Quantifying physiological differences between *Brassica napus* cultivars to identify traits to improve phosphorus uptake.

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Abstract

Phosphorus is an essential macronutrient for plants however many soils are phosphorus deficient. This leads to large scale application of phosphorus fertilisers which are non-renewable. An alternative to increasing phosphorus application would be to breed plants that are more efficient at taking up phosphorus from the soil. *Brassica napus* is the source of the second most produced vegetable oil worldwide and the primary source of edible vegetable oil in the European union and China. Its importance globally makes it important to future-proof it against declining availability of phosphorus fertilisers, this could be achieved through breeding new, more phosphorus efficient varieties.

Previous element analysis work done on winter *Brassica napus* from the Renewable Industrial Products from Rapeseed (RIPR) diversity population of inbred *Brassica napus* lines showed that there was a wide variation in phosphorus content of the leaf tissues. It was thought that root architecture may play a role in phosphorus acquisition and that characteristics could be identified that correlate to increased phosphorus acquisition. The aim of this work was to identify physiological characteristics that correlate to improved phosphorus uptake in different *Brassica napus* cultivars. 6 *Brassica napus* accessions were chosen to be screened, three with low phosphorus in their leaf tissues, Prince, Caramba and Gefion and three with high phosphorus, Pacific, Montego and Musette. The varieties were screened for root architecture and anatomical traits that correlate to phosphorus uptake using the pouch and wick growth system, hydroponic growth, histological sectioning and element analysis.

The pouch and wick and hydroponic experiments showed that the varieties with high phosphorus in the RIPR population correlated to an increased number of lateral roots, a higher root biomass and decreased convex hull area. These results indicate that root architecture is linked to increased phosphorus uptake in these varieties. These results could be used as the basis for future work that expands the number of accessions and root architecture traits studied to further quantify the role of root architecture in phosphorus acquisition.

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Musette (blue) had significantly total longer lateral root length than the low lines Prince, Caramba and Gefion (yellow)(t-test p=0.012).

Additional one-way ANOVA analysis indicated that there was a significant difference between some of the individual varieties. Post hoc analysis showed that Montego (B) was significantly different from the other varieties (A) but that there were no other significant differences between individual varieties.

Figure 26 - Figure showing the total root length of *Brassica napus* varieties grown on ¼ strength MS media. The t-test showed that there was a significant difference in total root length between the low (yellow) and high (blue) varieties (p=0.009) with the high phosphorus varieties having significantly longer roots than the low varieties

There was however no significant difference between any of the individual varieties of *Brassica napus*

Figure 27- Shows the Convex hull area (mm^2) of 6 varieties of *Brassica napus* when grown with a ¼ strength MS media treatment. A t-test showed that the convex hull of the low (yellow) varieties had a significantly lower area than that of the high (blue) varieties (t-test p=0.001).

Post-hoc analysis using an ANOVA showed that there were significant differences between some of the individual varieties (p=0.009). Pacific (B) had a significantly larger convex hull area compared to the other varieties.

Figure 28- shows the maximum width of the roots of 6 varieties of *Brassica napus* when grown with a ¼ strength MS media treatment. The two groups, low (yellow) and high (blue) were significantly different when compared using a t-test (p=0.004) with the high group having roots that spread significantly wider than the low group.

Further analysis with a one-way ANOVA showed that there was a significant difference in the width of root spread between the individual varieties (p= 0.017). Post hoc tests revealed that Montego (B) has significantly wider roots than Caramba, Gefion and Musette (A*). The width of the roots wan not, however,

3.86mg/L p=0.042, 7.72mg/L p=0.027, 15.45mg/L p=0.003). The high group had significantly greater leaf biomass than the low group in all of the treatments

ANOVA and post-hoc analysis also showed differences between the individual varieties (p=<0.001) Prince (A) was significantly different from all except Caramba (A) and Musette (C). Gefion (B) was significantly different from Prince (A) and Musette (C). Pacific (B) was significantly different from Prince and Caramba (A) and Musette (C). Montego was significantly different from Prince and Caramba (A)….133

Figure 48 - t-tests showed that the SPAD values were only significantly different between the two groups in the 15.45mg/L treatment (p=0.045). The high group had significantly lower SPAD values than the low group.

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Chapter 1: Introduction, background and aims.

1.1 The issue with phosphorus

Phosphorus is an essential macronutrient for plants, making up about 0.2% of total plant dry weight (Akhtar et al, 2009). It is a vital element for many key biological processes including cell wall formation, DNA synthesis and ATP production. With an increasing global population putting ever greater demands on agriculture the use of phosphorus fertilisers is increasing (Cong et al, 2020). While estimates of phosphorus fertiliser use are inexact, it is estimated that in the UK farmers applied 90kt of phosphorus per year in the years leading up to 2011 via fertiliser use (Dawson & Hilton, 2011).

Only 15-30% of the applied phosphorus is taken up by plants in the year that the fertiliser is used, which leads to a surplus of phosphorus in 71% of arable land globally (Qin et al, 2022). These high levels of phosphorus application are necessary due to the low efficiency of plants at acquiring phosphorus from the soil. Not only are phosphorus resources being wasted, the overuse of fertilisers can have an adverse effect on the biodiversity of the environment, as well as negatively affecting soil, air and water quality (Tian et al, 2016). A well-known environmental problem is that the run-off from overuse of fertilisers, including phosphorus fertilisers, causes eutrophication which can have a devastating effect on downstream water environments (Lambers & Plaxton, 2015). Agriculture in Britain already contributes more than 12000 tonnes of phosphorus to surface water every year (Hammond et al, 2009). Overuse of different types of fertilisers can also directly damage the crops themselves as it increases the incidence of lodging which reduces the quality and yield of crops, the opposite of the aims when applying fertiliser (Tian et al, 2016).

Not only do fertilisers present a source of pollution but the application of phosphorus fertilisers is also an unsustainable practice. Phosphorus fertilisers are produced using phosphorus containing rocks, which are a non-renewable resource, as such there is a limit to the supply of phosphorus obtained in this fashion. Estimates of phosphorus reserves vary, the International Fertiliser Development Centre (IFDC) estimates that the easily accessible phosphate rock resources will last

for another 300-400 years while other estimates believe that inexpensive sources of phosphorus could be depleted as soon as 2050 (Heuer et al, 2017)(Kumar et al, 2018). While the exact length of time the current resources will last is unknown we can be certain that the phosphate rock reserves will run out and it is therefore necessary to prepare for this. There are several approaches that can be taken to combat the future scarcity of phosphorus. New approaches and technologies are being developed that provide a source of phosphorus without the need for phosphate rocks (Mnthambala et al, 2021). It is however also important to focus not only on the production of phosphorus but also on its usage, therefore, strategies to reduce phosphorus use and extend the lifetime of our current phosphorus reserves need to be developed. As stated a key use of phosphorus is in fertilisers. Potentially increasing crop efficiency at acquiring phosphorus could decrease the need for phosphorus applications and extend the lifetime of the resources available (Wang et al , 2020).

Improving phosphorus uptake may also improve the use of marginal soils and therefore expand the farmland available for use. The nitrogen fixing properties of legumes mean they can be grown in soils with poor nitrogen availability. However, legumes are vulnerable to low P conditions due to the high P requirement for the growth of N2 fixing nodules both for the tissues, phosphorus is particularly required for the plant to synthesis mitochondrial and symbiosome membranes (Sulieman & Tran, 2015). This makes P an important constraint for legume growth especially in marginal soils which could benefit from the nitrogen fixing properties of the plants, improving P uptake may make these soils viable for farming without needing a large input of phosphorus from fertilisers (Sulieman & Tran, 2015). This could be particularly useful to farmers in parts of the world where soil is poor quality and fertilisers are unavailable as it could expand their potential farming land.

To improve phosphorus uptake in plants we need to study how the roots interact with the soil environment, both physiologically and chemically. The work presented in this thesis focuses on *Brassica napus* roots.

1.2 *Brassica napus*

Brassica napus, also known as Oilseed Rape or Canola is grown worldwide with the main production regions being Canada, Europe, China, India and Australia which combined produced over 71.4 million metric tons of *Brassica napus* in 2018 (Zheng et al, 2020). Over the last 20 years the production of rapeseed oil has increased by 2-4 times in the countries that produce it most (Kirkegaard et al, 2018). Rapeseed oil is the second most produced vegetable oil globally after soybean oil (Chew, 2020). Canola is the primary source of edible vegetable oil used in the European Union and China. In addition to its primary use as an edible oil, *Brassica napus* is also used as animal feed and for the production of bio-oil and biodiesel in Europe (Chew, 2020). The by-products of the oil extraction process are also in demand. There are two by-products of oil extraction depending on the method of extraction used; when the oil is extracted from the seeds using a press the by-product is known as a press cakes, if the seeds undergo further oil extraction using a solvent after pressing then the by-product is usually known as meal although there is some ambiguity in the use of both terms (Arrutia et al, 2020). Both of these by-products are high in protein and have been widely used as animal feed, however, since 2010 there has also been increasing demand for the by-products as a source of plant based protein for human consumption (Arrutia et al, 2020). While humans cannot directly eat either the press cakes or meal the protein in both can be isolated using an alkaline extraction process to produce protein products comparable to those extracted from soy beans in both quality and versatility (Singh et al, 2022).

The demand for *Brassica napus* is going to increase going forward due to a number of factors, increasing demand for the edible oil, the hunt for “green” alternatives to fossil fuels and the increasing use of its by-products for both human and animal consumption. Increasing demand means that it is vital that work is done to improve yields and future proof *Brassica napus*, addressing issues with phosphorus uptake is part of this.

1.3 Phosphorus

1.3.1 Phosphorus in soil

How plants interact with phosphorus is a direct result of the chemistry of phosphorus in the soil. While abundant phosphorus may be present in the soil only a small portion of this phosphorus will be biologically active and available to plants. Phosphorus is constantly cycling in the soil, however, there is little net change in the biologically available phosphorus (Stewart & Tiessen, 1987).

What the plant actually takes up is phosphate, the fully oxidised form of phosphorus. It is because of the chemical interactions between phosphate (Pi) and soil that the element is so difficult for plants to take up. The availability of Pi is dependent on many reactions including dissolution and precipitation of P bearing minerals, hydrolysis of organic matter and the adsorption or desorption of phosphate on the surface of soil particles. It is also affected by the composition of the soil, the pH and the presence of competing anions (Akhtar et al, 2009).

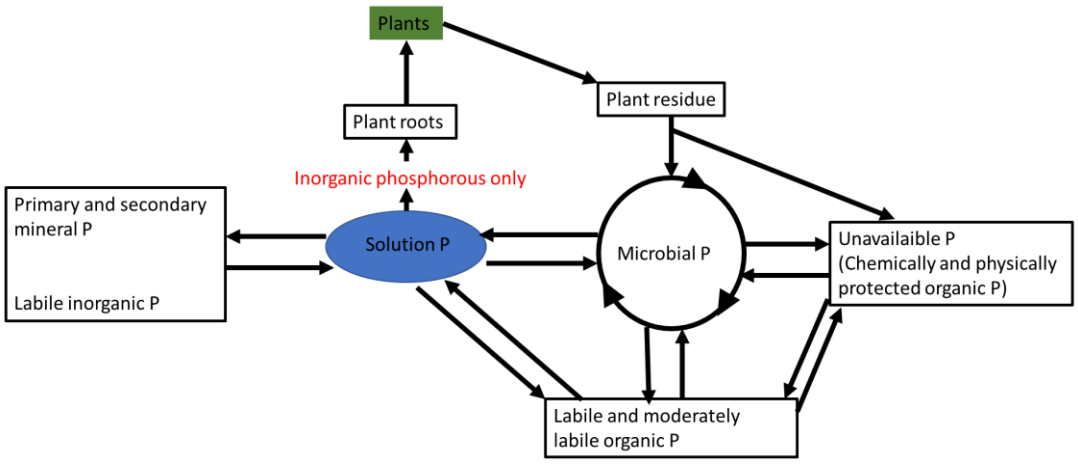

Figure 1: Key interactions in the phosphorus cycle in the soil. Redrawn from Stewart & Tiessen, 1987.

Primary P minerals dissolve slowly which creates phosphate ions that are either in labile, easily broken-down compounds or in solution. These phosphates fall into two categories, organic phosphates which are phosphates of esters or inorganic

phosphates which are salts of phosphoric acid (Stewart & Tiessen, 1987). Plants can only take up inorganic phosphates while microbes can make use of both inorganic and organic phosphate. When, dead plant matter, manure or other fertiliser is added to the soil some of the phosphorus in the manure becomes inorganic phosphorus in solution making it available to plants (PhosphorusDynamics | UNL Water,) Not all of the phosphate ions are taken up by plants or microbes, some precipitate into secondary minerals which may then be broken down again and become soluble (Stewart & Tiessen, 1987).

Phosphate ions also become stabilised and therefore unavailable to plants by interacting with minerals in the soil. Compounds which contain iron, aluminium or calcium can have phosphorus form a thin layer on the surface of the compound in a process called adsorption. pH plays a key role in determining the bonding of adsorbed phosphorus in the soil, phosphorus can be fixed by aluminium or iron in acidic soils or by calcium in alkali soils (Lyu et al, 2016). Figure 2 shows the established view of how pH affects phosphorus solubility in the soil. As pH changes the relative levels of phosphorus fixation by key elements in the soil also changes and on average phosphorus is most abundantly available at a pH of approximately 6.5 (Penn & Camberato, 2019). There are exceptions to this to this rule of thumb depending on the balance of phosphorus sorption mechanisms in the soil, however in general this rule applies in most situations (Penn & Camberato, 2019).

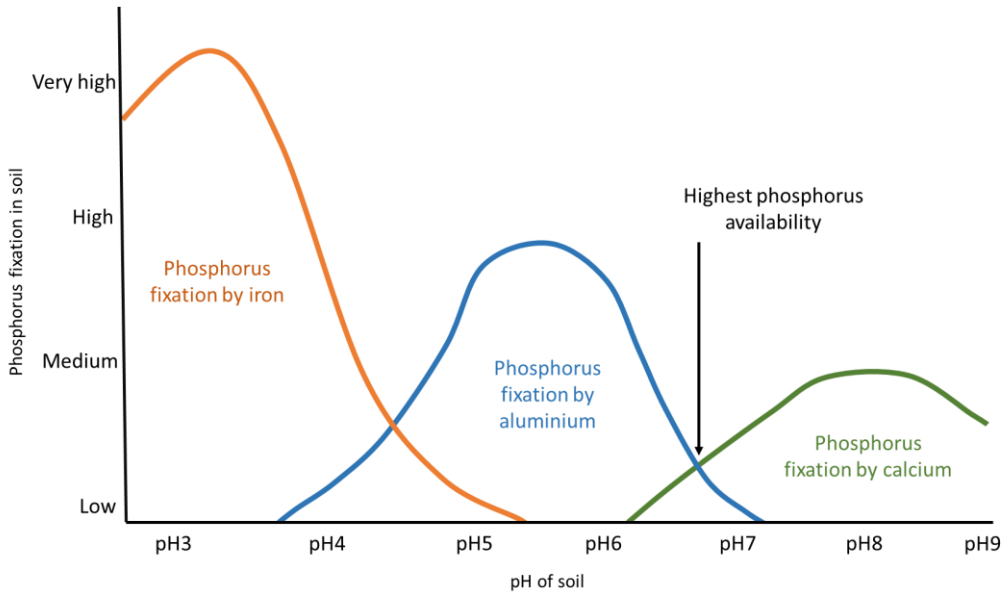

Figure 2: shows relative phosphorus fixation in the soil in by three key elements and how this is affected by the pH of the soil. On average phosphorus is usually most available at a pH of about 6.5 when it is least bonded to any of these minerals although there are exceptions to this. Redrawn from (Penn & Camberato, 2019).

The many interactions involved in the phosphorus cycle mean that only a small percentage of the phosphorus present in soil is available in a form that plants can make use of at any given time. The interactions between phosphorus and other elements in the soil also mean that phosphorus is not very mobile in soil, this means that plants have to actively seek it out.

In general the topsoil strata have a greater amount of phosphorus than the subsoil strata as the topsoil is enriched with phosphorus from dead plant matter, and in the case of farmland from manure or other fertilizer added to the soil (Lynch, 2011). Because of the immobile nature of phosphorus, it remains in the topsoil.

1.3.2 Phosphorus in plants – an overview of roots

Due to their sessile nature plants have to be able to respond to the environment around them. Roots are an excellent example of the high level of developmental plasticity shown by plants. This plasticity in plant growth means they can change their root growth patterns to respond to their environment including responding to water availability, nutrient levels, mechanical stresses from the soil and interaction with other organisms (Péret et al, 2014).

As phosphorus is relatively immobile in the soil, plants have to forage for it and as such root show a highly plastic response to phosphorus levels in the soil (Williamson, 2001). Nutrient heterogeneity is not only an issue of where in the soil the nutrients are but also when they are available, as such plants have developed mechanisms that allow them to respond to nutrient availability both in root establishment and in later growth (Rogers & Benfey, 2015).

Root systems are made up of several root types which have specific functions within the overall root architecture. The root types can be separated into embryonic and post embryonic roots. Primary roots are embryonic roots formed at the basal pole of the embryo. Monocots often also have seminal roots which are formed at the scutellar node, however not all species have seminal roots for example maize does but sorghum and rice do not (Rogers & Benfey, 2015). The embryonic roots are key in the early life of the plant before the postembryonic roots are produced, the embryonic roots form the basis of the root system architecture and the initial framework for the plant to seek out water and nutrients.

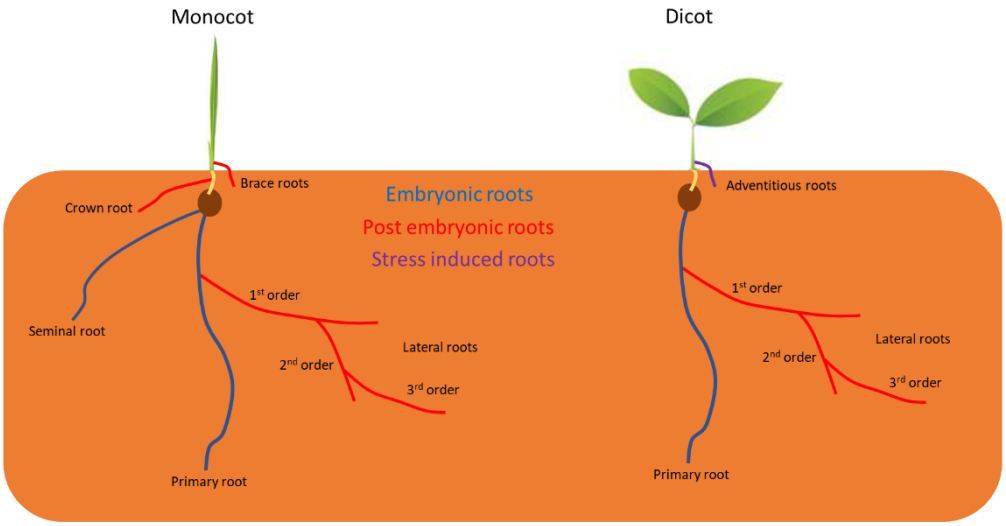

Figure 3 – shows the differences between monocot and dicot roots.

Roots fall into two categories, embryonic roots and post embryonic roots. During early growth monocot root architecture consists of primary roots and seminal roots which establish the seedling before the post embryonic crown and brace roots which are responsible for most nutrient and water uptake by the plant.

Dicot root architecture consists of a single primary root which then develops laterals which can grow further orders of lateral roots. Dicots can also produce shoot-born roots when under stress, these are called adventitious roots (Koevoets et al, 2016).

