the images were then made binary and the white content of the image measured (D). Scale bar = 20 mm.

7.3.4 Statistical analysis.

To calculate the recovery phase, the mean root:shoot ratio at day 7 (d7) for each line in the pruned treatment was compared to the mean root:shoot ratio for the same line at day 0 (d0) in the pruned treatment. Significant difference between treatments and individual lines was identified using ANOVA and multiple comparisons were made using Student's T test. All statistical analyses were done using Genstat.

7.4 Results.

The rapid rooting RILs that were selected for scoring high for all three of the "rapid rooting" phenotype traits; PRL, TLL and TNL from the growth pouch assay in chapter 5 were RILs 5, 35, 42, 66 and 87. The slow rooting RILs that had consistently low scores for all three traits were RILs 41, 73, 100, 111 and 114.

7.4.1 Shoot growth d0.

There was a highly significant difference ($P<0.001$) across lines at d0 for shoot area with commercial B (14895 mm^2) being the largest, which was significantly higher than all lines with the exception of commercial C and RIL 87 (12908 mm^2), which had a significantly larger shoot area than all RILs except RILs 66 and 5. The RIL 114 (5140 mm^2) had significantly lower shoot area at d0 than all other lines (Figure 7.4).

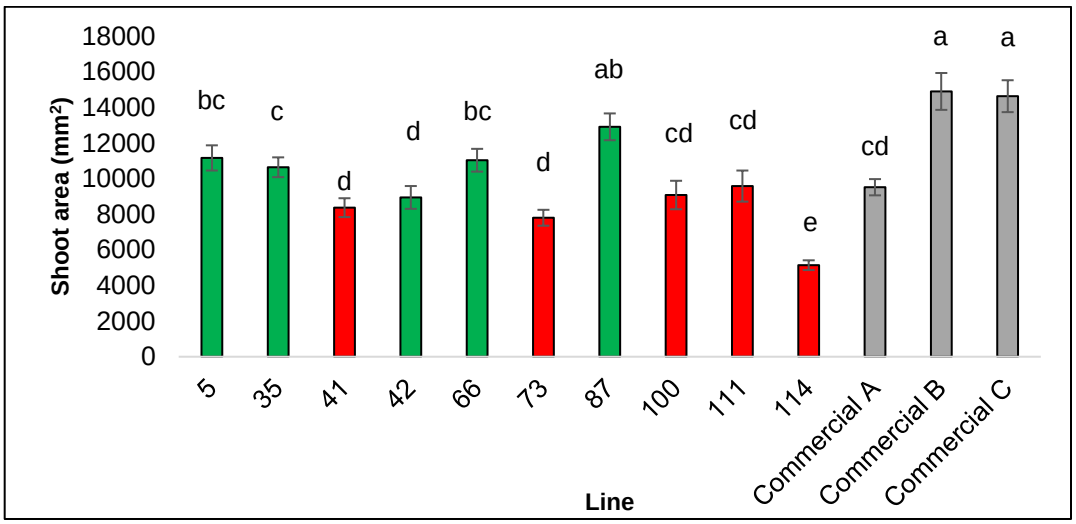

Figure 7.4: Shoot area at d0 for the mapping population RILs and commercial varieties (n=20). Error bars are SEM.
Green bars are rapid rooting lines from growth pouch assay. Red bars are slow rooting lines from growth pouch assay. Grey bars are commercial lines.

7.4.2 Shoot growth d0-d7.

There was a significant difference (P=0.018) for shoot area increase d0-d7 in the unpruned treatment across all lines. RIL 73 had the greatest shoot area increase of 14634 mm^2 over the 7-day period and was significantly greater than RILs 114 and 111 and commercial A. Commercial A had the least shoot growth between d0 and d7 in the unpruned treatment with a total increase of 10789 mm^2 (Figure 7.5). Between d0 and d7 in the pruned treatment, there was no significant difference (P=0.186) identified for shoot growth across the lines. RIL 66 had the greatest shoot area increase of 10780 mm^2 (Figure 7.5).