In dicots the post embryonic roots consist of lateral roots only unless the plant is under stress in which case some dicots can be induced to grow shoot born adventitious roots (Koevoets et al, 2016) In monocots the post embryonic roots are lateral, crown and brace roots. The lateral roots form on all roots below the surface of the soil, they increase the surface area of the root system which increases water and nutrient uptake. Lateral roots form the majority of root length in most plants, although not always the majority of root weight due to the smaller diameter of lateral roots compared to primary roots (Postma et al, 2014). In monocots the crown roots emerge from underground stem nodes and brace roots emerge from aboveground nodes. These roots play a role in water and nutrient uptake but are also a defence against lodging (Rogers & Benfey, 2015).

1.3.2.1 Phosphorus uptake strategies in plants

Plants have evolved multiple strategies for P acquisition. These strategies include, changes in root morphology and architecture, symbiotic relationships with mycorrhizal fungi, improving internal phosphorus economy through metabolic changes and altering root physiology to promote the exudation of chemicals that increase phosphorus availability in the rhizosphere (Cong et al, 2020).

1.3.2.1.1 Exudates.

As noted above, the pH of the soil has an impact on the solubility of phosphorus. Plants such as alfalfa, spinach and radish have been shown to increase the secretions of organic anions in response to low phosphorus availability (Pantigoso et al, 2020). In tomato, chickpea and white lupin it was found that phosphorus deficient soils prompt an increase in proton release (Lyu et al, 2016). Lupins also form specialised cluster roots which secrete citrate and malate which lowers the pH in the soil (Pantigoso et al, 2020). These ions and organic acids diffuse into the rhizosphere due to the large concentration gradient between the cytoplasm of the root cells and the soil solution. The pH changes caused by these exudates can liberate phosphate that the plant can then take up (Akhtar et al, 2009).

Exudates which change soil pH are not the only ones produced by plants. Phosphorus deficiency can also prompt the release of phosphatase enzymes into the rhizosphere which convert organic phosphorus compounds into available inorganic phosphorus compounds (Redel et al, 2019). Legumes generally enhance root chemical processes more than cereal crops through the exudation of carboxylates and phosphatases (Lyu et al, 2016).

As well as enzymes plants can also produce compounds which promote microbial growth and activity in the rhizosphere, these microbes can digest insoluble phosphorus and convert it to a soluble from available to plants (De Andrade et al, 2022). This links to another method of phosphorus acquisition plants use.

Symbiotic mycorrhizal fungi.

Arbuscular mycorrhizal fungi have a relationship with the majority of plant species, the fungi provide the plant with mineral nutrients in exchange for carbohydrates from the plant (Püschel et al, 2021). The hyphae of the fungi can reach from the roots of the plants several centimetres into the surrounding soil. This means that the fungi can access phosphorus that is beyond the phosphorus depletion zones around plant roots. Interactions with mycorrhizal fungi can increase the area from which phosphorus can be absorbed by as much as two orders of magnitude compared to that accessed by plant roots alone (Püschel et al, 2021).

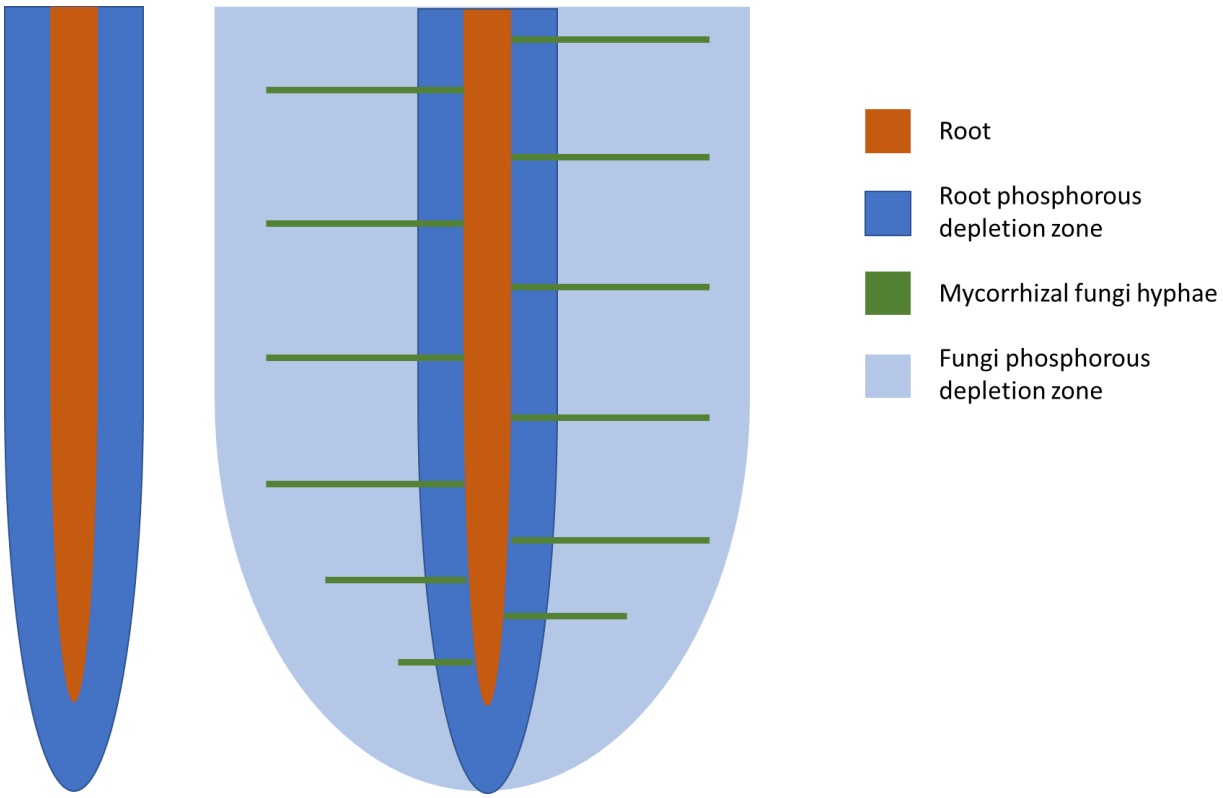

Figure 4: shows (not to scale) a representation of the difference between the area phosphorus can be absorbed from by the root of a plant compared with the area that can be foraged when the root associates with mycorrhizal fungi. The fungal hyphae hugely increase the area that can be foraged for phosphorus in exchange for carbon from the plant.

Economical use of phosphorus.

As well as strategies that improve phosphorus uptake plants can also make internal changes to improve the efficiency of phosphorus use (Ding et al, 2010). This can be through replacing phosphorus containing molecules with alternatives. Phospholipids contain around 30% of cellular organic phosphorus, most of the cell membrane is comprised of a variety of phospholipids (Zhu et al, 2022). These phospholipids can be hydrolysed to release phosphorus when the plant is experiencing phosphorus deficiency. The phospholipids are replaced with either galactolipids or sulfolipids synthesised in plastids so that membrane function is maintained (Zhu et al, 2022).

Increasing phosphorus use in tissues with a high phosphorus demand can also improve overall phosphorus use efficiency. Some legume varieties have more efficient use of phosphorus because of adaptations in the efficiency of their nitrogen fixing root nodules. As these nodules require a lot of phosphorus to grow plants which have more efficient nitrogen fixing require fewer root nodules improve the phosphorus use efficiency of the plant (Sulieman & Tran, 2015).

Plants can also remobilise phosphorus from older organs to young and actively growing ones. During vegetative growth of broad-leaved plants lower leaves on the plants become shaded and therefore less photosynthetically efficient, rapid translocation of phosphorus from these leaves to newer ones occurs (Cong et al, 2020). Phosphorus is not only relocated from leaf tissues, root systems can also be a source of phosphorus later in the plant's life cycle. Phosphorus uptake usually decreases when the plant reaches the reproductive stage as the roots mature and then senesce (Dissanayaka et al, 2018). This means that a large root system can potentially benefit plants in two ways; by improving phosphorus acquisition from the soil and by acting as a reservoir of phosphorus that can be remobilised (Dissanayaka et al, 2018). There may also be changes in root diameter in response to low phosphorus. The specific root length changes in plants that are growing under poor phosphorus conditions, the root are longer and thinner requiring a reduced input of phosphorus while still exploring the soil (Lyu et al, 2016).

As well as changes to the whole root structure to maximise efficient phosphorus use there can also be changes to the internal anatomy of the roots. Aerenchyma, air spaces within plant tissues, reduce the metabolic cost of root tissues allowing greater root growth (York et al, 2022). Computer modelling has suggested that aerenchyma could improve the acquisitions of phosphorus in beans by up to 14% and in maize by up to 70% (Lynch, 2015). Large aerenchyma like those seen on the left-hand side of figure 5 fill out the root space without the need for phosphorus to create cell walls or cell contents which reduces the nutrient cost of the root compared to the denser cells with little to no aerenchyma that are seen in the right hand root.

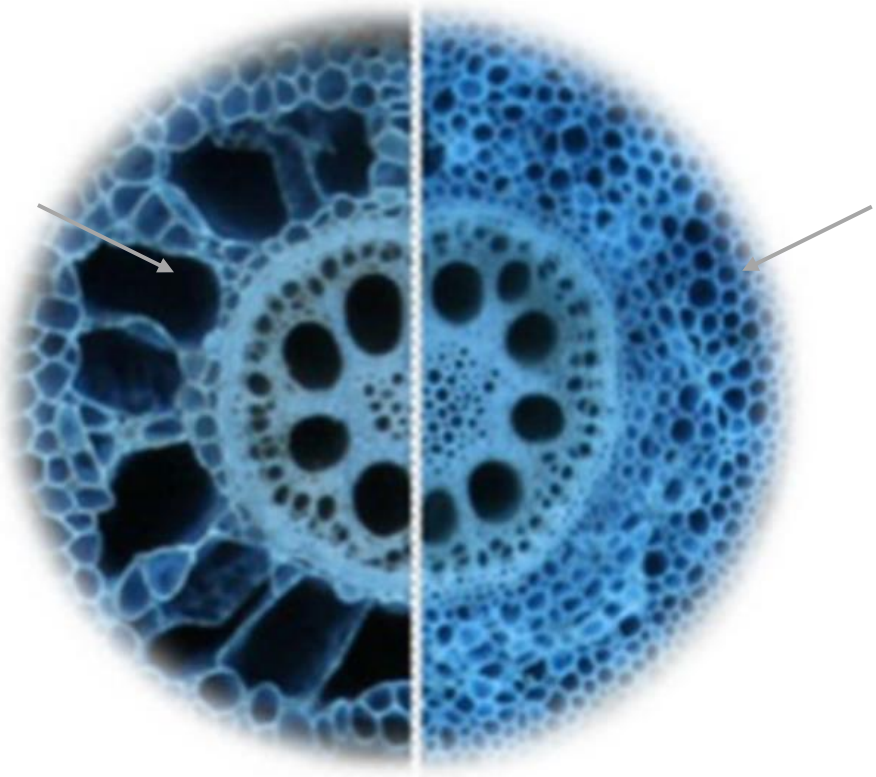

Figure 5: shows the phenotypic variation in maize root cortical aerenchyma formation. (Arrows indicate aerenchyma)

On the left a root with large aerenchyma which have been demonstrated to improve phosphorus usage efficiency. On the right a root with much smaller aerenchyma.

Image from Lynch, 2015.

Root morphology and architecture.

Root morphology refers to the characteristics of an individual root as an organ, for example root diameter, root hair cells and cortical senescence (Nguyen & Stangoulis, 2019). Root system architecture (RSA) refers to the whole root system and the spatial distribution of the roots within the soil (Nguyen & Stangoulis, 2019). RSA is not only controlled by the plant's nutrient foraging adaptations, but also determined by the interaction of soil conditions and plant foraging responses. Altering their root system architecture can provide plants with advantages in certain growth conditions for example low nutrient availability or drought (Rogers & Benfey, 2015). Both morphology and architecture play a role in phosphorus foraging.

An example of this can be seen in work done on Arabidopsis. It was found that in Arabidopsis thaliana plants grown on plates low phosphorus availability caused the plants to adapt their root system architecture. The plants experiencing low phosphorus formed a shallow root system which could promote foraging in the topsoil. The plant is prompted to change its root architecture when the primary root encounters soil with low phosphorus, primary root tips are sensitive to phosphorus availability in the soil (Thibaud et al, 2010). Root system architecture can also be altered due to low phosphorus availability in the shoot (Williamson et al, 2001). This suggests that Arabidopsis root architecture changes in response to phosphorus deficiency, however, it should be noted that these experiments used plants grown on agar plates not in soil. Iron concentration in media can affect the root responses to low phosphate by inhibiting primary root growth (Dong et al, 2017) (Gutiérrez-Alanís et al, 2017). There is also evidence that plants grown on phosphorus limited agar plates can be susceptible to iron toxicity which may cause extreme changes in roots such as severe reduction of the primary root length (Janes et al, 2018). These experiments may therefore not be fully representative of the effect that phosphorus deficiency in the soil has on root architecture.

Other experiments have found that the root surface area was expanded by reducing primary root elongation rate, promoting lateral root development and increasing root hair formation (Abel, 2017). In Arabidopsis experiencing low

phosphate conditions the meristem of the primary root has been found to undergo meristem exhaustion, wherein cell elongation is reduced, cell division is arrested, and cells differentiate prematurely, this results in primary root elongation being inhibited (Gutiérrez-Alanís et al, 2018). As well as increasing lateral root development root hair differentiation and elongation is stimulated under low phosphate conditions (Williamson et al, 2001). This further increases the surface area available to forage for phosphorus, at both a cellular and architectural level lateral growth is being promoted over primary growth.

It can be seen from these experiments that under low phosphorus conditions Arabidopsis inhibits the extension of its primary root in favour of promoting lateral and root hair cell formation. By increasing the root mass in the topsoil, the plant's roots are foraging in the area when phosphorus is most abundant (Lynch, 2011). The inhibition of primary root growth is not a direct result of the plant being starved of phosphorus as this would inhibit all root growth, rather the changes are a deliberate alteration of the root growth in order to maximise phosphorus uptake.

Root morphology in crop plants

Model organisms are extremely important to biological research; however, it is important to also experiment with crop species to confirm that the results from experiments in Arabidopsis carry over into the crops which we eat. Many crop species have been examined for their root-phosphorus interactions and the results of those studies have shown that there are variations in responses between species of plants although some common responses have been found.

In field trails common beans (*Phaseolus vulgaris*) cultivars with shallow root systems with dispersed basal roots (roots close to the shoot) and those with deeper root systems were grown on both high phosphorus fertilised soil (160kg P per hectare) and medium phosphorus unfertilised soil in monocultures and in polyculture. Both soils had marked increase in concentration in the topsoil. The unfertilised soil had 6.7, 2.4 and 1.2 mg/kg in the 0- 10-, 10- 20-, and 20- 30-cm soil depths respectively and the fertilized soil had 35.9, 2.3, and 1.2 mg/kg in the 0-10-, 10-20, and 20-30-cm soil depths respectively. The plants were grown for 35 days

after planting. It was found that the shallow-rooted cultivars had greater yield on low phosphorus soils when compared to cultivars with a deeper root system (Rubio et al, 2003). This was increased when the cultivars were grown in competition, with the shallow rooted cultivars reaching almost double the yield of the deep-rooted cultivars when grown in competition with each other (Rubio et al, 2003).

The benefits of a shallow rooted system for phosphorus foraging were also seen in an experiment where a mixture of sand, perlite and vermiculite were used as the growth media. Three phosphorus treatments representing different phosphorus spatial distribution in the soil were created by mixing solid phase-buffered phosphorus with solid media. Two of the treatments where homogenous, one with high (50µM) and one with low (0.2 µM) phosphorus and the third treatment had stratified phosphorus with 50µM in the top 7cm and 0.2 µM below 7cm. The plants were grown for 28 days and then harvested, and the dry biomass of the plants and the root lengths were measured. When grown in a homogenous media shallow rooted cultivars had no growth advantage over deep-rooted cultivars. However, when the phosphorus was concentrated in the topsoil, as is seen in nature, the shallow-rooted cultivars had a growth advantage (Rubio et al, 2003). The shallower root system means that there is a greater root mass in the topsoil where phosphorus is most abundant.

This shows that in the common bean the root architecture is an important factor in phosphorus uptake regardless of mycorrhizal interactions or other environmental factors that could occur in the field. It was suggested that a shallow root system could have difficulty accessing water which is usually found deeper in the soil. Anchorage also has the potential to be a problem, however, in these experiments they did not find this and hypothesised that the greater role of the taproot in anchoring the plant may offset the shallower root system (Rubio et al, 2003).

The cereals *Triticum aestivum* and *Zea mays* altered their root growth in response to low phosphorus. These species showed an increased root to shoot ratio in low phosphorus, more roots grew to hunt for phosphorus. *Triticum aestivum* also showed an increased specific root length (SRL) (Lyu et al, 2016). Specific root length is a measure of fine roots that is used to characterise the resource use of the plant,

it is the ratio of root length to root mass. A higher SRL means the roots are longer and thinner. This is believed to allow the plant to explore the soil at a lower metabolic cost (Ostonen et al, 2007).

1.3.2.3 *Brassica napus* root morphology responses to phosphorus

Brassica napus has shown a strong root morphology response to phosphorus deficiency across a number of experiments. In a hydroponic experiment two genotypes of *Brassica napus* were grown with either high (1000µM/L), low (5µM/L) or no phosphorus in the solution, one genotype was considered phosphorus efficient and the other in-efficient. After 45 days both genotypes showed a higher shoot biomass under high phosphorus conditions and lower biomass under low phosphorus conditions, while the inverse was seen for root biomass. This means that roots make up a significantly larger percentage of total biomass when under low phosphorus conditions, suggesting the plant is investing proportionally more resources to the roots (Zhang et al, 2011). Plants from both genotypes in the low P conditions showed significant increase in total root length, root surface area, root volume and had a significant increase in the number of lateral roots compared to those in high phosphorus conditions, however the response by the phosphorus efficient genotype was significantly stronger than the inefficient genotype (Zhang et al, 2011).

In soil experiments using low phosphorus soils from unfertilised sites in China that had 2.6mg/kg phosphorus naturally and was then treated with either 100mg/kg phosphorus or 0mg/kg phosphorus *Brassica napus* again allocated a higher proportion of its biomass to roots when under low phosphorus conditions. *Brassica napus* also showed an increase in specific root length which would indicate that the roots are longer and thinner under low phosphorus conditions (Lyu et al, 2016). Under low phosphorus conditions *Brassica napus* also showed an increase in the production of root hair cells and the root hair cells produced were longer than those under low phosphorus conditions (Lyu et al, 2016). This is potentially to

increase the surface area of the root system which could increase phosphorus uptake.

Further experiments have shown that the seed yield, shoot dry weight and total phosphorus content of *Brassica napus* plants was strongly correlated with the root length and root surface area in the top 10cm of soil. However there was no correlation between the shoot dry weight and root exudates or between total phosphorus content and exudates (Duan et al, 2020). This suggests that in *Brassica napus* root morphological changes may have a stronger effect on the uptake of phosphorus and the eventual yield of the plants than the biochemical processes in the rhizosphere.

1.4 Tools to study roots

1.4.1 Studying root architecture

None of the physiological changes in roots could be investigated without the use of a variety of tools both in the lab and in field trials. The methods used vary in the speed with which data can be collected and in how closely they mimic real-world conditions, but each method has a role to play in studying root responses to phosphorus.

Historically root structure is not something that plant breeders have selected for unless it is a root crop, for example carrots or cassava (Thomas et al, 2016). This has been in part because detailed, high throughput methods of analysing root architecture to inform breeding have not existed (Zhu et al, 2011). While root architecture has probably been indirectly selected for by selecting for above ground characteristics to improve yields in the future it would be more beneficial to be able to select for specific root traits to breed for (Voss-Fels et al, 2018). This is especially true if farmers want to try to make use of marginal land, where root architecture is a vital component of plant survival (Zhu et al, 2011).

While phenotyping crops grown in the field gives the truest and most comprehensive information on the root architecture in a proper agricultural setting it is difficult to visualise roots in place in the soil and it is also hard to remove the

roots from the soil due to the extensive networks they can form (Zhu et al, 2011). The development of complementary laboratory and greenhouse methods for analysing the roots have allowed the architecture to be visualised more easily (Zhu et al, 2011). Growing the plants in controlled outdoor experiments can replicate the conditions of the normal soil environment well, however this does not address the problem of imaging the roots without risking damaging them by removing them from the soil, therefore several methods have been developed to tackle this issue (Zhu et al, 2011). Here I will cover a range of methods for studying roots both in the lab and in the field.

1.4.1.2 Phenotyping in the lab

2D imaging

Several lab methods have been developed which do not require soil to grow the plants in, one of these is the Pouch and Wick system. In this method the seeds are placed between a sheet of paper and a sheet of plastic stood upright in trays of media so that the paper absorbs the media. After the seed germinates the roots grow down the paper and can then be photographed and analysed to obtain data about the root architecture (Thomas et al, 2016).

This method has some advantages in that it is a very high throughput method, many plants can be grown in a single experiment allowing a lot of data to be collected. It is also easy to monitor what nutrients the plants receive without needing to test the soil since the paper is placed in pure media. This also means it is easy to experiment with different media. The 2D structure also has the advantage of being easy to analyse, some software such as RootNav is already semi-automated which reduces the time needed to map the root structure. It has been estimated that the total cost for each plant is less than £1 and that the total processing time approximately 2 minutes per plant for sowing, care, imaging and analysis, which is extremely fast compared to the estimated 20 minutes required for extraction, washing, imaging and analysing plants grown in field conditions (Thomas et al, 2016).

This method does have some limitations as the way to study plants. It only shows the 2D structure of the roots, not the more complicated 3D structure that would be seen in the soil, therefore some root architecture features might be altered by forcing the roots to grow in 2D. In addition to this, because the plants are grown without soil, there is no interaction between the roots and the microbes, fungi and other factors in the soil. There is also a time limitation on the experiments as if left too long the roots can grow off the end of the paper, therefore this method can only be used to image very young plants (Trachsel et al, 2010). Depending on the species and rate of growth the plant may only be able to grow for about two weeks, meaning that other methods are needed if the root architecture at a later stage of growth is to be examined.

3D imaging

As already stated, when growing plants in a 2D system some of the root architecture features that would normally be seen in the 3D growth in the soil are lost. It is therefore useful to have a lab-based method that allows the roots to grow in a 3D structure while still being easier to image than plants grown in the field.

X-ray computer tomography (X-ray CT) and Magnetic Resonance Imaging (MRI) have both been used to image roots grown in soil (Pflugfelder et al, 2017). X-ray CT works by passing an X-ray beam through the sample, the bean is partially absorbed reducing the intensity of the beam, this is called attenuation (Metzner et al, 2015). The attenuation values of materials within the soil vary allowing an imaged to be produced showing the roots (Metzner et al, 2015). By rotating the sample and taking multiple 2D images a 3D reconstruction can be produced. This method has been used to study a wide range of species including Arabidopsis, Barley, Wheat, Maize and Chickpea roots (Zhu et al, 2011).

MRI uses a strong magnetic field to detect the magnetic field of protons which are abundant in living tissue particularly in water molecules (Metzner et al, 2015). The density of the protons can be used to produce a strong contrast between “root water signal” and “root soil signal” allowing the detection of roots grown in a soil

core (Metzner et al, 2015). This method has been used to study phytopathology, root structure and even the movement of water within roots (Metzner et al, 2015).

Both methods are affected by the substrate the plants are grown on. Roots and soil have overlapping X-ray attenuation values making automatic computer modelling difficult. Additionally, root attenuation values vary depending on water retention and on the root density which changes as the root ages (Mairhofer et al, 2013). Using artificial substrates or controlling the soil moisture can make X-ray CT image processing easier but may be limiting since it does not accurately represent natural soil environments (Pflugfelder et al, 2017). MRI faces a different problem with substrate choice as the magnetic properties of soils which contain ferromagnetic particles deteriorate the images produced (Pflugfelder et al, 2017). Additionally, soil moisture affects the resolution of the images with high water content potentially preventing the detection of thin roots (Pflugfelder et al, 2017). To counter these problems labs which specialise in MRI often use particular soil substrates with known properties (Pflugfelder et al, 2017). This allows for more reliable image capture but may not represent the conditions in the field accurately.

These methods are limited by the equipment required for the image capture as both X-ray CT scanners and MRI machines are large and expensive. There has been some progress in producing a portable MRI for imaging above-ground plant tissues in the field which could be a very useful tool for researchers, however, it is not currently applicable to studying roots (Meixner et al, 2021). However, because these methods show the 3D structure of roots grown in soil the results produced are more comparable to the environment that crops would encounter in the field than techniques which grow plants without soil.

Another method for 3D imaging of plants does not involve soil, instead the plants are grown in a clear gel which can then be imaged (Piñeros et al, 2016). There are currently two methods of imaging the roots in the gel. The first uses a laser to scan the roots in the gel and builds up a 3D structure. This method gives very precise measurements of the root architecture; however, it is limited by a long scanning time and by the fact that only a small area can be scanned at once (Zhu et al, 2011).

The second gel-based method uses a cylinder of clear gel as the substrate for the plants to grow on. The cylinder is rotated and photographs are taken every 9° to give 40 images per 360° rotation (Piñeros et al, 2016). These images are processed to create a 3D model of the root system which has been shown to be able to quantify 27 different root traits across four species, maize, rice, sorghum and soybean (Piñeros et al, 2016).

These methods have the advantage of being able to see the root structure directly however there are some issues with growing plants in gel. For example, the roots are exposed to light which would not normally be the case, and which could potentially have an effect on the root traits being studied. An additional concern is that the gel would not exert the same mechanical pressure on the roots as the soil. The gel will be homogenous in its texture and the difficulty which the roots will face passing through it will also be largely homogenous. Soil however varies in texture and may contain obstacles to plant growth. Additionally, the chemical composition of the gel will be homogenous whereas in soil the distribution of phosphorus is heterogeneous. In the soil the roots will seek out areas which have a higher phosphorus content, the homogeneity of the gel may therefore affect the growth of the roots in a different way to the growth seen in soil.

The type of gel used has also been shown to have an effect on the growth of plant roots, for example gellan gum has been shown to affect root growth in some species (Piñeros et al, 2016). While rice was able to grow well maize and sorghum had smaller, stunted root systems when grown on gellan gel compared to when grown in hydroponics or sand media (Piñeros et al, 2016).

Another lab-based method that does not involve soil is hydroponics. Hydroponics is the growth of plants in liquid nutrient solutions. The plants are usually, but not always, supported by an inert medium for example rock wool, gravel, vermiculite or other material which provides mechanical support (Sharma & Singh, 2019). The pouch and wick method described above is one form of hydroponics with the paper providing the mechanical support. There are a few benefits to using hydroponics for research, the first is that it provides easy control of the nutrients that the plant is exposed to. The media that the plant is grown in can be customised more easily

than soil could be which is ideal when researching the effect of a particular element on plants. The second is that the roots do not need to be excavated for study, reducing damage that could potentially affect results, however, as the roots are floating in water the architecture of the roots is difficult to image and may be different from that of plants grown in a solid medium. The third advantage is that commercial-style hydroponics can grow plants for much longer than other lab methods such as the pouch and wick method. A key drawback for several of the high throughput lab methods, for example the pouch and wick system, is that these methods can only show root architecture while the plants are young. This means that the results may be affected by seed germination and initial growth and may not show the same results that would be seen in a more mature plant (Trachsel et al, 2010). However, hydroponics is used for commercial growth of plants for example lettuce (Bian et al, 2020), herbs (Chen et al, 2020) tomatoes, cucumbers, sweet peppers and strawberries (Gómez et al, 2019). It has even been reported as being used for illicit cannabis growing operations in Europe and North America (Gómez et al, 2019). The range of plants that can be grown in hydroponics shows the ease of applying the technology to different species and that it can be used to grow even large plants like sweet peppers and tomatoes to maturity.

1.4.1.3 In the field phenotyping

Field trials of crops have existed at least since the 1860s when Mendel grew and crossed pea plants in his monastery's garden. There are benefits and drawbacks to studying plants in the field particularly when studying roots. As noted in the section on studying plants in the lab, methods such as pouch and wick, hydroponics, or growing plants in gel are high throughput, however, these methods are not going to accurately emulate soil conditions. Growing plants in real world conditions will give the most accurate data as to how these plants are likely to grow for farmers. However, by growing in the field you lose control over a number of variables, for example water availability, soil composition and temperature. As temperature has been shown to regulate root development it would be important to monitor these conditions so that they can be factored in to analysis of results (Hund, 2010).

There are also specific challenges when analysing roots grown in the field. The most basic method of phenotyping roots in the field is to dig up the plants and measure the roots manually (Zhu et al, 2011). However, this results in the loss of some of the root mass during excavation and washing, particularly the loss of smaller and therefore more fragile roots. There are methods to account for the loss of roots, for example “Shovelomics” is a method of predicting root architectural traits from excavated root crowns (Trachsel et al, 2010). Their method was tested on maize crowns and allowed them to use ten architectural traits to describe a simple architecture of maize root crowns. Depending on the soil the excavation and cleaning on the plants took between three and eight minutes while scoring the traits took two minutes regardless of conditions (Trachsel et al, 2010). Shovelomics has since been adapted to study cowpea and beans (Rogers & Benfey, 2015)(Burridge et al, 2016). It has also been used to study *Brassica napus* (Arifuzzaman et al, 2019). This method does lose some of the root mass and the architecture is only based on the crown roots, but it is a useful tool. Regardless of how the roots are analysed once excavated this is a destructive method which doesn’t allow for multiple measurements of the same plant.

There are alternatives to digging up roots for analysis. One alternative is the use of clear tubes inserted into the soil called minirhizotrons (Zhu et al, 2011). These tubes can be inserted at various angles depending on what is needed from the experiment. Roots that grow down the outside of the tubes can be imaged with cameras passed down the tubes. This method allows repeated measurements at different points in the growth season which would not be possible with excavation techniques. As technology has advanced the resolution of the imaging has improved, recently it has become possibly to image not only fine plant roots but also fungal mycelium (Defrenne et al, 2021). Only roots directly against the tube can be imaged which leaves much of the root system hidden, however what is visible through this method is unusual in that it will show the finest of roots that would be destroyed by excavation.

An alternative approach to either excavation or minirhizotrons is to use ground penetrating radar or electrical resistivity tomography, these are low resolution

options that have been adapted to measure the biomass of roots (Zhu et al, 2011). Ground penetrating radar releases electromagnetic pulses into the soil which are reflected, transmitted or scattered depending on the material the pulse encounters (Delgado et al, 2017). It is a very rapid technique compared to other in soil measures however it can only image thick roots, around 0.5cm and up, and these roots need to be relatively close to the surface for the radio waves to be able to image them (Zhu et al, 2011). The depth to which this method can image roots depends on the composition of the soil with homogeneous sandy soil being the most optimal conditions for measuring biomass using this technique (Giambastiani et al, 2022). Additionally, the resolution of this method means that only the coarse roots can be imaged accurately while the fine roots can be missed.

Electrical resistivity tomography is used for measuring the biomass of roots in the soil, by applying an electric current to the soil. The electrodes can be on the surface of the soil or can be in boreholes in the soil, when a current is applied the resistivity of the soil gives an estimated measurement of the biomass of roots in the soil (Giambastiani et al, 2022). This method was originally used to measure tree roots but has since been adapted and used to measure the low density roots of alfalfa (Zhu et al, 2011). The composition of the soil needs to be taken into account in analysing the data, however, this method has been successfully used on both clay and sandy soils to measure root biomass (Giambastiani et al, 2022). This method however is only useful for measuring root biomass, it does not provide information on the root architecture in the soil.

One major advantage of these methods compared to destructive methods like Shovelomics is that they can be used repeatedly to image roots over time. Ground penetrating radar has been proposed as a way to monitor root bulking as in cassava to aid breeders in selecting cultivars with high early root bulking (Delgado et al, 2017). However, these methods do not measure as many aspects of the roots as excavation or the use of minirhizotrons. It is also possible for false positives to be returned, particularly when encountering solid objects like stones or clumped clay. This can be mitigated by pre-sieving the soil which, while possible in greenhouse trials, becomes impractical for large scale field trials (Delgado et al, 2017). Electrical

resistivity tomography can likewise be used for repeated measurements of root biomass; however, it is something of a blunt instrument since the only data it gives is the biomass. It has been suggested that a 3D electrical resistivity tomography method, which could give further information on mean root depth, could be developed in the future (Giambastiani et al, 2022).

1.4.2 Tools to study root anatomy

Several techniques exist for imaging root anatomy, varying in the clarity of the images produced, the speed with which they can be acquired, and the degree of technical knowledge needed to produce clear images. Histological sectioning by hand requires a high degree of technical ability in order to produce consistent high-quality sections for imaging. This is particularly true for fragile tissues such as thin roots (Atkinson & Wells, 2017). There are alternative methods to sectioning by hand such as fixing the sample in paraffin wax or resin and sectioning the sample on a microtome however embedding samples in this way is time consuming. A more high-throughput alternative is to embed the sample in an agarose gel for sectioning on a microtome (Atkinson & Wells, 2017). Confocal microscopy of these sections can yield high quality 2D images quickly. However creating 3D images from confocal microscopy is very time consuming (Strock et al, 2019).

An alternative to sectioning is laser ablation tomography (LAT), which uses a laser beam to cut away a sample being fed through the beam while a camera continuously images the cut portion of the sample (Hall & Lanba, 2019). The images from this technique can then be reconstructed into a 3D image using computer software (Strock et al, 2019). The images produced are high resolution and the technique is high throughput, however, because of the technology involved this method is not widely available.

Both sectioning and LAT cut the sample, an alternative method is clearing the samples, a method which makes the samples transparent. Clearing has been extensively done on animal tissues for example investigating the neural network of animal brains (Nagaki et al, 2017). Although this clearing has been used less on

plants there are techniques to clear plant tissue samples for example the PEA-CLARITY method which was developed from the CLARITY method of clearing animal tissues (Nagaki et al, 2017). This method is slow however, with many preparation steps taking 7-9 weeks to complete (Nagaki et al, 2017). Another method which only requires 2-3 days of preparation work is labour intensive, with many changes of solution, and includes toxic reagents including, methanol, xylol and dimethlsulfoxide (Nagaki et al, 2017).

Recently a new technique has been developed for clearing roots using methyl salicylate, this method has been tested on both monocots and dicots including *Brassica napus* (He et al, 2021). This method takes 3 days to prepare the samples so while it is not as high throughput as sectioning it is faster than previous methods of clearing. Additionally it allows both 2D images and 3D reconstructions of the plants to be made easily (He et al, 2021). The cleared samples could also be stored for up to a year without damage to the cell structures (He et al, 2021). This method is not suitable for very thin or small root samples however, as the clearing process could cause small samples to shrink (He et al, 2021).

1.5. – Improving P uptake

Modifying roots is potentially one way for the supply of phosphorus to be extended. By increasing the efficiency of phosphorus uptake by the roots there would be a reduced need for phosphorus fertilizer applications by farmers. It is not simply a case of breeding plants with a larger root system. As noted before plants respond to phosphorus deficiency in a variety of ways. In the case of root system architecture and root morphology plants respond to phosphorus deficiency through specific changes to their roots, not simply increasing the root biomass.

We already know that there are variations in the efficiency of phosphorus uptake between different plant species and also differences between genotypes within a species (Tian et al, 2012). Breeding strategies could potentially look at increasing the efficiency of P uptake from the soil or reducing the amount of P needed by the plant, both of which would reduce the amount of P containing fertilisers that it

would be necessary to apply to the soil (Hammond et al, 2009). These strategies require the identification of traits related to better uptake of, or more efficient use of, phosphorus, and the identification of varieties of plants that have these traits and could therefore be used as part of a breeding program to create new, phosphorus efficient varieties of the crop.

An alternative to breeding would be to use genetic engineering to create lines of plants which could use less phosphorus, or which are better at taking it up from the soil. This would require the identification of genes responsible for traits which improved phosphorus acquisition or utilisation. As genetic engineering faces criticism from many people it is more likely that breeding would be more easily accepted although this is potentially a slower process.

Regardless of the method chosen for creating more phosphorus efficient varieties of plants work is needed to identify varieties which have desired traits. As described above there are a number of established methods for studying root structure and using several of these methods will give the most complete picture as each had strengths and weaknesses.

Modification of plants is not the only course of action that should be taken to extend the availability of phosphorus, taking steps to improve the efficiency of phosphorus fertilizer usage is also important (Ros et al, 2020). This is an important strategy to extend the lifetime of the phosphorus reserves, particularly since breeding or modifying plants takes time.

1.6 Aims

The aim of this work was to establish if there is a correlation between root architectural and morphological traits and increased phosphorus uptake in *Brassica napus*.

There is natural variation in the traits of different varieties of *Brassica napus.* In 2009 an international research consortium selected approximately 450 homozygous inbred lines to form the basis for studying the genetic and phenotypic variation in *Brassica napus*, this set of accessions is called the ERANET-ASSYST

consortium diversity population ("Multinational Brassica Genome Project (MBGP) Meeting notes – PAG 2013) The varieties were selected to be both genetically and geographically diverse (Schiessl et al, 2014). The aim was to provide a set of publicly available information on these varieties and to provide researchers with seeds to do further research.

Using a subset of the ERANET-ASSYST consortium diversity population and some further lines 387 accessions; 163 winter, 127 spring and 7 semi-winter oilseed rape, 35 swede, 15 winter fodder and 40 exotic or unspecified accessions were selected to form the Renewable Industrial Products from Rapeseed (RIPR) diversity population (Alcock et al, 2017). These lines were grown and had their seed and leaf ionomic traits analysed (Alcock et al, 2016). 28 elements including phosphorus were measured using inductively coupled plasma mass spectrometry (ICP-MS) Using the data on leaf phosphorus content from this work I intend to identify several accessions with particularly high or low phosphorus content and compare the root traits of those with high phosphorus to those with low phosphorus. The aim is to identify correlations between root traits and phosphorus content of plants which could then be used to inform further research into the mechanisms underlying these traits.

Chapter 2: Element analysis of different cultivars of *Brassica napus* to investigate the role of root architecture in nutrient uptake.

2.1 Introduction

The aim of this project was to establish if there is a correlation between root architectural and morphological traits and increased phosphorus uptake in *Brassica napus*. To do this it was first necessary to establish that the variation in phosphorus is significant and second to establish if there is reason to believe that the root architectural and morphological traits are contributing to this variation.

2.1.1 RIPR line selection

The original basis for choosing the six varieties to study was the difference in the phosphorus content of the plants from the Renewable Industrial Products from Rapeseed (RIPR) population. This is a subset of accessions from the larger ERANET-ASSYST consortium diversity population which was established to give researchers a geographically and genetically diverse set of *Brassica napus* varieties to study. Element analysis was carried out on the RIPR population in 2016 which provided the data on the phosphorus content of the accessions (Alcock et al, 2016).