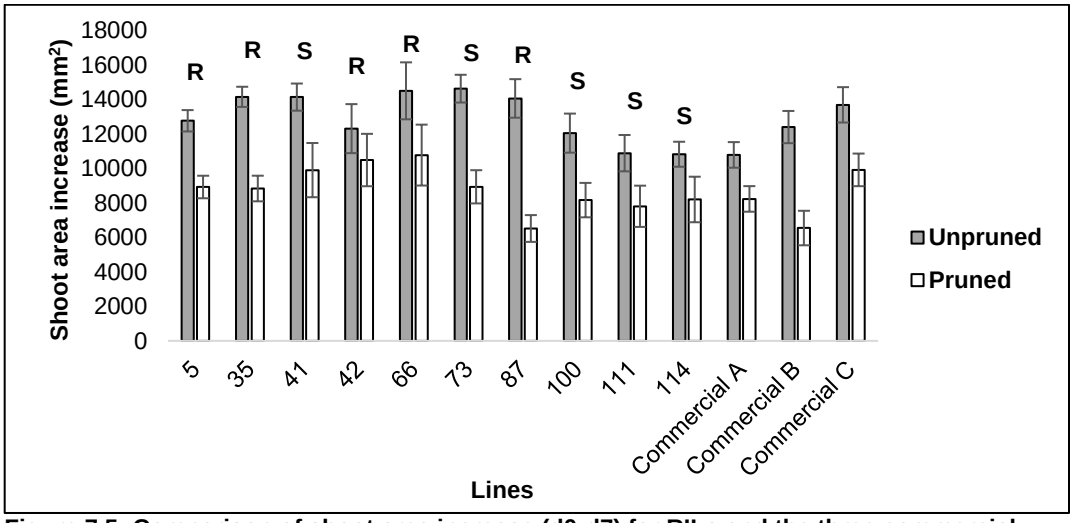

Figure 7.5: Comparison of shoot area increase (d0-d7) for RILs and the three commercial varieties in the pruned and unpruned treatments (n=10). Error bars are +/- 1 SEM.
Data labels represent the rapid rooting (R) and slow rooting (S) extreme RILs from the growth pouch assay.

7.4.3 Root growth d0-d7.

There was a significant difference (P=0.001) found across all lines for root growth between d0 and d7 in the pruned treatment. The RIL 35 had the greatest root growth (2235 mm^2), which was significantly greater than RILs 5, 73 and 100 and commercial A. RIL 114 had the lowest root growth with a mean of 1019 mm^2 (Figure 7.6).

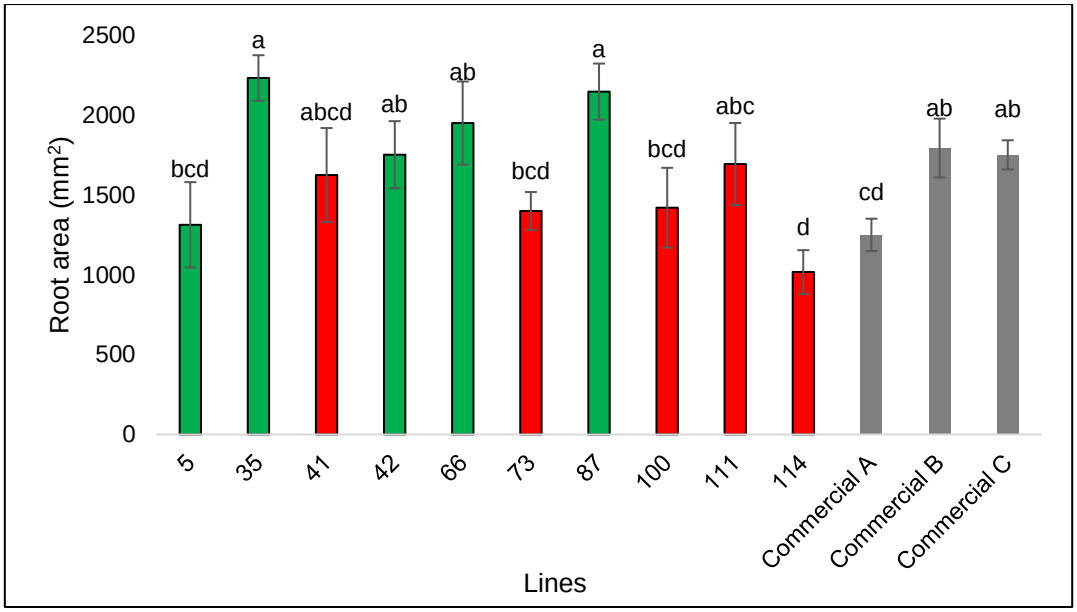

Figure 7.6: Comparison of root growth (d0-d7) for RILs and the three commercial varieties in the pruned treatment (n=10).
The red bars represent the slow rooting RILs identified in the growth pouch assay. The green bars are the rapid rooting RILs, the grey bars are the commercial lines. Error bars are SEM.

7.4.4 Root:shoot comparisons.

There was no significant difference (P=0.342) in the root:shoot ratio across lines at d7 in the unpruned treatment with the ratio ranging from 0.15 – 0.2, however there was a high significant difference (P=0.002) in the root:shoot ratio across all lines when considering total root growth of the unpruned treatment (Figure 7.7).

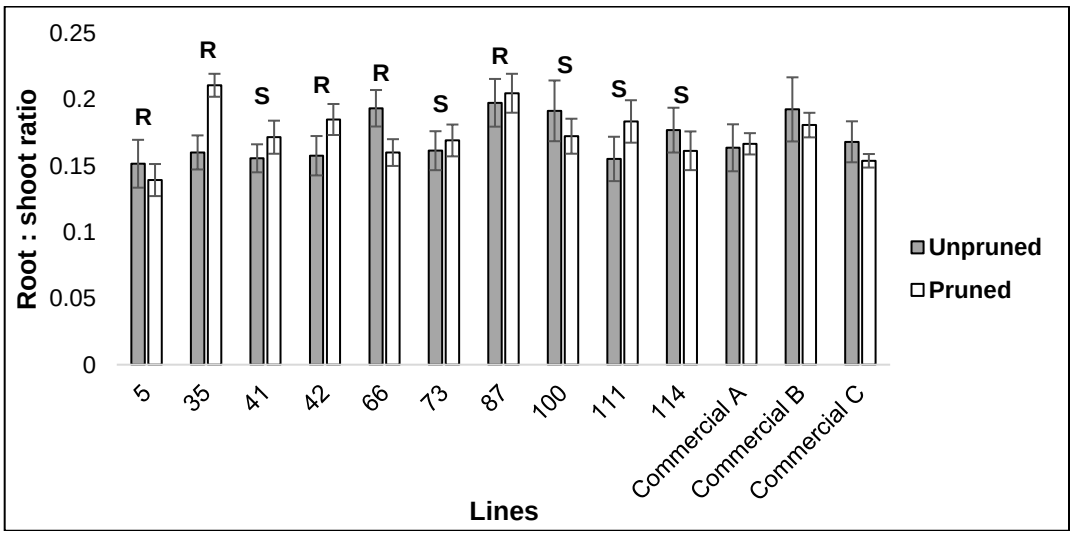

Figure 7.7: Root:shoot ratio for total growth over the experiment for pruned and unpruned treatments (n=10).
The data labels represent the extreme rooting lines from the growth pouch assay. Rapid rooting RILs (R) and slow rooting RILs (S). Error bars are SEM.

There was a significant difference (P=0.006) across all RILs for root:shoot ratio recovery phase completion. RIL 87 was the closest to recovering the root:shoot ratio at d7 to that at d0 in the pruned treatment with a mean of 63.1% recovered, which was significantly higher than those of RILs 5, 41, 42, 100 and 114 along with commercial A and commercial C. RIL 35 had the second largest recovery, recovering 57.7% of the root:shoot ratio of that of d0 pruned (Figure 7.8).

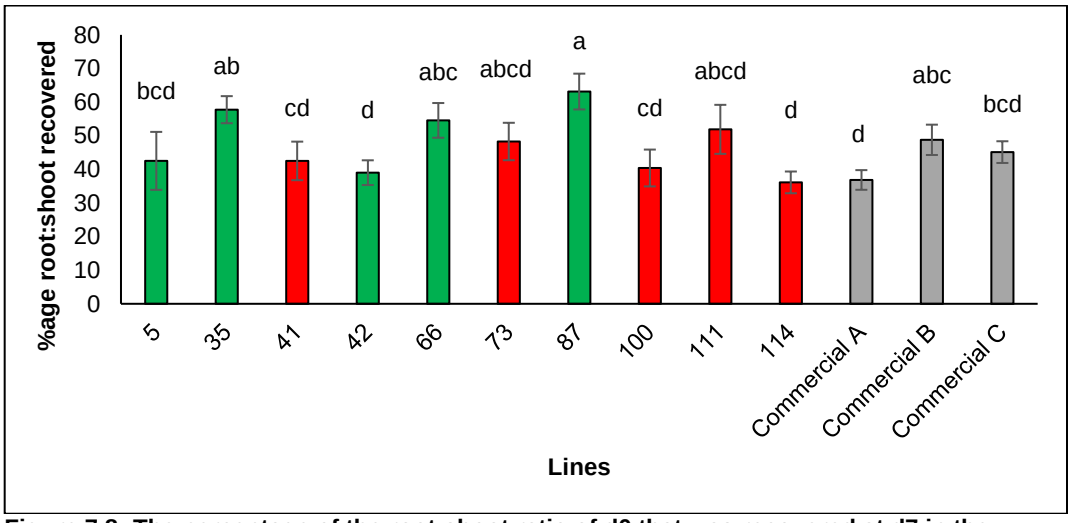

Figure 7.8: The percentage of the root:shoot ratio of d0 that was recovered at d7 in the pruned treatment (n=10).
Green bars are the RILs identified a srapid rooting in the growth pouch assay.The grey bars are the commercial lines. The red bars are the slow rooting RILs. Error bars are SEM.