The RIPR population is made up primarily of spring oilseed rape (OSR), winter modern OSR and winter OSR with some swede, fodder and other Brassicas included. I chose to focus on the winter modern varieties initially as these are usually planted in August or September in the northern hemisphere which aligned with when my experiments began (Gourrion et al, 2020). Additionally winter OSR is grown more widely in Europe than spring OSR which is more often grown in Canada and China (Gourrion et al, 2020).

For the element analysis that produced the data used to select plants for study the RIPR plants were grown on soil. Seeds from all the accessions were sown on fine-grade (<3 mm particle size) compost (Levington Seed & Modular + Sand -F2S; Everris Ltd., Ipswich, UK) in modular propagation trays to germinate. The compost was covered with perlite and transferred to a glasshouse vented at 15 °C with supplementary lighting to ensure day lengths of 12 h. The plants were watered once daily by hand as required until transplantation. Five plants of each accession were transplanted into individual 5 L pots (internal Ø 22.5 cm; height 18 cm) which contained Levington C2 compost (Scotts Professional, Ipswich, UK). The pots were

then arranged within two single-skinned polytunnels (with a Visqueen Luminance Skin, Northern Polytunnels, Colne, UK) with no additional lighting or heating, at the Sutton Bonington Campus of the University of Nottingham. The plants were arranged in a randomised block design with 5 replicates each containing one plant from each accession. Three replicate blocks were assigned to one poly tunnel and two to another. The 5L pots were automatically irrigated with 133ml of water at 8am, 12pm and 6pm by a Hunter Irrigation Controller (Hunter Industries, San Marcos, CA, USA, provided by Hortech Systems Ltd., Holbeach, UK) (Alcock et al, 2016).

The leaf samples were collected when the plants reached the rosette stage (when 6-8 leaves are showing) Three fully expanded leaves were cut from the plant, stored in separate paper bags and then freeze dried for 48-60h. The samples were homogenised using a mortar and pestle before element analysis via inductively coupled plasma-mass spectrometry (ICP-MS) (Alcock et al, 2016).

There are 80 Winter OSR Modern varieties in the RIPR population. The phosphorus content of the leaves of these varieties had been measured and ranged from 3290mg/kg to 5698mg/kg. To establish if this was a significant difference in the phosphorus content a T-test was carried out on the highest and lowest 20 varieties leaf phosphorus data for these varieties. The t-test showed that there was a significant difference between the highest and lowest phosphorus accessions ($p=3.51226E-17$).

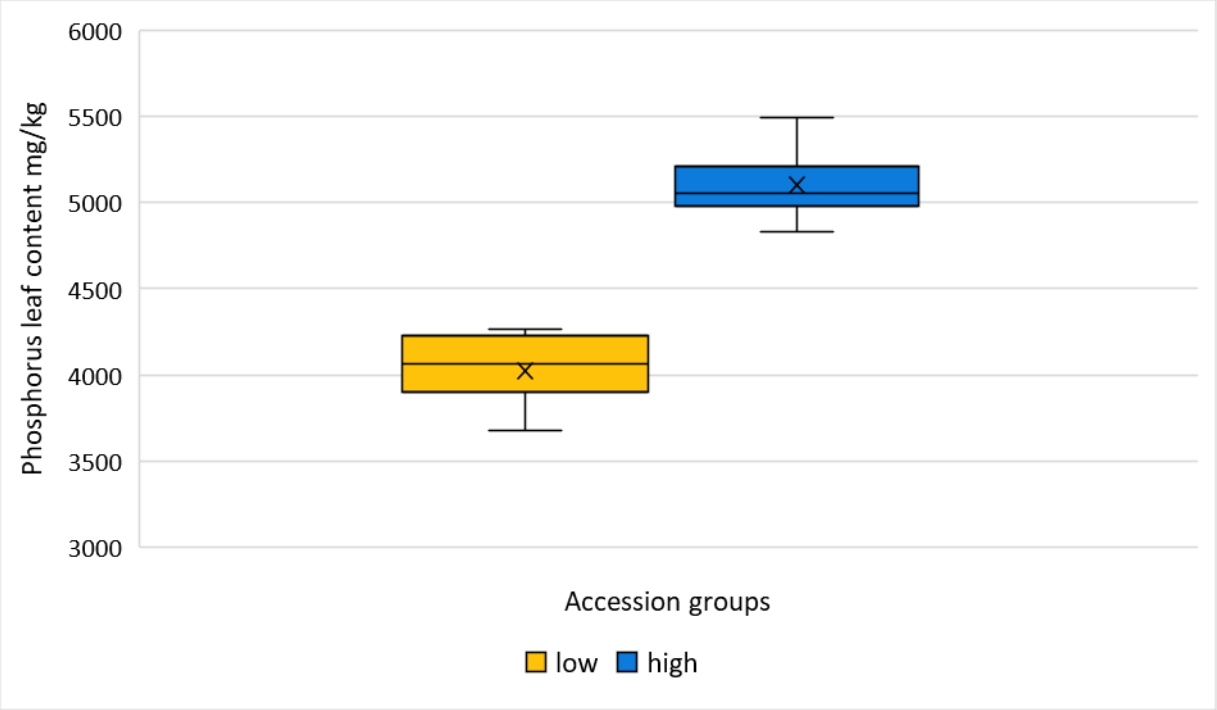

Figure 6 - shows the data from the 20 highest and 20 lowest phosphorus Winter OSR Modern accessions from the RIPR population. There was a significant difference in the phosphorus content of the two groups (p= 3.51226E-17) when they were compared with a t-test.

Based on this difference in phosphorus content these accessions appear to be suitable for further investigation into whether the root architecture is influencing the phosphorus uptake in *Brassica napus*. 6 varieties were chosen from the highest and lowest phosphorus groups. These varieties were Prince (3290mg/kg), Caramba (3675mg/kg) and Gefion (3720mg/kg) as the low phosphorus content accessions and Pacific (5307mg/kg) Montego (5698mg/kg) and Musette (5231mg/kg) as the high phosphorus accessions.

2.1.2 Testing effect of root architecture on phosphorus uptake

While there is a significant difference between the high and low phosphorus content of the accessions this may not be solely due to root architecture. Phosphorus uptake is affected by several factors in addition to root architecture. Exudation of ions and enzymes, associations with mycorrhizal fungi and altering phosphorus use efficiency are all strategies that plants use (see chapter 1). Before attempting to quantify the root architectural traits of the varieties chosen it was

necessary to establish whether root architecture is having a significant effect on phosphorus uptake.

Growing the plants in a hydroponic system removes root architecture as a factor in phosphorus uptake, since the roots are freely floating in water rather than exploring a solid media. In the RIPR population the varieties Pacific, Montego and Musette had higher phosphorus levels in their tissues than the varieties Prince, Caramba and Gefion, if the difference between the phosphorus levels of the two groups changes when the plants are grown in hydroponics rather than in the soil it would indicate that root architecture has an impact on the uptake of phosphorus in these accessions of *Brassica napus*.

Growing plants hydroponically could also affect the phosphorus levels by removing mycorrhizal fungi colonization of roots and affecting the plant's ability to use exudates in phosphorus acquisition. It is unclear how the loss of mycorrhizal fungi interactions will affect *Brassica napus*, however, a comparison of 6 week old maize plants colonised with mycorrhizal fungi and uncolonized plants showed that when grown in hydroponics the colonized plants took up phosphorus more quickly but there was no correlation between colonization and plant growth or phosphorus accumulation in the shoots (Higaki et al, 2017). Exudates could also be complicated by the use of a hydroponic growth system. In the soil exudates will be concentrated around the roots however in the hydroponics where there is a constant flow of media the exudates may be washed away. Hydroponic growth of plants has been used to collect and measure exudates, however, it is unclear whether the effect of flowing media on root exudates has been studied (Williams et al, 2021). Potentially any effects of removing mycorrhizal fungi and root exudates will be mitigated by the phosphorus already being in solution since these adaptations to phosphorus acquisition are to access insoluble phosphorus.

The RIPR plants were sampled at the rosette stage (approximately 8 weeks), my intention was to sample at the same stage at 8 weeks old, however after the first 8 week experiment it became clear that I would not be able to separate and weigh the roots due to tangling in the hydroponic channels so I chose to conduct shorter experiments in order to be able to both measure the roots and sample the leaf

element makeup (Alcock et al, 2016). This resulted in two sets of element analysis data, the first done on samples from the 3-week experiment and the second on samples from the 8-week experiment.

2.1.3 Additional element analysis

Multi-element of plant samples results in profiles of the elements present in the plant, this allows the comparison of levels of nutrients across varieties or treatments. Multi-element analysis is done by inductively coupled plasma (ICP) spectrometry, either ICP-mass spectrometry (ICP-MS) or ICP-optical emission spectroscopy (ICP-OES) (Hansen et al., 2013). ICP spectroscopy is able to detect elements ranging in concentration from below 0.1 μm/g to over 50,000 μm/g or in other terms is accurate to 0.1 parts per million (Hansen et al., 2013). In addition to measuring the phosphorus it was possible to measure 10 other elements and analyse these for trends that relate to the high or low phosphorus varieties. In other research it was found that phosphorus deficiency in *Brassica napus* resulted in reduced concentrations of calcium, iron, magnesium, manganese and zinc in the seeds produced by the plant so changes in leaf phosphorus levels may also affect other elements (Wang et al, 2020).

2.1.4 Aims

To compare the phosphorus levels in the plants without the effect of root architecture to establish if these varieties are suitable candidates to study root architectural traits. How the phosphorus level in the plant tissue responds to having the root architecture removed as a factor in phosphorus uptake will indicate if root architecture is a significant factor in the different phosphorus levels seen in the RIPR population data. Additionally, as the data is available a further 10 elements will be analysed to see if there are any trends related to either phosphorus treatment or variety.

2.2 Methods

2.2.1 Plant materials

The plants were grown in glasshouses at the Sutton Bonington campus of the University of Nottingham. Glasshouses had additional heating to ensure the temperature did not fall below 20°C and had additional lighting to ensure a minimum day length of 16h.

Seeds were sown on rock wool in propagation trays in the glasshouse to allow germination. The seeds were watered but not provided with any nutrients. After 7 days seedlings were selected to be sown in a hydroponics set up and were then grown for a further 3 weeks or 8 weeks depending on the experimental run. The hydroponic system used the nutrient film technique (NFT) and was built by technician Rory Hayden. The set up consists of a 5m long bench with 4 guttering pipes set on a slope with a 10cm difference from beginning to end. Each of these gutters is covered to prevent media evaporation but has the option for up to 10 spaces to be left open for the rockwool blocks containing the plants to be immersed in the media. The low end of the gutters are connected to 4 black water tanks with a capacity of 84L each. The tanks are black to prevent the growth of algae. Figure 7 below shows a diagram of this set up.

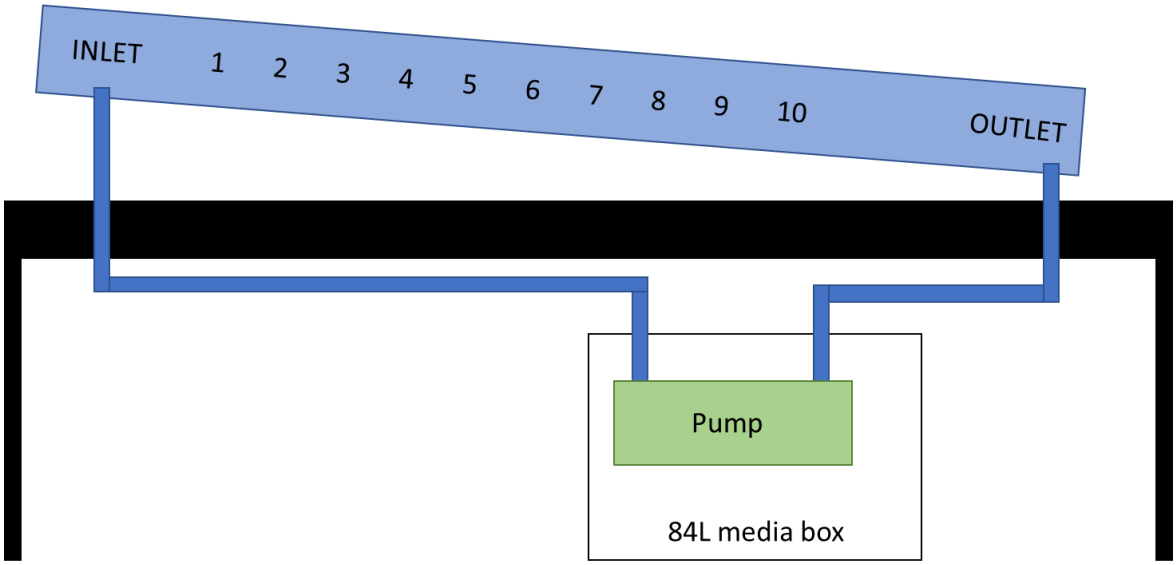

Figure 7: Shows the side view of hydroponics setup. The bench is showing in black, and the guttering and its connected pipes are shown in blue
Media is pumped from the box to the inlet at the higher end of the guttering.
Media flows down to the outlet at the lower end of the guttering and returns to the media box.
Note only one media box is shown here but each bench has four boxes so each gutter has an individual media supply, this allowed up to four different treatments to be used on each bench.
Seedlings are placed at regular intervals down the gutter in rock wool blocks which provide an anchor for the seedlings in place of soil and absorb the media.

The Hydroponic system components on each bench were 4 100mm square white gutters of 4m length and two stop ends for each, 100mm square white gutter all supplied by John A. Stephens LTD (Nottingham, UK). All pipes and pipe fixtures and fittings were supplied by Hortech Systems LTD: ¾ inch. Polyethylene tank connector outlet bulkhead fitting, ¾ inch. Polypropylene back nut threaded fitting, 20mm nutlock elbow for low density polyethylene (LDPE) pipe, 20mm x ¾ inch. Nutlock valve for LDPE pipe, 16mm x 20mm nutlock connector joiner for LDPE pipe, 20mm LDPE pipe. In each 84L tank there was an Aquarius Universal Classic 1500L/hr max flow rate pump also supplied by Hortech Systems LTD (Spalding, UK).

2.2.2 Plant positioning in the hydroponics

Each bench had 4 gutters with 10 spaces for plants in each. For the element analysis experiments 3 benches were used. This gave 12 gutters which had the 4 phosphorus treatments randomly assigned to them. Since there were 3 gutters for each treatment each treatment had a total of 30 spaces for plants, 5 per variety, and the plants were randomly assigned to these 30 spaces. This was repeated for

each treatment so that there were 5 plants of each variety receiving each of the 4 phosphorus treatments. Table 1 shows the treatments and varieties present in each gutter.

Table 1 shows the randomised placement of the treatments and *Brassica napus* varieties across the 3 benches. First the 4 treatments were randomly assigned to the gutters the 5 of each variety of *Brassica napus* were randomly assigned positions within each treatment.

Bench 1					**Bench 2**					**Bench 3**			
Gutter 1	**Gutter 2**	**Gutter 3**	**Gutter 4**		**Gutter 5**	**Gutter 6**	**Gutter 7**	**Gutter 8**		**Gutter 9**	**Gutter 10**	**Gutter 11**	**Gutter 12**
3.86mg/L	**7.72mg/L**	**3.86mg/L**	**15.45mg/L**		**1.9mg/L**	**3.86mg/L**	**7.72mg/L**	**15.45mg/L**		**15.45mg/L**	**1.9mg/L**	**7.72mg/L**	**1.9mg/L**
Gefion	Pacific	Prince	Prince		Gefion	Caramba	Caramba	Musette		Gefion	Pacific	Caramba	Musette
Caramba	Musette	Prince	Gefion		Montego	Musette	Gefion	Prince		Pacific	Pacific	Gefion	Prince
Pacific	Prince	Pacific	Caramba		Caramba	Musette	Montego	Musette		Prince	Caramba	Prince	Prince
Gefion	Musette	Gefion	Montego		Gefion	Musette	Gefion	Caramba		Caramba	Gefion	Pacific	Pacific
Caramba	Prince	Pacific	Gefion		Musette	Montego	Prince	Pacific		Caramba	Caramba	Caramba	Musette
Montego	Gefion	Musette	Pacific		Pacific	Montego	Montego	Montego		Caramba	Gefion	Musette	Prince
Caramba	Pacific	Gefion	Gefion		Pacific	Montego	Gefion	Pacific		Montego	Prince	Prince	Musette
Pacific	Pacific	Prince	Prince		Prince	Montego	Caramba	Musette		Gefion	Montego	Montego	Caramba
Pacific	Montego	Prince	Musette		Montego	Gefion	Pacific	Montego		Pacific	Gefion	Caramba	Montego
Prince	Montego	Musette	Montego		Caramba	Caramba	Musette	Prince		Musette	Musette	Musette	Montego

2.2.3 Media preparation.

This method was based on that of a previous PhD student Josefina Lozano (Lozano, 2021). Using this method 4 phosphorus treatments were made 1.9, 3.86, 7.72 and 15.45 mg/L. there were 4 treatments used so that these plants could also be used for another experiment (see chapter 4) to study the effect of different phosphorus concentrations on the varieties.

The nutrient solution is a combination of seven stock solutions. These stock solutions were prepared to the concentrations detailed in table 4. To prepare the four treatments for the variable phosphorus experiments solutions 2-5 were combined according to table 2. These solutions provide the essential nutrients for the plants. Solutions 1 and 7 are added in accordance with the amounts detailed in table 3. These two solutions provide the variable phosphorus levels across the treatments.

Table 2: Seven stock solutions are required to create the variable phosphorus media. The chemical content and g/L required to make the solution are shown in the table below. The amounts of solutions 1 and 7 are not given in this table as these are variable depending on the treatment.

Solution number	Chemical content	Mol Wt	Molarity (M)	g/L in stock solution	ml/L in treatment	Final solution molarity
1	KH2PO4 KOH	136.09 56.1	0.2667 0.5333	36.30 29.92	**Variable**	
2	MgSO4.7H2O CaCl2.2H2O	246.47 147.02	0.375 0.0125	92.43 1.84	**2**	0.75 0.03
3	FeNaEDTA	367.05	0.05	18.35	**2**	0.10
4	Ca(NO3)2.4H2O	236.15	1	236.15	**2**	2
5	NH4NO3	80	1	80	**2**	2
6	 H3BO3 MnSO4.4H2O ZnSO4.7H2O CuSO4.5H2O Na2MoO4.2H2O	 61.83 223.06 287.55 249.68 241.95	(mM) 30 10 1 3 0.5	 1.85 2.23 0.29 0.75 0.12	 **1**	(uM) 30.00 10.00 1.00 3.00 0.50
7	K2SO4 KOH	174.26 56.11	0.1333 0.5333	23.227 29.62	**Variable**	

Table 3: Amount of solutions 1 and 7 required for the variable phosphorus treatments.

Treatment	ml/L solution 1	ml/L solution 7
1 = 1.9mg/Lw (0.0625mM KH2PO4)	0.23	1.64
2 = 3.86mg/L (0.125mM KH2PO4)	0.47	1.41
3 = 7.72mg/L (0.25mM KH2PO4)	0.94	0.94
4 = 15.45mg/L (0.5mM KH2PO4)	1.88	-

When preparing the treatments solutions 1, 2 and 7 should be added to the water first. The pH should then be adjusted to 6. The other solutions can then be added. Finally, the pH should be checked again and adjusted to a final pH of 6.4. Adding solutions in this order and with the pH changes prevents the formation of precipitates.

Other than the concentration of phosphorus, which was intentionally varied there will be slight variation in potassium and sulphur due to the composition of the variable solutions which used KOH, KH2PO4 and K2SO4, all other elements should be present in the same amounts for each treatment.

2.2.4 Harvesting and element analysis.

The plants in the 3-week experiment were still in the leaf development phase of growth between stages 16-19 on the BBHC-scale of plant development where the plants have 6-9 leaves. The plants from the 8-week experiment were at the rosette stage, 30 on the BBCH-scale, like the plants from the RIPR population apart from a few individuals which had begun to bolt (Meier, 2001). The figure below shows a simplified version of the growth stages of *Brassica napus*. The 3-week plants were at the 4-6 leaf stage of growth while the 8-week plants had all reached the rosette stage that the majority of the 8-week except for a few which had begun to bolt. The growth stages are outlined in figure 8 which is a modified figure from the Canola Council of Canada website (Canola growth stages , 2022)

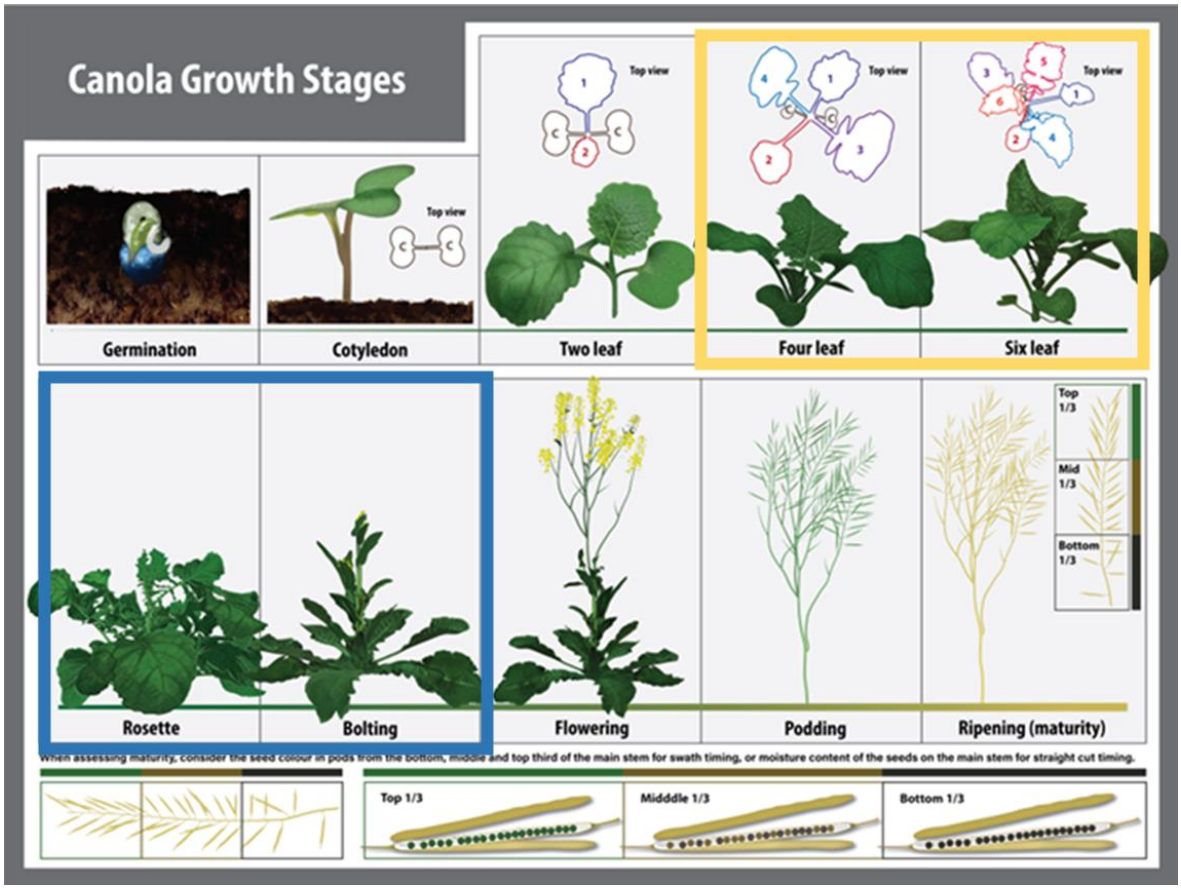

Figure 8 figure showing *Brassica napus* growth stages. This figure does not show all stages on the BBCH-scale however is does show the difference between the four leaf, six-leaf, rosette and bolting stages. Plants in the 3-week experiment were between the four-leaf and six-leaf stages of growth when harvested (outlined in yellow). Plants from the 8-week experiment reached the rosette stage except for a small number of plants which reached the bolting stage (outlined in blue) (Modified figure taken from the Canola Council of Canada website, 2022).

Three plants per variety and per treatment were selected at random. The leaves of these plants were harvested and dried in paper sacks for one week at 50°c. After a week they were checked to ensure they were completely dry. Once completely dried plants were then ground to a rough powder in a mortar and pestle. Samples were then individually bagged to be sent for analysis by the company Lancrop which carries out plant and soil analysis.

Lancrop use ICP-MS mineral analysis to measure the level of elements in plant samples. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) is an element analysis technology capable of detecting most elements at the level of milligram to nanogram per litre. Inductively coupled plasma (ICP) is an ionisation source this is

used to decompose a sample into its constituent elements and to transform these elements into ions the relative amounts of which can then be measured.

Each experiment had 24 treatment and variety combinations (4 treatments and 6 varieties) and each of these combinations had 3 samples sent for analysis. A total of 144 samples was sent for analysis by Lancrop, 72 samples from the 8-week experiment and 72 samples from the second 3-week experiment.

The samples were analysed for a total of 11 elements. The elements nitrogen, phosphorus, potassium, calcium and magnesium were analysed as percentages of total dry weight. The elements boron, copper, iron, manganese, molybdenum and zinc were measured as parts per million.

2.2.5 Statistical analysis

Initially a t-test was run in excel to determine if there was a significant difference between the two groups of varieties, high and low phosphorus. A p value below 0.05 was considered to be significant. The t-tests were run for the results of each treatment separately.

To gain a clearer understanding of the relationships between the individual varieties the results from all of the 6 varieties and 4 treatments were also analysed using a 2-way ANOVA using GenStat. A p value below 0.05 was considered to be significant. The results of the 8 week and 3-week experiments were considered separately.

Additional post hoc analysis was carried out in excel by comparing the mean of each variety to the mean of every other variety. If the means of the varieties varied from each other by more than the mean standard error of difference, then they were considered to be significantly different from each other. This same method was used to compare the means of the treatment groups to establish if the results of different treatments were significantly different from each other. This additional analysis shows which of the varieties or treatments are significantly different from each other and therefore shows if there are trends between the high and low phosphorus groups or between particular individual varieties.

2.3 Results

2.3.1 Plants grown in hydroponics had no differences in phosphorus content of groups.

In the 3-week experiment none of the t-tests comparing the two groups were significant regardless of the treatment the plants received. There was no correlation between the high or low phosphorus groups from the RIPR population and the uptake of phosphorus when grown in hydroponics. When the 6 varieties were compared with a two-way ANOVA the variety of *Brassica napus* did have a significant effect (p=<0.001) on phosphorus percentage showing that there is some variation in phosphorus uptake between some of the individual varieties.

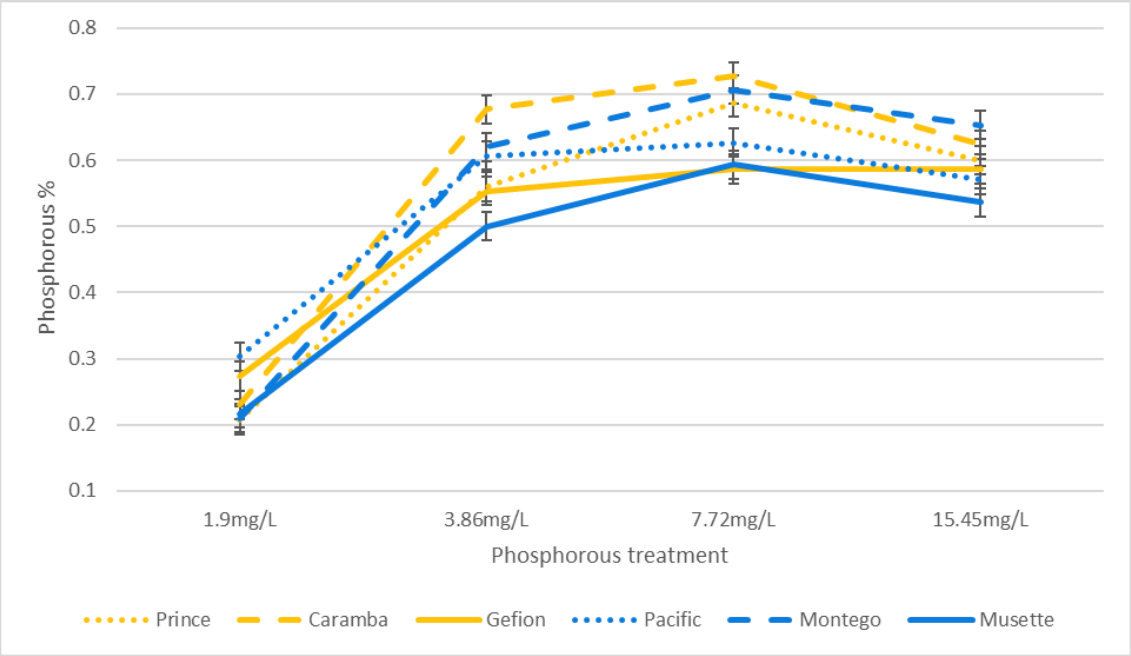

Figure 9 shows the phosphorus percentage in the leaves of 3-week-old *Brassica napus* seedlings grown under four different treatments.

There is no correlation between the high (blue) or low (yellow) phosphorus groups and phosphorus percentage in the leaves of 3-week-old plants grown in hydroponics when compared with a t-test (1.9mg/L p=0.845, 3.86mg/L p=0.589, 7.72mg/L p=0.442, 15.45mg/L p=0.434). Additional ANOVA analysis showed that the was a significant difference between some of the individual varieties (p=<0.001) but post-hoc analysis confirmed that this did not correlate to the two groups.

In the 8-week experiment when the plants are at the rosette stage like in the RIPR experiments then there is no significant difference between the two groups when compared with a t-test regardless of which treatment the plants received. When additional analysis was carried out with an ANOVA there was no difference between the varieties (p=0.166).

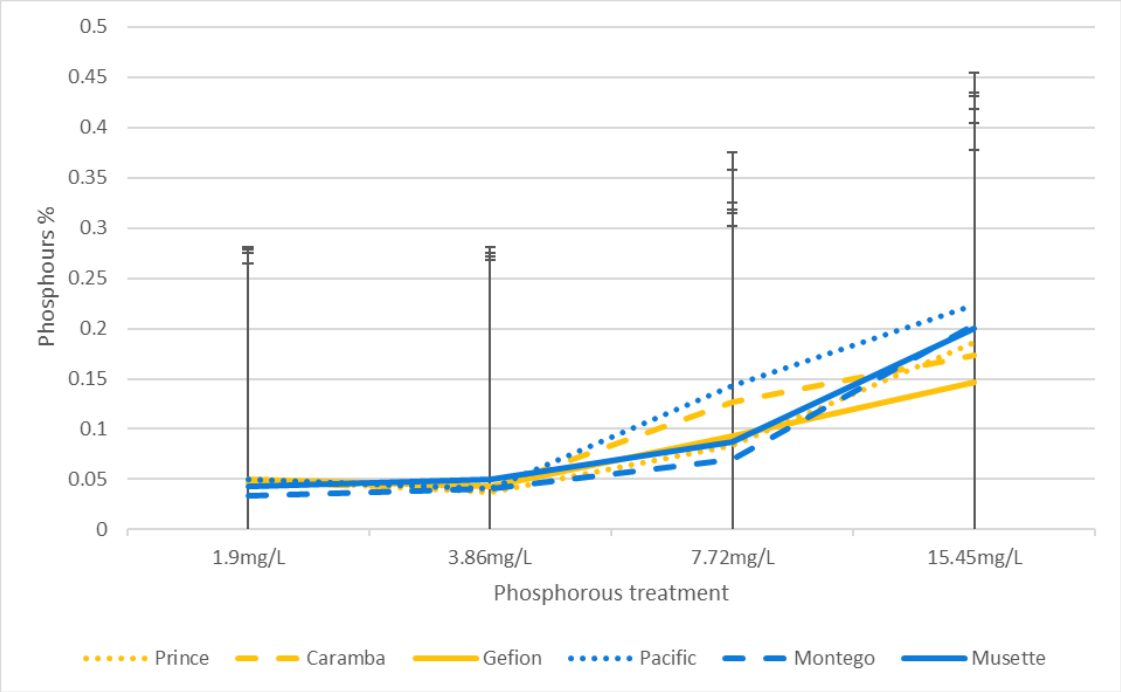

Figure 10 Shows the percentage of phosphorus in the leaves of 8-week-old *Brassica napus* seedlings under 4 different phosphorus treatments.

There is no correlation between the high (blue) or low (yellow) phosphorus groups and phosphorus percentage in the leaves of 3-week old plants grown in hydroponics when compared with a t-test (1.9mg/L p=0.25, 3.86mg/L p=0.612, 7.72mg/L p=0.954, 15.45mg/L p=0.059). Additional ANOVA analysis showed that the was also not any significant difference in the phosphorus percentage of the individual varieties (p=0.166).

2.3.2 Results of other element analysis

2.3.2.1 Elements other than phosphorus did not correlate to the accession groups in the 3-week experiment.

The treatments had a significant effect on all elements analysed except for calcium which showed no significant changes with treatment. Sulphur, and potassium were expected to be significantly affected by the treatment as these elements were part of the solutions used to change the phosphorus content of the treatments. Molybdenum and zinc do not have a clear pattern in how they respond to different treatments however the other elements do show a pattern. Nitrogen, potassium and magnesium show a positive correlation between the percentage of each element and the increasing phosphorus treatments. Manganese also shows an increase in parts per million as the phosphorus in the treatments increases. An increase in the phosphorus in the treatments correlates to a decrease in the parts per million of boron, copper and iron.

The variety of *Brassica napus* only significantly affected the percentage of nitrogen, the percentage of potassium and the parts per million of copper and iron. There was no clear pattern between the low phosphorus plants and the high phosphorus plants with regards to the level of nitrogen, potassium or iron. There may be a slight difference between how much copper the two groups take up however this only seems to be the case for treatment 1 and treatment 2 where the high phosphorus varieties Pacific, Montego and Musette appear to have slightly higher amounts of copper than the low phosphorus varieties.

2.3.2.2 Elements other than phosphorus did not correlate to the accession groups in the 8-week experiment.

As in the 3-week experiment calcium was the only element that was unaffected by either treatment or variety of *Brassica napus*. Treatment had a significant effect on all of the other elements. The effect of treatment did not have a clear pattern in the elements sulphur, boron, copper and manganese. The magnesium results showed a trend of increasing in response to phosphorus increasing in the treatments, this is the same trend as seen in the 3-week experiment.

Some of the trends seen in the 8-week data are the opposite of those seen in the 3-week data. Nitrogen and potassium showed a decrease in percentage of dry weight in response to the increasing phosphorus in the treatment, this is the opposite trend to the samples from the 3-week experiment. The parts per million of iron in the samples increased in response to the increasing phosphorus treatments. This is the opposite trend to that seen in the 3-week hydroponics experiment.

Some of the elements that had no trend in the 3-week experiment showed a trend in this experiment. Molybdenum parts per million increased with the increasing phosphorus treatments in this experiment whereas in the 3-week experiment there was not a clear trend in the data. Zinc decreased in response to the increasing phosphorus treatments in the 8-week experiment, again the 3-week experiment showed no clear pattern in the amount of zinc.

Only 4 of the elements, nitrogen, potassium, copper and iron were significantly affected by the variety of *Brassica napus* being studied. These were the same 4 elements that had a significant difference between the varieties in the 3-week experiment. However, none of these elements showed a trend in the results between the low phosphorus varieties (Prince, Caramba and Gefion) and the high phosphorus varieties (Pacific, Montego and Musette).

2.4 Discussion

2.4.1 Growth in hydroponics instead of soil resulted in no significant difference in phosphorus content.

The results of the element analysis showed that the phosphorus percentage increased as the treatments phosphorus content increased as expected. However unlike in the RIPR population there was no significant variation between the two groups of varieties of *Brassica napus*. There was variation between the phosphorus levels of individual varieties in the 3-week experiment, however this was not correlated to either group, and in the 8-week experiment there was no significant variation in phosphorus levels at all.

In the RIPR population the varieties Pacific, Montego and Musette had almost double the mg/kg of phosphorus of the lower phosphorus varieties Prince, Caramba and Gefion (Alcock et al, 2016). My aim with this experiment was to establish if root architecture plays a role in the differences in phosphorus levels seen in the RIPR plants. The plants in the RIPR experiment were grown in soil whereas my plants were grown in hydroponics (Alcock et al, 2016). By growing the plants in the hydroponics, the effect of the root architecture on phosphorus foraging would be negated as the roots are freely floating in a constant stream of liquid media. The results of this experiment suggest that differences in root architecture between the varieties may be part of the reason for the difference in phosphorus.

Root architecture is not the only factor that may be affected by growing the plants in hydroponics, there may also be an effect on root exudates and mycorrhizal fungi interactions due to the loss of soil. However, these mechanisms of phosphorus acquisition rely on liberating phosphorus from the soil around the root rather than foraging for phosphorus. Exudates release phosphorus from the soil into solution either through direct digestion in the case of phosphatases or by altering the soil chemistry which breaks the bonds between phosphorus and other elements (Redel et al, 2019). In the hydroponic system the phosphorus is already in solution and available to the plants. This may compensate for the loss of the exudates. Mycorrhizal fungi digest phosphorus that plants cannot and extend the foraging area beyond the roots (Püschel et al, 2021). However, the loss of mycorrhizal fungi may be compensated for in hydroponics because the solution is constantly refreshed as it cycles through the system and therefore the plant has a constant supply of phosphorus directly at the roots.

2.4.2 Significant differences in elements other than phosphorus did not correlate to the groups.

The percentage of nitrogen was significantly affected by the variety of *Brassica napus* being studied this could be because plants of different varieties had significant differences in their leaf biomass (see hydroponics chapter 4), meaning the plants grew to different sizes which could have affected the uptake and usage of nitrogen in their tissues.

Potassium varied as expected in response to the treatment changes. The solutions used to vary the phosphorus in the treatments were solution 1 which was made using KH2PO4 and KOH and solution 7 made using K2SO4 and KOH, therefore as well as variations in the phosphorus levels the changes in the treatments were expected to also cause changes in the level of potassium and sulphur in the tissues of the plants which was seen. Additionally, potassium was also significantly affected by the variety of *Brassica napus* being sampled. However the measurements of potassium were within the normal range as potassium can be up to 10% of plant dry weight (Réthoré et al., 2021). The plants in both the 3-week experiment (3.43%-6.74%) and the 8 week experiment (0.21%-2.22%) were not outside the normal range for potassium. There was also no pattern between the high phosphorus varieties and low phosphorus varieties which suggests the differences may be due to natural variation in the levels of potassium in the individual accessions rather than due to differences related to the phosphorus acquisition groups.