There was a highly significant difference between the root:shoot ratio at d7 in the unpruned treatment compared with the root:shoot ratio at d7 in the pruned treatment observed in RIL 35 (P<0.001), RIL 87 (P=0.005) and commercial B (P=0.022) (Figure 7.9).

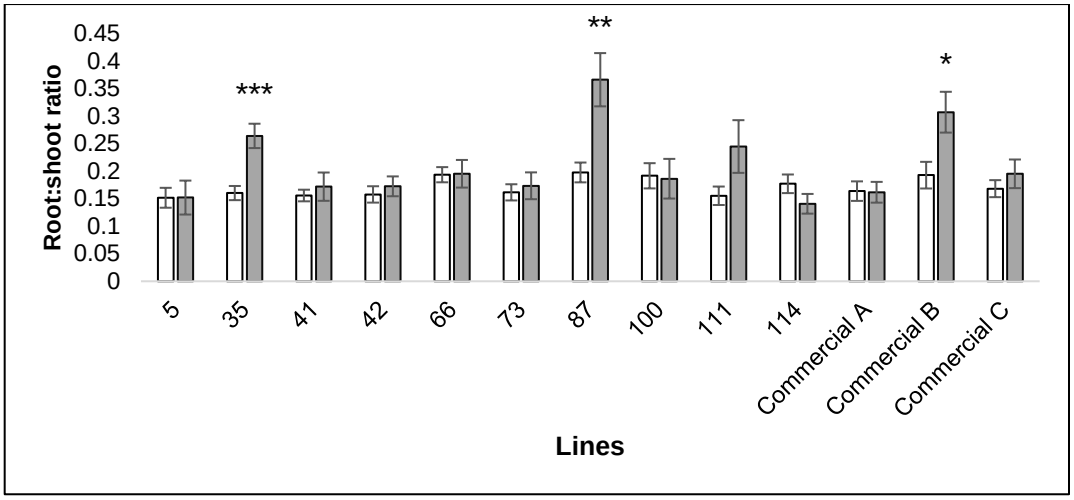

Figure 7.9: Mean of the root:shoot ratios of growth in lettuce transplants 7 days after transplanting in the pruned and unpruned treatments (n=10).
Comparison of growth allocation following transplanting. The open bars represent the mean of the root:shoot ratios after 7 days in the unpruned treatment. The grey bars are the mean of the root:shoot growth of the lines between day 0 and day 7 in the pruned treatment. Error bars are SEM. Rapid rooting RIls from the growth pouch assay were RILs 5, 35, 42, 66 and 87. Slow rooting RILs were RILs 41, 73, 100, 111 and 114 (***=<0.001, **=<0.01 & *=<0.05).

7.5 Discussion.

In this controlled environment study, we compared ten lines of a Saladin X Iceberg RIL population, previously identified as being the extremes for root growth traits, with three current commercial varieties. We pruned the roots from the surface of commercial propagation blocks as a treatment to represent root damage in a commercial planting system. The post pruning root growth was taken as a measure of root growth and hence plant establishment.

Seven days after pruning treatment and transplanting, root re-growth showed genotypic variation and the previously identified rapid rooting RILS from the growth pouch assay (chapter 5), RILs 87 and 35 had significantly greater root re-growth than the RILs 114, 5, 73 and 100. This indicates that RILs 87 and 35 maintained higher root growth than lines 114, 5 and 73 following root pruning. This is further supported as no significant difference across lines was identified for relative root re-growth, i.e. lines that had a greater root area at d0 had recovered a greater root area by d7.

The study identified a difference across all lines at the pre-transplanting stage for shoot area. However, no difference was found among lines for shoot area increase 7 days after root pruning and transplanting in the pruned treatment. This suggests that the shoot growth in all the lines in the 7 days following pruning was relatively similar regardless of the absolute amount of root pruned and re-growth, indicating a rapid rooting phenotype has no

detrimental impact on shoot growth regardless of the larger amount of root that can potentially be pruned at transplanting.

There was a strong relationship between root and shoot growth at d0 where lines with low root areas had low shoot areas and lines with large root areas had high shoot areas. However, significant differences occurred in root:shoot at d0 and d7 and there was no significant relationship between these two (data not presented). It was notable that the commercial lines had some of the lowest root:shoot ratio values suggesting that breeders have selected for visible top growth, at the expense of root growth traits, which would have been an un-noticed trait in high input cultivations, however as pressures to reduce inputs greaten this phenotype may not remain un-noticed.