All of the elements except calcium were significantly affected by the treatment being applied to them. This could be because of the increase in biomass in response to increasing phosphorus treatments (see hydroponics chapter 4). The increasing size of the plants could affect the uptake and distribution of the elements in the tissues.

It is also important to consider whether a statistically significant result corresponds to a biologically significant change. Element analysis is extremely accurate and can detect very small changes in element concentration, potentially this level of accuracy is producing statistical significance where no biological significance exists,

particularly in the elements that are measured in parts per million. In both the 3 week and 8-week experiments copper and iron were both significantly affected by both changes in treatment and the variety of plants however the changes were very small on the scale of parts per million.

For example, in the 8 week experiment the lowest result for copper was 2.1 parts per million and the highest was 11.3 parts per million. While statistically this is significant, biologically a difference of 9.2 parts per million of copper is unlikely to have any effect on the plants. In experiments into phytoremediation of heavy metal contaminated soils the shoots of *Brassica napus* plants grown as controls on soils with a normal amount of copper were found to have between 3.15 and 5.97 mg/kg or 3.15-5.97 ppm (Marchiol et al, 2004). In general the average copper content of plant tissues is 10µg/g of dry weight (Yruela, 2005). Therefore, while statistically significant the amount of copper in the samples is within normal range for the amount of copper usually found in plants and is therefore unlikely to be biologically significant.

Similarly a review of the iron concentration in different plant tissues found that the median iron concentration in leaf tissues is 167.0 mg/kg and the mean is 489.4mg/kg which is equivalent to 167ppm and 489.4ppm respectively (Ancuceanu et al., 2015). In the 8 weeks experiment the range of results for iron was between 39ppm and 77ppm and in the 3 week experiment it was between 77ppm and 266ppm. Again, the statistical significance is not biologically significant as the element analysis results are in a normal range. Element analysis is a powerful tool, but the results need to be examined for more than simple statistical significance, especially with relatively small sample sizes.

2.5 Conclusion

In conclusion the element analysis of my hydroponically grown plants showed that the phosphorus levels were not significantly different between the accessions when the effect of root architecture was removed. Since the previous work done on the RIPR population showed a difference it appears that root architecture plays a significant role in phosphorus uptake in these accessions of *Brassica napus*,

therefore, these accessions are good candidates for further investigation of root architecture.

Other elements are significantly affected by the variety however this may be explained either by variation in plant biomass that does not appear to be linked to phosphorus uptake or by a mathematically significant result that does not translate into a biologically significant effect.

Chapter 3: Variation in root architecture and anatomy is not associated with phosphorus uptake in six *Brassica napus* lines when treated with quarter strength MS media.

3.1 Introduction

Based on the results of the element analysis experiment root architecture appears to have a significant effect on the amount of phosphorus that the 6 accessions take up. Growing the plants in hydroponics where root architecture is removed as a factor in phosphorus uptake caused the two groups of accessions to have no significant difference in their phosphorus content. This is unlike the results of the 2016 study where the plants were grown on soil and where there was a significant difference between the phosphorus content of the accessions (Alcock et al, 2016). As removing the root architecture removed the difference in phosphorus between the groups this suggests that root architecture is a significant factor in modulating phosphorus uptake in *Brassica napus*.

Root architecture refers to the shape; angle, branching and distribution; of roots within the soil. As phosphorus is relatively immobile in the soil, any change to the root architecture is expected have a significant effect on the plant's phosphorus foraging as it allows the roots to access different parts of the rhizosphere. A shallower root system has been seen as an adaptation to phosphorus deficiency in other species (Lynch, 2011). *Brassica napus* has also been shown to alter root architecture under low phosphorus conditions, increasing root length and number of lateral roots (Lyu et al., 2016). There have also been increases in specific root length seen in *Brassica napus* (Lyu et al, 2016). Specific root length is the ratio of the root length to the root biomass of fine roots, in general a high specific root length is associated with a resource-acquisition strategy with long roots with low biomass maximising soil exploration for low metabolic cost (Wen et al, 2019). By quantifying the root architecture of the accessions, it may be possible to establish a correlation between individual architectural traits and the high or low accession groups.

As well as root architecture, root anatomy may also be a factor in phosphorus uptake. Maize plants from different genotypes showed highly plastic root anatomy which correlated to nitrogen acquisition (Yang et al, 2019). Enhancing the formation of root cortical aerenchyma reduces the metabolic cost of soil exploration in maize plants experiencing drought or nitrogen limited conditions (Vejchasarn et al, 2016).

The number of cortical cells in maize roots have also been shown to improve drought tolerance by reducing the respiratory cost of roots, increasing root depth which leads to greater water capture (Vejchasarn et al, 2016).

Phosphorus uptake may also be affected by root anatomy. A study in rice plants grown on a mix of vermiculite, sand and solid phase buffered phosphorus found that plants grown with low (2µm) phosphorus had changes in their root anatomy (Vejchasarn et al, 2016). Both the total root cross section area and total area of the cortical cells was reduced under low phosphorus treatments. There was also variation in how much different genotypes were affected by the low phosphorus treatment, suggesting that different rice genotypes may have different responses to phosphorus starvation (Vejchasarn et al, 2016).

However, the increase in aerenchyma as a response to stress described above is in monocots where the cortex is persistent whereas in dicots the cortex is destroyed during secondary growth (Lynch et al, 2021). In dicotyledonous plants the roots undergo secondary growth through cell division of two cylindrical meristems, the vascular cambium and the cork cambium, this division results in the destruction of the epidermis, endodermis and cortex (Strock & Lynch, 2020). Therefore, since the cortex is destroyed during secondary growth it is unclear how much effect changes in the cortex such as increased aerenchyma formation during primary growth will have on phosphorus uptake in *Brassica napus*. There may, however, still be some differences in root diameter as radial root expansion has a high nutrient requirement which may not be advantageous when the plant is experiencing phosphorus deficiency (Strock & Lynch, 2020). Examining the root anatomy of the 6 accessions may show if there are any differences in root anatomy that could contribute to phosphorus acquisition.

3.1.2 Aims

The aims of these experiments were to compare the root architectural and anatomical traits of the 6 varieties under low phosphorus conditions and identify correlations between the high and low phosphorus lines. I predict that if root architecture and anatomy are affecting phosphorus acquisition as suggested by the

work in the previous chapter that the high phosphorus group of varieties, Pacific, Montego and Musette will display an increase in traits linked to improved phosphorus acquisition when compared to the low group varieties. For example, I would expect the high group to have, a significantly higher root length, increased number of laterals and increased root area compared to the low group as there are all root architecture traits that have been linked to increased phosphorus acquisition. Likewise in the anatomy experiment I would expect to see a decrease in cortical cell number and root diameter in the high group as these have been seen to be adaptations to low phosphorus stress and therefore may increase phosphorus acquisition.

The plants grown in the pouch and wick experiment will be used for both experiments, first having the root architecture photographed and then being sectioned to study the internal anatomy of the roots. By identifying correlations between increased phosphorus acquisition and specific root traits it is hoped this could inform future work on the mechanisms behind the traits associated with improved phosphorus uptake.

3.2 Methods

3.2.1 Designing an experiment set up to compare root architectural and anatomical traits.

Analysing roots while in the soil is difficult to do in a high throughput method and can result in the loss of roots during extraction from the soil. To enable faster and more accurate analysis of the root architecture I chose the pouch and wick method as used by Alcock (Alcock et al, 2016). This is a higher throughput method which allows many samples to be studied quickly without the need for extraction from soil or washing instead the root architecture can be photographed without interfering with the roots at all. This method also allows for easier alteration of nutrient availability as all nutrients are provided via liquid media giving greater control over the nutrient supply.

3.1.3.2 Method selection for anatomy experiment

My experiment was based on the method set out by Jonathan Atkinson and Darren Wells in their paper, An Updated Protocol for High Throughput Plant Tissue Sectioning (Atkinson & Wells, 2017). In this method the plant tissue of interest is fixed in an agarose gel, sectioned on a vibratome and imaged using a confocal microscope. This method is high throughput and adaptable to different plant tissues making it ideal for this work as it would create many images quickly. It also did not require a multi-step process to stain the tissues, a single stain in combination with autofluorescence of the plant tissues was enough to show the cells.

3.1.3.3 Soil phosphorus content and media selection.

For this experiment it was decided to start with a low phosphorus media as the long-term aim is to identify traits that may be advantageous to foraging in low phosphorus conditions. In the EU and UK agricultural topsoil, soil 0-20cm below surface level, have a range of 50-150kg/ha of phosphorus (Panagos et al, 2022).

Determining how much of the phosphorus in soil is available to plants is complicated. While most soils contain phosphorus in a range of forms only a small fraction of that phosphorus is available to plants. It is generally accepted that approximately 1%-5%, of the total soil phosphorus is available for plants to take up (Jing Zhu et al, 2018). However often data on soil phosphorus is based on soil tests such as the Olsen P test or the Morgan soil test phosphorus (STP) index. These are two widely used indices to estimate phytoavailable phosphorus in the soil which are then used to inform fertiliser use. For example when using the Morgan STP index system to test arable soils 1 0–3.0 mg/L of phosphorus is considered very low, 3.1–6.0 mg/L is low, 6.1–10 mg/L is considered optimum and soils over >10 mg/L are considered to have high phosphorus and pose an increased environmental risk due to phosphorus runoff (McDonald et al, 2019). Both tests are affected by the type of soil being tested, with the Morgan extraction overestimating the amount of available phosphorus in alkaline soils due to the extraction method breaking chemical bonds between phosphorus and minerals in the soil which the plants

would not be able to access (Vero et al, 2022). Olsen P test on the other hand often shows a poor relationship between the availability estimate and phosphorus actually taken up by the plants (Recena et al, 2015).

Regardless of which method is used these tests are not absolute measures of how much phosphorus in the soil is available to plants. This means that it is not possible to directly translate much of the data on soil phosphorus in agricultural soils to the creation of solutions for use in the pouch and wick system.

Even when these tests are not used it remains difficult to compare liquid media, which will be used for these experiments, to the phosphorus found in soil as converting between kg/ha or mg/kg to µm/L is not possible since the actual availability of phosphorus is dependent on many factors relating to the soil chemistry. Therefore, instead of trying to convert between field experiments and liquid nutrients it was decided to use an existing nutrient solution recipe as a starting point for these experiments.

Murashige and Skoog Basal Medium (MS media) has been widely used media for plant tissue culture since it was first described in 1962 (Murashige & Skoog, 1962). This media is also suitable for use in the pouch and wick growth system. It contains all of the macro and micronutrients needed for plant growth and is easily obtained from suppliers in either power or liquid form. For the first experiments examining root architecture and root anatomy it was ideal.

The powdered media used came from sigma Aldrich. Following the standard instructions nutrient media solution is made using 4.4g/L. MS media gets its phosphorus content from KH_2P0_4 170mg/L which means that MS media made up to standard strength has 38.7mg/L phosphorus when dissolved. As noted above for these experiments a low phosphorus content was desired. Therefore, the decision was made to use quarter strength media which would have 9.68mg/L of phosphorus.

3.2.1 Plant materials

The same 6 winter type *Brassica napus* plant accessions from the RIPR population were used in this experiment a (Alcock et al, 2016). The accessions Prince, Caramba and Gefion (3290mg/kg, 3675mg/kg and 3720mg/kg of leaf phosphorus in the RIPR population respectively) are the low phosphorus lines. Pacific, Montego and Musette (5307mg/kg, 5698mg/kg,5231mg/kg of leaf phosphorus in the RIPR population respectively) are the high phosphorus lines.

These 6 selected lines were grown using the pouch and wick high throughput phenotyping system based on work described previously (Alcock, et al, 2018). This system uses growth pouches which comprise a sheet of A4 blue germination paper (SD7640; Anchor Paper Company, St Paul, MN, USA), clipped together with an A4 sheet of black polythene on either side of the paper (Cransford Polythene Ltd, Woodbridge, UK). The paper and polythene were clipped together on one short edge using bulldog clips which also secure the sheets to an acrylic rod (Acrylic Online, Hull, UK). Once assembled the pouches were suspended in aluminium frames which contain plastic drip trays. Each drip tray was first filled with 2L of a 25% strength Murashige and Skoog Basal Medium (MS media) solution (9.68mg/L phosphorus) made using deionised water (Sigma Aldritch, Gillingham). Before the seeds were sown the pouches were left overnight to absorb the MS media solution to ensure the paper was fully saturated. Once the paper is fully soaked then two seeds are trapped between the paper and black plastic, one on each side of the paper. The roots grow down the paper and can later be photographed. The water in the drip trays was replenished with 1L of deionised water every 4 days.

Each of the frames used contains nine drip trays, each of which holds 10 pouches allowing a maximum of 90 pouches per frame. The frames were located in a growth room (2.2 m width, 3.3 m length, 3.0 m height). Photosynthetically Active Radiation (PAR; measured at plant height with a 190 SB quantum sensor; LI-COR Inc., Lincoln, NE, USA) was approximately 207 µmol m−2 s−1, generated by 400 W white, fluorescent lamps (HIT 400w/u/Euro/4 K, Venture Lighting, Rickmansworth, UK). The growth room was set to 20°C days, 18°C nights with a 16hr photo period to

mimic conditions that the seed would experience at sowing which for winter oilseed in the UK occurs in late August (Brown et al, 2019).

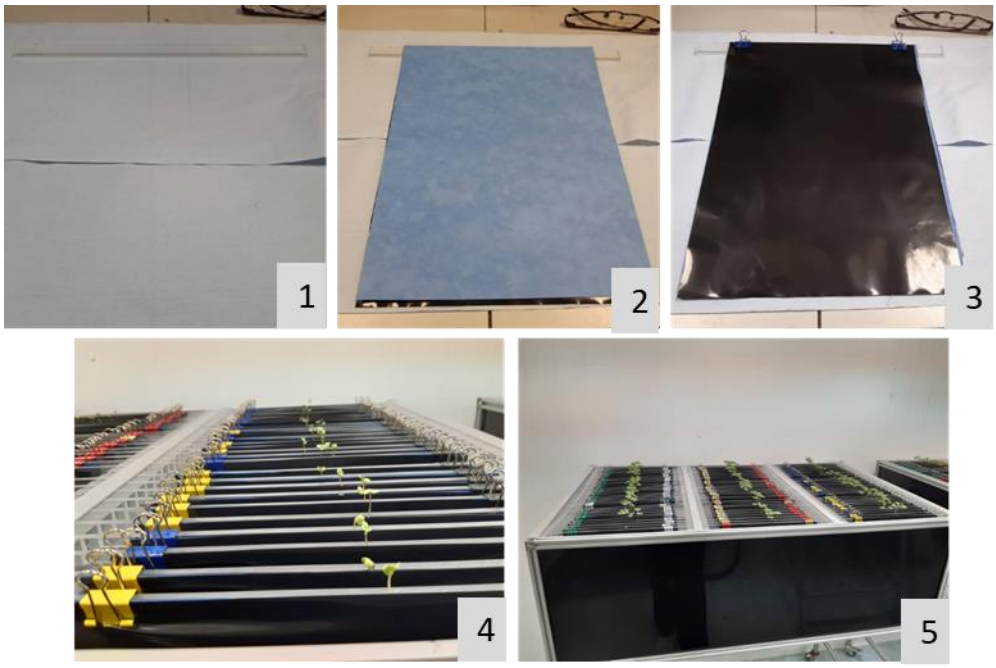

Figure 11: Step by step pouch and wick set up. Blue paper is sandwiched between two sheets of black plastic and bulldog clipped to the fibreglass strip (pictures 1-3). The sheets are placed into the racks with the lower end immersed in the media trays, the media is absorbed by the paper. Once the paper is fully soaked then two seeds are trapped between the paper and black plastic, one on each side of the paper. The roots grow down the paper and can later be photographed (pictures 4 and 5).

3.2.2 Plant positioning in Pouch and Wick

The placement of seeds in the pouches was randomised and the seeds were sown by trapping them in between the plastic and paper approximately 1cm down from the top edge of the sheet. Figure 12 shows an example plan of the randomisation of the accession placement within the rack. Each pouch had two seeds, one on each side of the sheet of paper to maximise the data collected from each experiment. Both seeds in a pouch were the same accession.

	1	E		31	D		61	B
	2	D		32	C		62	E
	3	D		33	F		63	D
	4	C		34	F		64	B
Tray 1	5	A	Tray 4	35	D	Tray 7	65	D
	6	E		36	C		66	C
	7	F		37	C		67	A
	8	E		38	B		68	C
	9	D		39	E		69	A
	10	D		40	F		70	F
	11	C		41	D		71	D
	12	F		42	A		72	A
	13	D		43	A		73	B
	14	D		44	D		74	B
Tray 2	15	A	Tray 5	45	F	Tray 8	75	F
	16	B		46	C		76	B
	17	E		47	E		77	C
	18	F		48	E		78	F
	19	E		49	A		79	F
	20	E		50	E		80	B
	21	E		51	A		81	A
	22	F		52	C		82	A
	23	E		53	D		83	F
	24	B		54	E		84	B
Tray 3	25	A	Tray 6	55	B	Tray 9	85	D
	26	A		56	A		86	C
	27	C		57	B		87	F
	28	B		58	B		88	B
	29	E		59	C		89	A
	30	C		60	C		90	F

Figure 12 - This figure shows an example of one of the randomized plans of accession placement within the rack. Each of the trays contains 2L of media, in this experiment all of the trays contained 2L of ¼ strength MS media (9.68mg/L phosphorus). Each tray has 10 pouches absorbing media from it, each of which has a seed sown on each side of the paper. The letters in the plan correspond to the 6 accessions Prince (A), Caramba (B), Gefion (C), Pacific (D), Montego (E) and Musette (F). A new randomized plan was created each time the experiment was repeated.

3.2.3 Root architecture photography method

The plants were grown for 2 weeks after sowing and were then photographed using a stand to maintain a set distance of 55cm from the sheets images were taken using a Digital Single Lens Reflex (DSLR) camera (Canon EOS 1100D, Canon Inc., Tokyo, Japan) once the camera had been attached to the stand and automatically focused for the first image the auto focusing was turned off to ensure the camera settings did not change for subsequent photos. The photographs were then analysed with a

program called RootNav to obtain a map of the roots and to get quantitative analysis of root traits (Pound et al., 2013). The traits analysed were total root length, primary root length, lateral root length, number of lateral roots, convex hull, maximum width, maximum depth, width/depth ratio and the y coordinate of the centroid.

3.2.4 Using RootNav to measure root architecture.

RootNav is a semi-automated program developed at the University of Nottingham which speeds up the analysis of the roots considerably over an entirely manual method (Pound et al, 2013). There are settings for analysing, Rice, wheat and Arabidopsis, and an additional custom setting which can be used for any plant and was the setting used for analysing the *Brassica napus* plants grown using the pouch and wick method. Computer learning has been used to improve the automation of RootNav, however, the neural network has to be trained for new plant species and unfortunately had not been used on *Brassica napus* before (Yasrab et al, 2019). Attempts to train the neural network using my images were unsuccessful so I was unable to fully automate the process.

The program requires a measurement of pixels per mm to be input if the output is to be measured in mm. The measurement was obtained through ImageJ averaged over several images per experiment, since the camera was on a stand and the settings were the same for each image the pixels per mm did not change for images in a single experiment.

There are several steps to analysing images in RootNav the initial steps are usually fast and easy to accomplish. Figure 13 shows the first steps in analysing a root image in RootNav. First the image must be loaded into the program, usually the image is initially shown as a probability map, it is however easier to analyse the images using the source images and this can be found by going to the view menu and selecting source image.

The first step after changing to source image is to map the primary root, in my plants this is relatively simple as the plants have a single primary root. To map the root a source point must be determined, this is where the primary root joins the stem, and a source marker is placed. Then a primary marker needs to be placed at the tip of the primary root. Then the program can be made to map the path of the primary root. Sometimes the map will not quite correspond to the root and can be adjusted manually by clicking and dragging the line the program has mapped however in figure 13 you can see that the program has mapped the primary root perfectly.

Analysing the lateral roots follows almost exactly the same process as the primary root except that adding a source point is not necessary, the lateral markers are simply added at the tip of each lateral root. As you can see from the images this can sometimes be difficult due to the laterals growing close together and may result in several of the lateral markers overlapping. This is not a problem as long as there is a lateral marker for each lateral root.

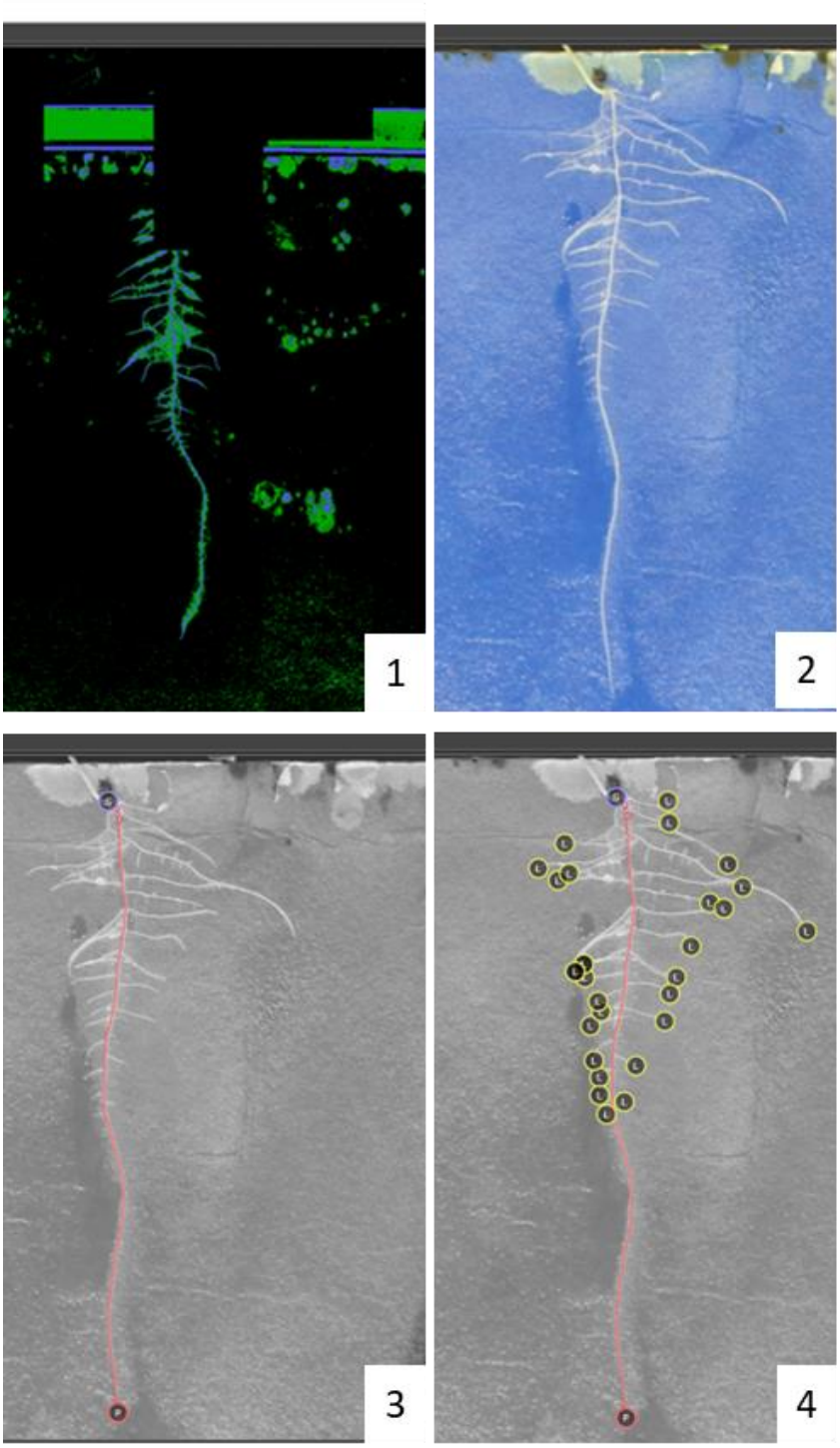

Figure 13 - This shows the initial steps to analyse a root image in RootNav.

1. The initial view of RootNav when first loading an image. The program automatically shows the image as a probability map.
2. The image when converted from probability map to source image.
3. By manually selecting a source point for the roots and selecting the end of the primary root the program will automatically map the primary root.
4. Each lateral root has to have its tip manually marked out which can become complicated when multiple lateral roots overlap. Some judgement is required on the part of the user to determine how many roots are present.

As shown in Figure 14 the program is often unsuccessful at mapping laterals accurately particularly those where the laterals overlap. This is easily corrected by moving the lateral lines to the correct place by clicking and dragging.

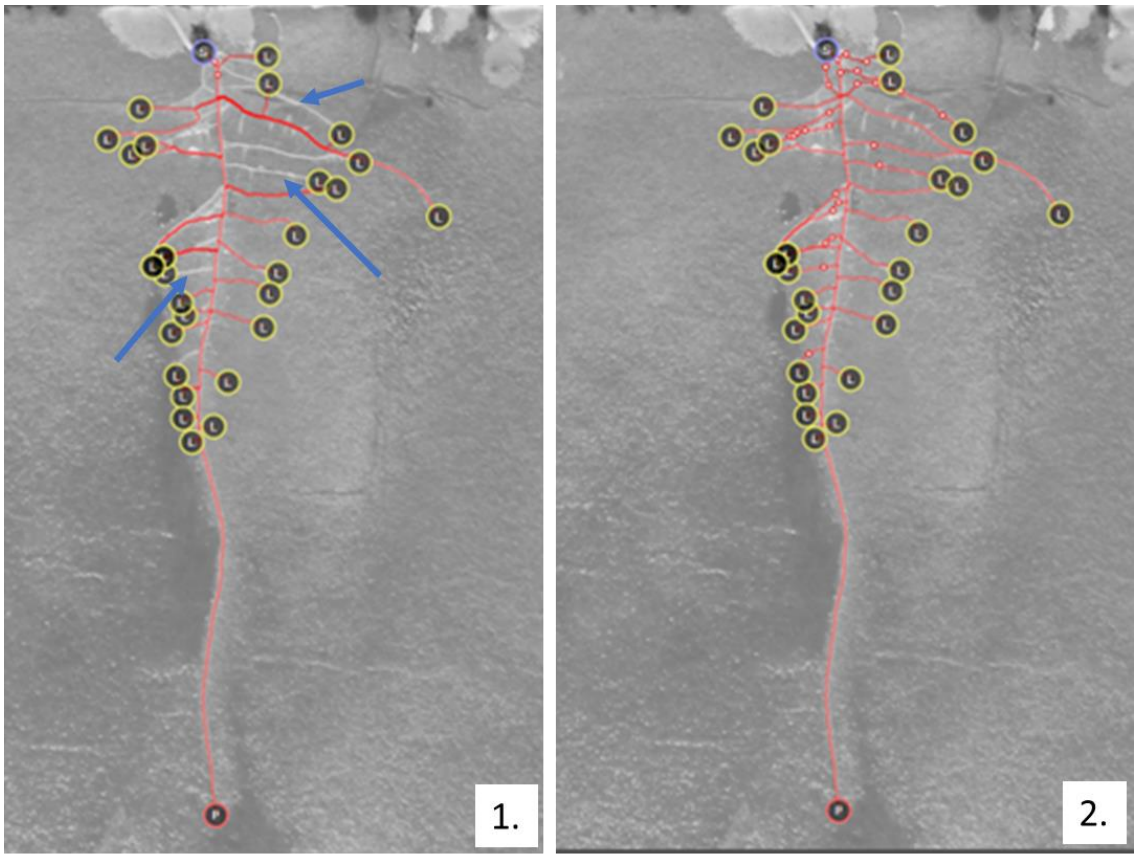

Figure 14 RootNav will attempt to map the roots automatically however it is usually only moderately successful in this.

1. shows the automatic mapping of the lateral roots. The automatic root map has failed to map several of the roots, examples of this have been indicated with arrows.

2. The root map can be corrected by clicking and dragging the map lines to the correct place as can be seen in this image where the map has been adjusted to cover the missed roots.

Once the laterals have been mapped either automatically or with some user intervention then the final step is to click measure which will present you with the root map seen in figure 15. This root map can then be saved to a folder to be analysed later. The program also produces a file which can be used with a secondary program called RootNav viewer to produce tables of data on a large variety of variables including the nine variables I have selected for analysis.

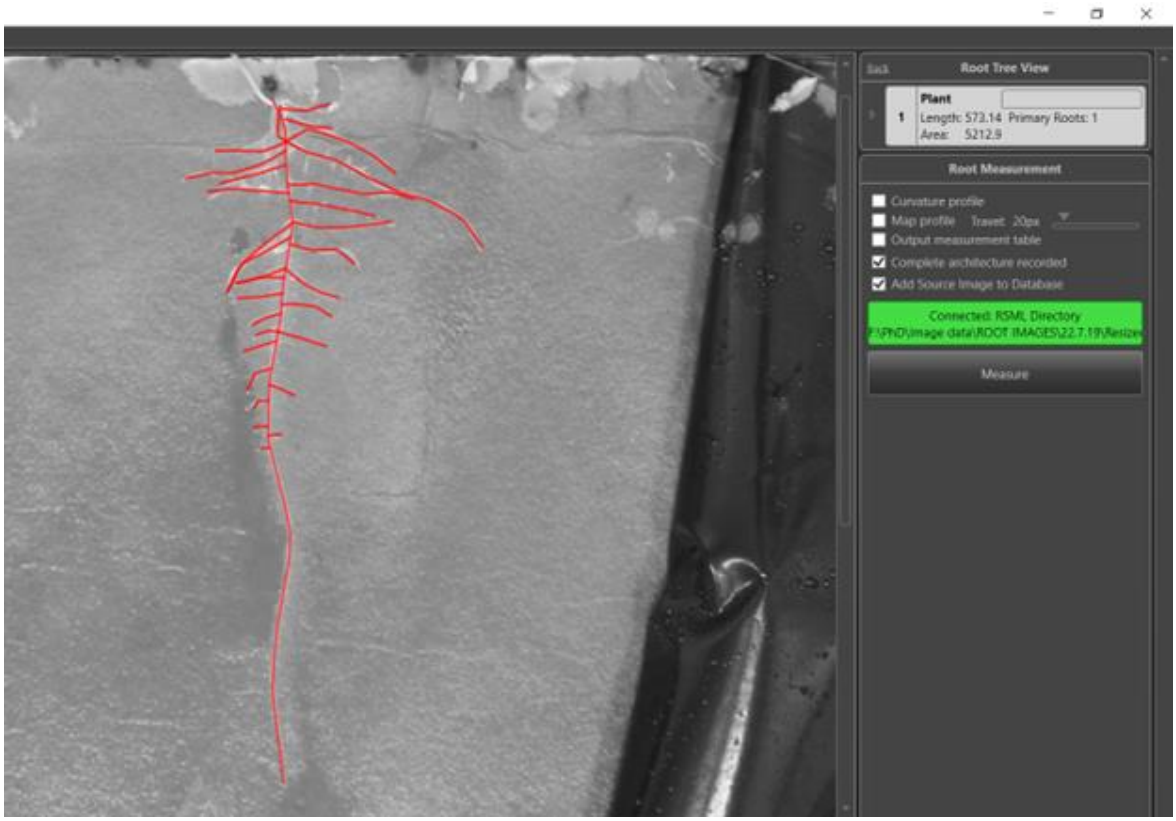

Figure 15 - shows the final root map produced after all primary and lateral root adjustments have been accepted.

There were 351 photos of seedlings grown on MS media in this experiment that were able to be analysed using RootNav. These images were analysed for nine root architectural traits that may affect nutrient foraging: Primary root length, lateral root length, total root length, lateral root number, convex hull area, maximum width of root system, maximum depth of root system, width/depth ratio and the y coordinate of the centroid. The plants were grown under the same conditions and were watered regularly to remove the effect of abiotic stress that could affect root growth.

3.2.5 Sample preparation for sectioning

A 5% low-melt agarose gel (analytical grade low gelling point agarose Sigma-Aldrich, UK) was then made and allowed to cool to approximately 28°c, stirring regularly so it cooled uniformly. While the agarose cooled a portion of the primary root, approximately 3cm long starting from 1cm below the stem was cut using a razor. The portion of the root taken can be seen in figure 16, the lateral roots were trimmed a short distance from the junction with the primary root to make it easier to remove the root from the paper and to prevent laterals from getting in the way during sectioning.

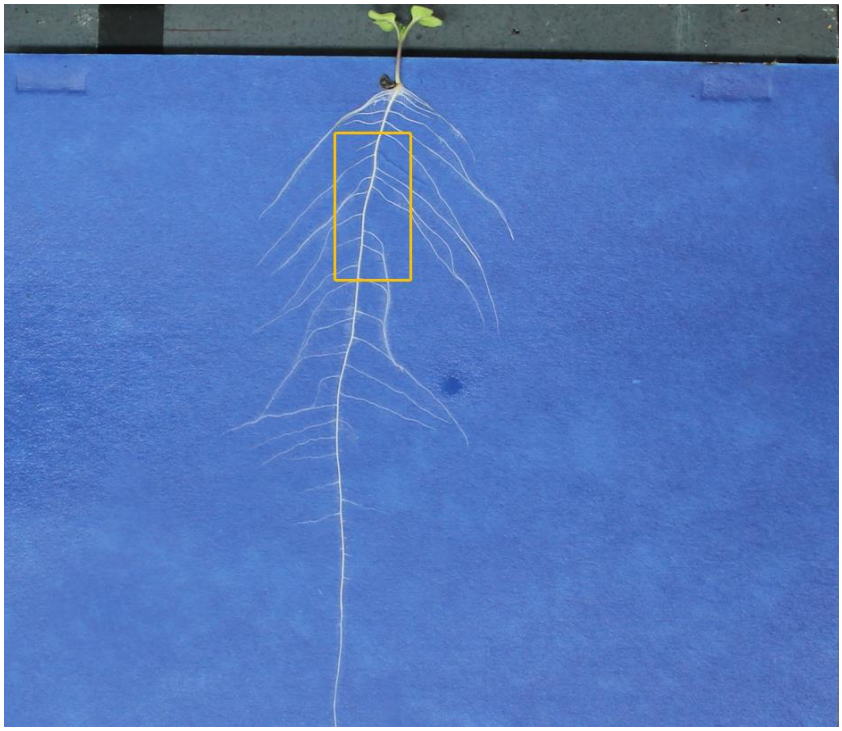

Figure 16: Shows the piece of root taken for imaging. The section was taken from 1cm below the start of the shoot and was approximately 3cm long. As part of the cutting the lateral roots were trimmed.

The root was then carefully removed from the paper and placed in a mould (5.5cm x 3cm x 1.3cm internal dimensions). Figure 17 shows a 3D rendering of the moulds used. The moulds were custom printed from polylactic acid for this purpose (Atkinson & Wells, 2017). The moulds allow several root sections to be set at once both speeding up the process and reducing waste. After placing the root segments between into the mould the mould was then wrapped with tape to both seal it and

hold it steady. Immediately after placing the roots and taping the mould the 5% low melt agarose which had been allowed to cool until nearly set, about 28°c, was then poured into the mould. It was important to pour as quickly as possible to prevent the roots drying out, it was also ensured that the agarose was not poured directly onto the roots as this could cause mechanical damage, the roots were fragile at this stage, and it was easy to destroy them via heat, dehydration or mechanical stress. The moulds have an angled section which directs the agarose into the well of the mould making it easier to avoid pouring directly onto the roots. Because of the constraints of needing to pour the agarose before it set 6 moulds were used at a time, one for each variety of *Brassica napus*, each mould can hold up to 5 root samples.

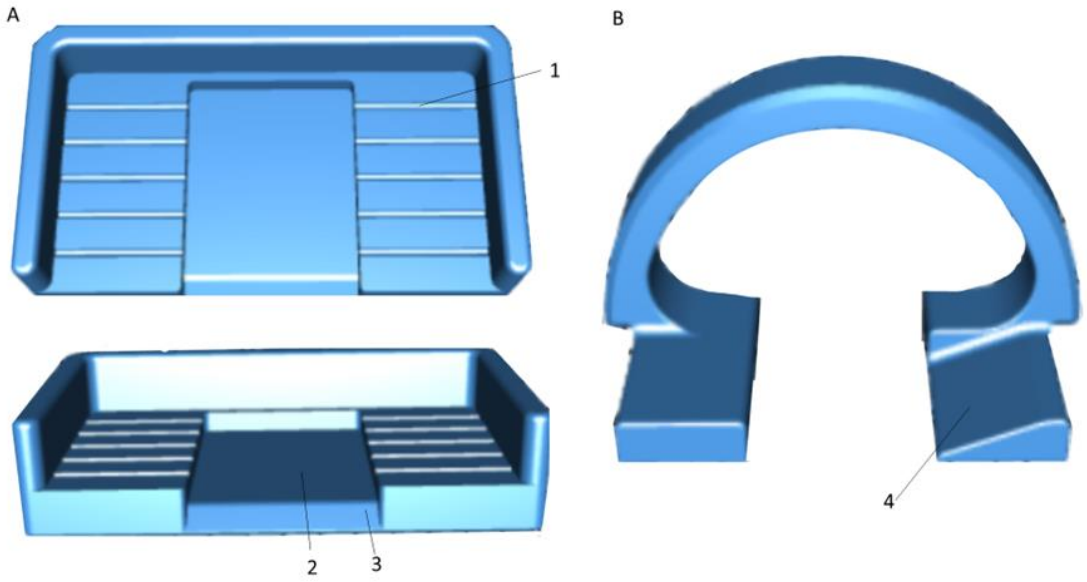

Figure 17 – Shows the mould used to prepare agar blocks for sectioning. The mould comes in two pieces A – the base of the mould which has (1) Grooves for root pieces to be placed into. (2) A central well for embedding media to be poured into. (3) Chamfer for block orientation.

B – The top of the mould which is used to trap the roots after placement in piece A. this has (4) an angled section to direct embedding media into the central well, avoiding media being poured directly onto the samples.

At every stage of the process the roots were handled carefully and had the minimal possible exposure to the air to prevent drying, this was to ensure the roots were in the best condition possible to provide the clearest sections. The figure 18 below shows the damage that can occur during sectioning.