The RILs 35 and 87 previously identified has having the “rapid rooting” phenotype in the growth pouch assay in chapter 5 produced more root growth during propagation compared to some of the other lines, but the removal of this relatively greater growth was not associated with a response in shoot growth relative to other lines. In fact, having a rapid rooting phenotype such as observed in RILs 87 & 35, which recovered a relatively greater proportion of the root:shoot allowed plants to recover from root pruning more quickly by temporarily reducing shoot growth to levels similar to other lines whilst rapidly restoring their root:shoot ratio. This suggests a rapid rooting phenotype has no negative impact on the transplant compared to relatively average rooting lines and would restore the root:shoot ratio more quickly. Further studies, which allow for a longer period following transplanting before destructive analysis are required to establish if a higher shoot growth phenotype was subsequently restored once the root:shoot ratio was fully recovered.

A potential mechanism to explain the different rooting phenotypes observed is GA and auxin mediated root growth. Auxin mediated in the shoot apex regulates Gibberellin (GA) in the root system. GA controls the concentration of several root growth inhibitors, known as Della proteins. By reducing the levels of these proteins lateral root initiation can take place (Fu & Harberd, 2003). Auxin is transported into the root cap from the shoot apex by the auxin transport protein, AUX1 expressed in the membrane of protophloem in the root system and the hormone accumulation in the root cap is involved with growth and root gravitropism (Swarup *et al*., 2001). The root pruning of the root tips in this study would lead to auxin accumulating in the remaining root tissue, increasing GA abundance, and leading to a reduction of Della protein expression, increasing lateral root initiation.

The Differentially expressed genes identidied in chapter six could also explain some of the differences seen in the pruning treatment. ACA7 and ACA8 were found to be upregulated in the rapid rooting RILs and the Iceberg parent compared to the slow rooting lines. The ACA7 and ACA8 calcium ATPases would allow the plants to respond quicker to the stress of root pruning as it has been found that during stress responses cytosolic calcium levels increase (Tuteja & Mahajan, 2007). This faster stress response coupled with the ability to upregulate the expression of genes involved with cell proliferation and elongation (as mentioned in chapter 6) would allow of the faster recovery of the root:shoot ratio, although differential expression studies on pruned roots would need to undertaken to identify if this is the case.

A further explanation could be the genetic variation in the expression of transcription factors involved with root cell proliferation and elongation, such as the BREVIS RADIX (BRX) transcription factor, which Mouchel *et al*., (2004) identified to have natural variation in a wild population of *A. thaliana* and contributed to increased root growth. Further studies would be needed to test these hypotheses and gain a better understanding of the genetic mechanisms involved.

It is proposed that if a transplanted lettuce crop had a rapid establishment phenotype, the act of root pruning may further alter the root architecture. The study by Biddington and Dearman (1984), observed an increase in lateral root initiation and root emergence following pruning of the root apex. A rapid rooting phenotype could utilise soil water and nutrients more efficiently, enabling reduced irrigation of the crop, reducing associated increases in soil salinity in some production systems. The same could also apply in a situation of mild phosphate deficiency in the soil where an increase in the number of shallow lateral roots of a plant would increase the acquisition of phosphate (Peret *et al*., 2014).

This study highlights that it may be possible to select for rapid rooting traits in transplanted crop systems. Breeding for a rapid rooting phenotype could also aid establishment of transplants raised in the smaller propagation cells now being used in automated transplanting systems of lettuce (Kerbiriou *et al*., 2013). It has been reported that smaller propagation modules/blocks lead to increased risk of transplant shock in lettuce (Gianquinto, 1991; Nicola & Cantliffe, 1996). Lines associated with a fast recovery phase of the root shoot ratio, such as that seen in RILs 35 and 87 after seven days could be beneficial in the new, small volume systems. Further work will be needed to establish if a rapid rooting phenotype benefits plant establishment in these systems.

7.6 Conclusion.

We have demonstrated in a controlled environment study that the rapid rooting trait in the lettuce lines studied was under genetic control. Significant genotypic variation was observed between lines in response to root pruning which could be a target for breeding programmes. Root pruning caused phenotypic variation with some of the selected Saladin x Iceberg RILs studied having a relatively high root growth rate and reduced shoot growth, whilst others showed a tolerance to root pruning with no reduction to root or shoot growth. This was not the case in the commercial cultivars, which all showed a reduction to both root growth and shoot growth following root pruning.

The rapid rooting trait in seedlings was maintained in transplants following root pruning and two RILs (35 and 87) displayed an increased rooting phenotype and recovered the root area post pruning more quickly than lines that did not possess the trait. The rapid rooting phenotype was associated with a reduced recovery phase in RILs 87 & 35, which recovered a greater relative amount of the root:shoot respectively than the other lines.

The rapid root growth trait is potentially of value in reducing establishment time in transplanted lettuce crops. The results of this study demonstrate that the Saladin x Iceberg RIL population will be useful to identify the genetic factors (QTL) that underlie the phenotype allowing breeders to select more efficiently for the rapid rooting trait.

8 General discussion.

The aims of the work in this thesis were to:

a) Quantify phenotypic variation in rapid rooting traits in seedlings of a lettuce diversity fixed foundation set (DFFS) consisting of 96 genetically fixed lines representing the entire gene pool of the *Lactuca* spp. (*Lactuca sativa*, *Lactuca serriola*, *Lactuca saligna* & *Lactuca virosa*).
b) Identify quantitative trait loci (QTL) and the associated flanking DNA markers associated with rapid rooting traits.
c) Utilise a targeted transcriptomic approach to identify candidate genes under the QTL that may contribute to the phenotype.
d) Identify if the rapid rooting phenotype observed in extreme lines of seedlings was maintained at the transplanting age
e) Establish whether the rapid rooting phenotype hastens recovery of the root:shoot ratio in a transplant system.

The first aim of the work was to optimise a high through-put assay for the use with lettuce seedlings. The 2D high through-put phenotyping assay was successfully applied to identify phenotypic and genotypic variation in the lettuce DFFS and the intra-specific mapping population. Between two studies over 1300 seedlings across two tanks in two single runs highlighted the high through-put capabilities of the assay. The assay could easily be used to quickly phenotype rooting traits from a very high number of Genebank accessions for lettuce spp. where the Centre for Genetic Resources, The Netherlands (CGN) has over 2500 lettuce accessions alone (van Treuren & van Hintum, 2009).

The study did not provide an answer to why lettuce did not grow well on the standard cassette that has successfully been previously used for *B. napus* and wheat in other studies. What was interesting was the fact pre-soaking the cassette increased root growth, which was most likely due to nutrient or water availability, although unpublished data showed a difference of only around 10% moisture content between the top and bottom of the anchor paper when the solution was simply allowed to diffuse as described in other studies (Atkinson *et al*., 2015; Thomas *et al*., 2016a,b; Wang *et al*., 2017b; Kenobi *et al*., 2017).

The study was able to quantify phenotypic variation in the lettuce DFFS for traits associated with what was termed in this work as a “rapid rooting phenotype”. The work identified significant phenotypic variation for rooting potential within the lettuce DFFS. Of all the lettuce seedlings observed across two experiments using the 2D high through-put phenotyping assay, the DFFS accession CGN04628 (cv. Kakichisha White) had by far the greatest growth for all three of the traits associated with the rapid phenotype (primary root length, total lateral root length & total number of lateral roots). What would be interesting from a breeding perspective would be to see how this line performs in a field experiment that can be treated with mild drought and/or nutrient deficiencies after transplanting compared with other cultivars to compare the phenotype with the work completed here. Kakichisha White was described by van Treuren & van Hintum, (2009) as being of Japanese origin molecular analysis grouped the accession with the stalk lettuce, although phenotyping suggested both stalk and cos characteristics.

Kakichisha White is a *L. sativa* spp. and introgression of the rooting traits from this line could have significant potential in improving lettuce yields under reduced inputs. The improvement of yields through breeding varieties with greater rooting potential has been accomplished before and was the case in “super rice” lines that were found to have higher root length density, which was contributing to the increased yields (Zhang *et al*., 2009). In a study by Uga *et al*., (2013) the yield of rice was improved in mild drought conditions through the introduction of the DEEPER ROOTING 1 gene, which promoted deeper rooting potential in rice and a larger root density at lower soil profiles improved seed yield in chick pea varieties in terminal drought conditions (Kashiwagi *et al*., 2006). Within the lettuce DFFS deeper rooting potential was identified in the wild *Lactuca* accessions compared to the *L. sativa* accessions, which corroborated with observations that Johnson *et al*, (2000) reported. The fact a deeper rooting potential exists in *Lactuca* offers breeding programmes the opportunity to breed lettuce cultivars with the ability to access water in times of mild drought with a reduced impact on yields, particularly at the establishment phase of crop development.

The extreme lines of the DFFS were found to segregate in a 3D sand assay in a similar pattern to the seedling assay, although to a lesser degree. What the study failed to report was the compaction level of the sand in each pot and whether that had any effect on the root growth of the lines. Substrate compaction has been shown to impact root growth of maize seedling roots (Iijima *et al*., 2000). It has also been shown through X-ray CT that the plasticity of the root system architecture in rice is influenced by substrate type and individual

lines respond in various ways (Rogers *et al*., 2016). This needs to be considered when comparing lettuce extreme in other conditions. What would be very interesting to see is how the extreme lines of both the lettuce DFFS and mapping population perform in a 3D platform that would allow non-destructive analysis of the root system in soil instead of sand, such as the X-ray CT platform, which would allow analysis of the root system in controlled conditions with reduced nutrient inputs and even soil compaction (Tracy *et al*., 2010; Tracy *et al*., 2012a,b).

The study in this work was able to identify phenotypic variation for root hair traits in the lettuce DFFS. This is the first known research in variation of root hair development in *Lactuca* spp. and offers an encouraging area of future research to identify accessions that may have improved uptake efficiency. Interestingly, the study identified accessions that produced relatively long root hairs at a low root hair density (*L. sativa* accessions) or short root hair length at a high density (wild *Lactuca* spp. accessions), but both traits were not observed together in any accession. This is in line with the findings by Wang *et al*., (2004) who found a negative correlation between root hair density and average root hair length in a soybean mapping population. The identification of these differences offers the chance to develop two inter-specific mapping populations for the segregation of root hair traits, one for root hair length and a second for root hair density.

An important point for a breeding programme is the identification of which, if any, of these traits increases the uptake efficiency of phosphorus in deficient soils for lettuce as studies in various crops have suggested that root hair length is responsible (Zhu *et al*., 2005; Gahoonia & Nielsen, 2004; Zhang *et al*., 2018). Others have suggested root hair density has no affect (Brown *et al*., 2013). Others report root hair density is responsible (Wang *et al*., 2004) or that both phenotypes are beneficial (Miguel *et al*., 2015), while an earlier study suggested neither phenotype increased uptake efficiency (Bole, 1973). The contrasting importance of root hair length and density on P acquisition in P deficient soils could be species specific. In addition, the interaction of mycorrhizal fungi within the rhizosphere and their role in phosphorus acquisition, which has been shown to be beneficial in the case of lettuce (Azcón *et al*., 2003) needs to be considered and will have been absent from the work carried out in this thesis.

This study identified QTL and DNA markers associated with rooting traits that make up the rapid rooting phenotype (PRL, TNL & TLL) along with further ratios of the three traits (LRND, LRLD & MLRL). It is important for plant breeders to identify if any of the QTL

identified in this work were stable across different environmental conditions. Further work should focus on analysis of the mapping population in a 3D assay, such as the sand assay used to analyse the segregation of the DFFS extreme lines. The work was limited to a choice of two mapping populations and if the QTL were not found to be stable a mapping population with greater segregation would be more promising. Kakichisha White has the greatest potential for rapid rooting and creating an F_2 mapping population with this accession and one of the poorer rooting lines, such as the parent line used (cv.Salinas) in this work for the QTL analysis, should be straight forward and this mapping population would interest breeders more as they would select for the greatest phenotypic difference to develop a mapping population.

The transcriptomic study in this work was able to identify several candidate genes associated with cell proliferation, cell expansion, ABA synthesis and cell wall synthesis through a combination of QTL mapping and transcriptomics approach. This approach has been used previously to identify a wound inducible gene associated with drought tolerance in rice (Patil *et al*., 2017). One issue that must be acknowledged is the fact the QTL identified in this work cover large genetic distances and identifying accurately the candidate genes associated with the QTL is a relatively low confidence approach (Norton *et al*., 2008).The QTL identified in the study need to be fine mapped prior to combining with a RNA-seq approach to minimise the chance of type I errors. This approach was used to identify a nodulin 26-like intrinsic gene that regulates boron deficiency in *B. napus* (Hua *et al*., 2016). To remove any chance of a type I or type II error in the absence of stable QTL across environments the whole transcriptome should be analysed. The approach used in this study allowed a focused search of differentially expressed genes and BLAST analysis of conserved domains within those genes as the lettuce genome is still not very well annotated compared to other model species. Future work should be considered to analyse the entire transcriptome results from the study to ensure important differentially expressed genes are not missed, which could aid the identification of homologous genes responsible for rapid root growth in other field grown transplanted crops, such as cabbage, broccoli, cauliflower and brussels sprouts or glasshouse grown transplanted crops such as tomato and bell peppers.

The transcriptomic study in this work pooled the total root tissue in the RNA-seq work. A further suggestion for future work would be to look at individual regions of the root system in the high and low rooting lines of the DFFS to gain a better understanding of the pathways responsible for the rapid rooting trait. It is clear that both cell proliferation and cell elongation are contributing factors to a rapid rooting phenotype (Beemster & Baskin, 1998),

but it is not known if the rapid cell division and rapid elongation are reliant on the expression of genes in other associated pathways i.e. is the rate of cell elongation governed by the expression of genes involved with cell wall synthesis or do genes involved with cell expansion directly cause the upregulation of genes involved with cell wall synthesis. From a breeding perspective the DNA markers associated with a beneficial rooting trait are the key information that give a competitive edge and are understandably more important than understanding the molecular processes behind the trait.

Fine mapping of the QTL regions associated with the rapid rooting phenotype identified in this study would allow for the development of a mapping population that segregates for markers at these alleles, which would allow for the analysis of the percentage of the rapid rooting trait contributed by the allele. Another interesting approach would be to analyse the DFFS line Kakichisha White to identify if any of the candidate genes identified in the study are up or down regulated to a relatively greater extent compared to the intra-specific mapping population parents and RIL extremes tested here.

Fertilizer inputs at some point in the future will need to be reduced and it has been stated that P input alone as fertilizer would need to be reduced by approximately 40% in European regions to have a zero imbalance in soils and rivers (Withers *et al*., 2020). Breeding varieties, not just in lettuce that have a root system with the ability to explore the soil profile and acquire nutrients from a deeper depth could help towards accomplishing this, without a negative impact on yields, although this relationship will need demonstrating in further work.

The transplant assay in this work was able to observe that within the extreme RILs of the mapping population the phenotype identified in the seedling assay was maintained in some of the lines, but not all at transplant maturity. Some lines had greatly reduced shoot growth to recover the root:shoot ratio, other lines appeared to have lost the phenotype altogether and some lines did not have much reduction in shoot growth following root pruning. The study by Kerbiriou *et al*., (2013) identified the cv. Nadine had a relatively smaller root system than other cultivars but a greater shoot dry weight and suggested resource use efficiency could be the explanation. This could explain the phenotype observed in these RILs in the study as root pruning did not influence shoot growth perhaps the uptake and use efficiency, is greater in these RILs or the root pruning triggered an increase in use efficiency. Further studies would be needed to fully investigate if this phenotype exists in these RILs.

The study showed that a rapid rooting phenotype led to greater root growth from the transplant block prior to transplanting, which would inevitably lead to an increase in root pruning at the point of transplanting at the suggested maturity of 5-7 leaf stage. The RILs that maintained the rapid rooting phenotype were closer to restoring the root:shoot ratio regardless of having more root pruned. This suggests a rapid rooting phenotype has no negative impact on the transplant compared to relatively average rooting lines and would restore the root:shoot ratio more quickly. The study by Kerbiriou *et al*., (2013) suggested that underdeveloped transplants (at the 3-leaf stage) were the most affected by transplant shock at transplanting. Kerbiriou *et al*., (2013) also observed in one field experiment the underdeveloped transplants had reduced N capture which correlated with reduced root length density, it would be intriguing to see how a rapid rooting phenotype line, such as Kakichisha White performs as a transplant at an earlier developmental stage and if this reduced the time spent in the glasshouse of plant raisers, or how lines with the phenotype perform in the Plant Tape system with a greatly reduced container. As with the sand seedling assay already mentioned further work should be undertaken to identify if the rapid rooting phenotype of the extreme RILs is maintained in soil.

8.1 Conclusion and further work.

In conclusion, this thesis has:

a) Quantified phenotypic variation in rapid rooting traits in seedlings of a lettuce diversity fixed foundation set (DFFS) consisting of 96 genetically fixed lines representing the entire gene pool of the *Lactuca* spp. (*Lactuca sativa, Lactuca serriola, Lactuca saligna* & *Lactuca virosa*).

b) Identified quantitative trait loci (QTL) and the associated flanking DNA markers associated with rapid rooting traits in a mapping population derived from the intra-specific Saladin x Iceberg cross.

c) Utilised a targeted transcriptomic approach to identify nine candidate genes under five of the 16 QTL that may contribute to the phenotype.

d) Identified that the rapid rooting phenotype observed in extreme lines of seedlings was maintained at transplant maturity in some of the extreme lines analysed.

e) Established that the rapid rooting phenotype hastens recovery of the root:shoot ratio in a transplant assay in the extreme lines that maintained the phenotype following root pruning.

The work in this thesis has identified phenotypic variation in *Lactuca* spp. for rooting traits including a rapid rooting phenotype. The work has identified QTL, and DNA markers

associated with a rapid rooting phenotype along with candidate genes. The rapid rooting phenotype offers breeders great potential to breed commercial lettuce cultivars that could, although not shown in this work, hasten establishment in the field and allow quicker access to water and nutrients under a reduced input and/or mild drought condition. Breeding for improved rooting traits in lettuce could help lettuce producers maintain yields against pressures of the future to reduce inputs. Further work is needed to understand the phenotype more closely.

8.2 Further work.

Firstly, the transcriptomic analysis in this work needs to include all the genes differentially expressed. Secondly, the QTL need to be fine mapped to identify markers for individual genes associated with the trait or a new mapping population that includes Kakichisha White developed and thirdly, field experiments with the potential breeding material, which must include Kakichisha White should be undertaken to identify any benefits of the phenotype. Only when these issues have been addressed could a breeding programme have the confidence to use marker assisted selections for a usually hidden trait.

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