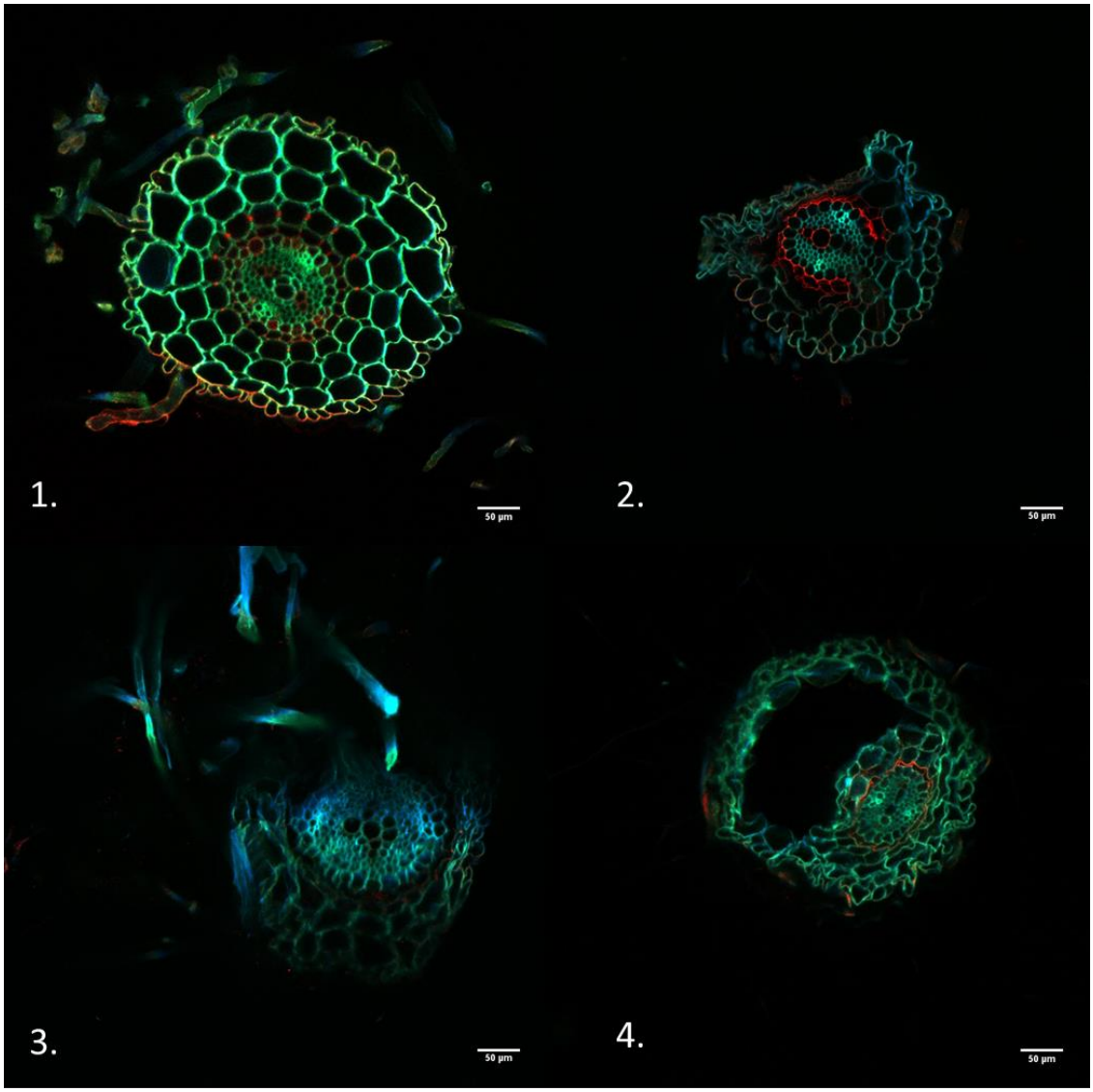

Figure 18: Shows four example sections. Section 1 is one of the clearest sections obtained, it even has an intact root hair cell. Sections 2-4 show three different issues commonly seen with the sections. Section 2 shows a root that has shrivelled, possibly from too-hot agarose gel or from over-exposure to the air between cutting and gel pouring. Section 3 shows a root section that has shifted in the agarose after cutting on the vibratome, the root is now at an angle so only part of it is in focus. This could have been because of rough handling of the slice of agarose. Section 4 shows damage to the root from an unknown cause which has damaged the centre of the section.

The setting temperature for this agarose is 25°C, however the moulds were allowed to cool completely to room temperature (approximately 20°C) to ensure it was fully set. The agarose containing the roots was then removed from the moulds and the excess agarose was trimmed using a double-edged razor blade (Wilkinson sword, United Kingdom). The agarose block was then fixed to one of the vibratome sample mounts using cyanoacrylate adhesive (Loctite). Three cross sections of the agarose

and roots were then obtained using a vibrating microtome [7000smz-2, Campden Instruments Ltd or VT1000s, Leica Microsystems (United Kingdom) Ltd]. The sections obtained are 250µm thick as this is thick enough to be stable when being handled, without being too thick for the confocal microscope to obtain a quality picture. All three sections taken from each root were imaged however only the clearest and least damaged of the 3 was used for analysis.

3.2.6 Imaging root sections using confocal microscopy.

The slices of agarose and roots are then left to soak for approximately 30s-1minute in a 0.3 mg/ml solution of fluorescent brightener 28 (Calcofluor White M2R, Sigma Aldritch) and were then rinsed with deionised water. The sections were then placed in a coverglass-bottomed cell chamber (Lab-Tek II Chambered Coverglass, Thermo-Fisher) and imaged on an Eclipse Ti CLSM confocal laser scanning microscope (Nikon Instruments). The microscope has three excitation lasers (405, 488, and 543 nm), three filter sets (450/35, 515/30, and 605/75), and four detectors. Images were collected using 20x objective. The confocal yields three images from separate channels, red, blue and green. These images were saved as TIFF files for future quantitative analysis. Before analysis the three channels had to be recombined in ImageJ to create detailed images of the internal structure of the roots. This was automated using a macro I wrote (see appendix 3). The use of the three channels, auto fluorescence and the single stain creates images where cell features such as xylem, phloem, Casparian band, exo-and endo- dermis can all be identified. ImageJ is a powerful platform for image processing and the ease of writing macros for it makes automating processes simple, my macro is a small example of this. ImageJ is widely used in the biological sciences (Rueden et al., 2017).

3.2.7 CellSeT analysis

CellSeT is a program designed to semi automate the analysis of cell sections (Pound et al, 2012). It speeds up the analysis of confocal images of plant tissue sections and produces data on cell area, cell number and cell diameter.

The steps for analysing images are detailed in figure 19 below.

1. Load image

2. Apply filters – I found that the default filters were sufficient for my analysis

3. Default segmentation – This causes the program to automatically outline the cells, the threshold for segmentation may require changing, as can be seen in figure 19 changing the segmentation threshold significantly improves the automatic outlining of the cells. For the sectioning images it usually needed to be changed from the default setting of 10 to a segmentation threshold of 5 or 6.

4. When segmentation is accepted there is the opportunity to manually adjust and correct the outline of the cells, this can be minimal on clear sections or may take some time when the section is damaged.

5. Once the outline is accepted and saved then the cells can be labelled. Once labelled the outline and cell map can be saved separately as show in figure 20 and the cell data can be saved as an excel document.

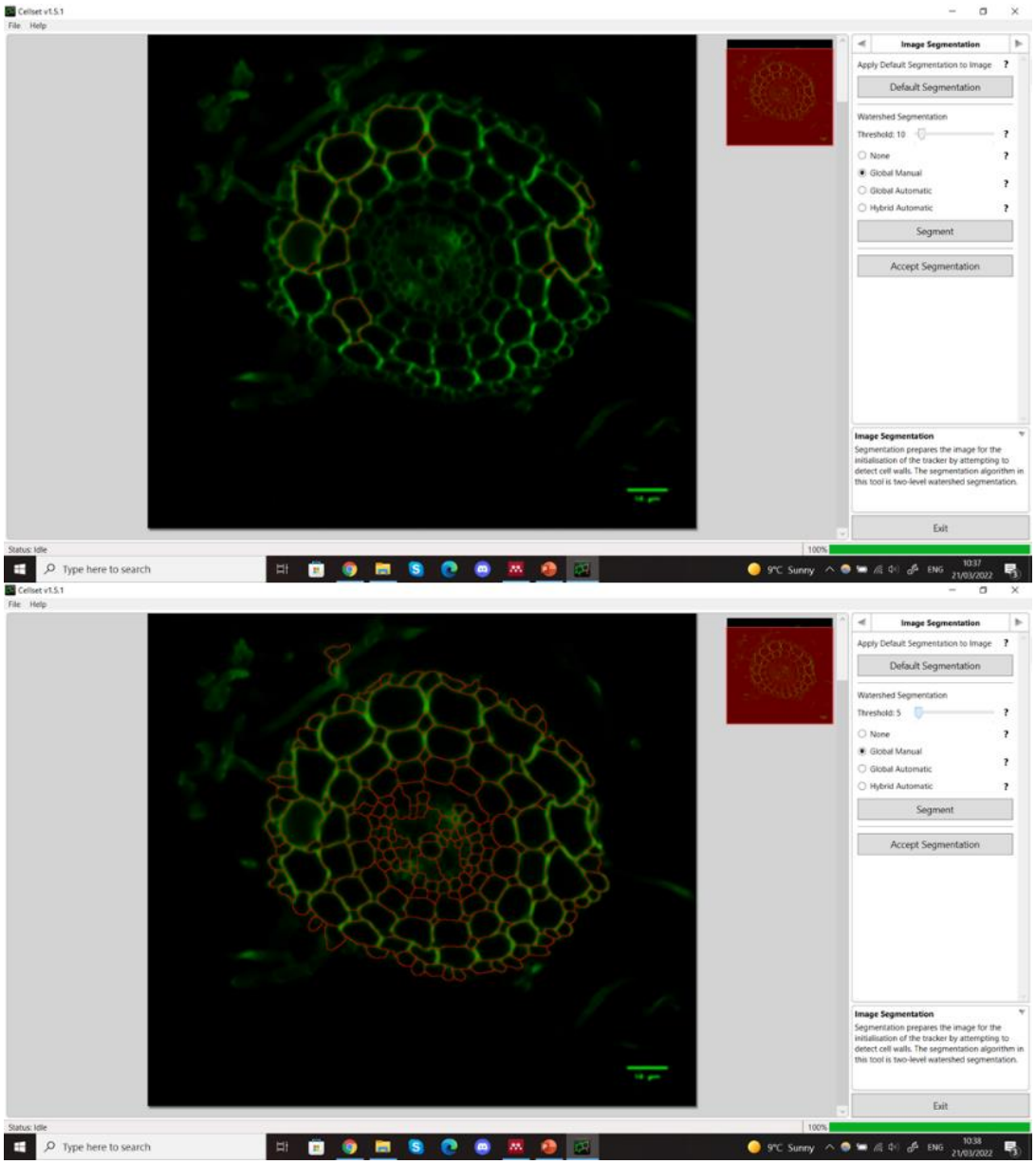

Figure 19: Altering the segmentation threshold markedly changes the automated outlining (in red). The top image shows the automatic outlining of the cells using the default settings, most of the cells have not been detected. Changing the segmentation threshold from the default to 5 or 6 results in a much greater rate of detection as shown in the lower image. Making this adjustment helped to speed up the cell labelling process although manual editing was usually still required.

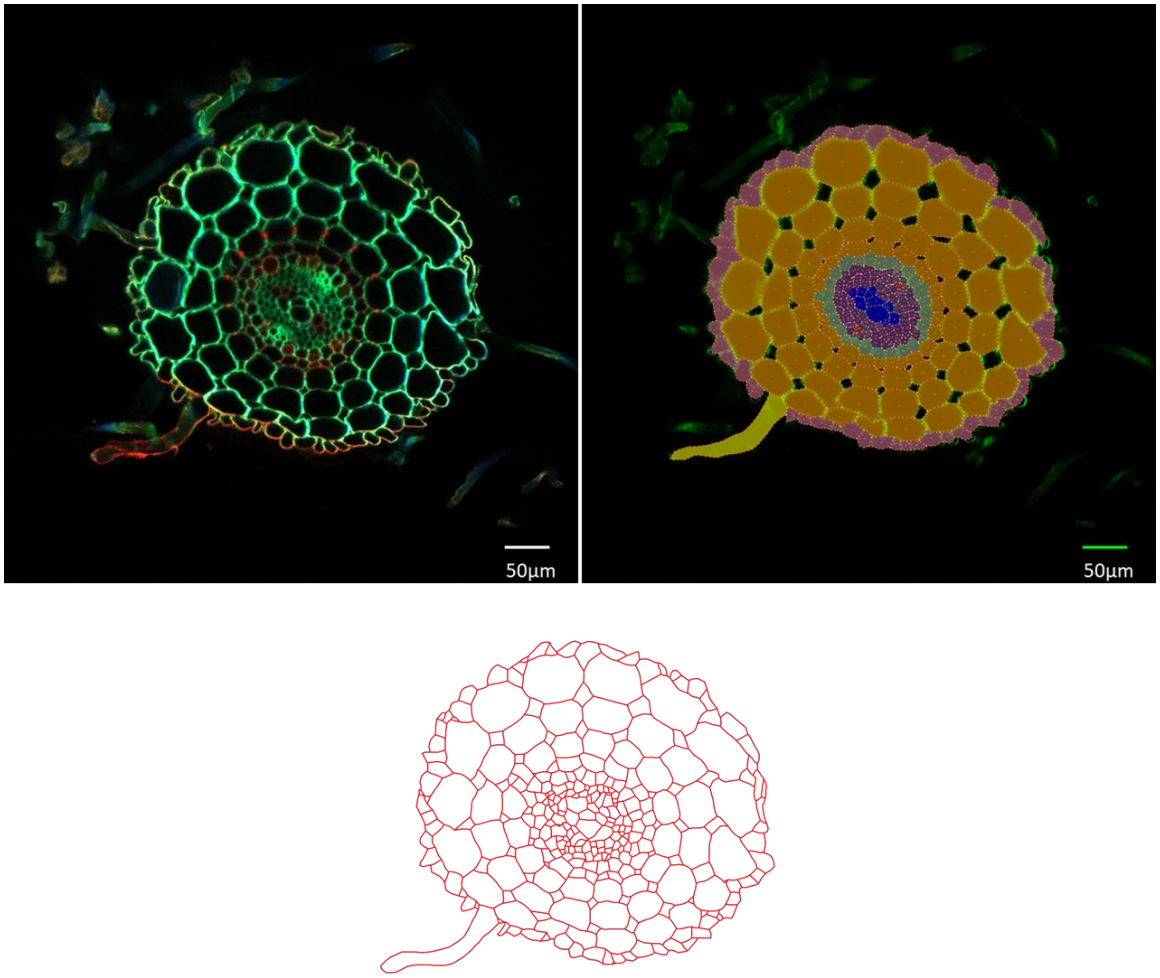

Figure 20: the original sectioning image (left), the labelled cell map (right), the outline (bottom). Cell colours in the central image have been chosen that maintain contrast for those with colour blindness to make the image accessible, an alternative would be to create a series of images with a single cell type highlighted followed by a composite image.

Colour chart: 1. (Yellow)- Root hair cell, 2. (Pink) – Epidermis, 3. (Orange) – Cortex, 4. (Turquoise) –Endodermis, 5. (Purple) – Stele, 6. (Blue) – Xylem, 7. (Red) – Phloem.

Figure 21 shows typical sections and cell maps for each of the varieties of *Brassica napus*, making a comparison between the varieties just from these photos would be difficult, potentially some measurements could be taken using a program such as ImageJ, however, analysis with CellSeT allows for a faster and much more in-depth analysis of the sections.

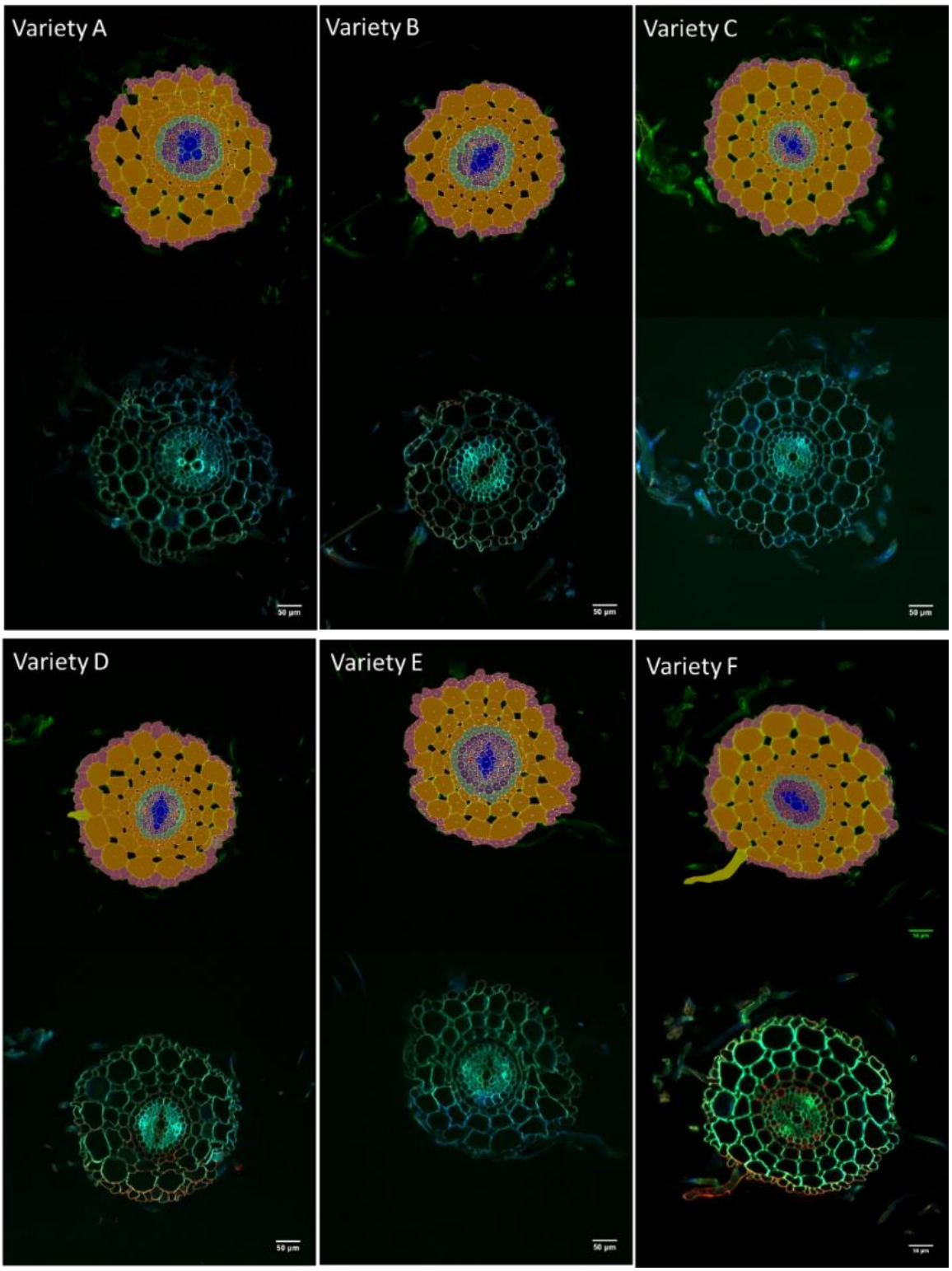

Figure 21: typical sections and cell maps for each variety. Varieties A-Prince, B-Caramba, C-Gefion, D-Pacific, E-Montego, F-Musette.

Colour chart: 1. (Yellow)- Root hair cell, 2. (Pink) – Epidermis, 3. (Orange) – Cortex, 4. (Turquoise) –Endodermis, 5. (Purple) – Stele, 6. (Blue) – Xylem, 7. (Red) – Phloem.

3.2.8 Root diameter analysis

ImageJ was used to analyse the root diameter by taking measurements across the widest point of the root section. As almost all sections showed some damage to the outer cell layers the diameter of the "core", comprising the endodermis, stele and vascular tissues, was also measured.

3.2.9 Cortical cell layers

The number of cortical cell layers, the cells between the epidermis and endodermis generally characterised as large cells with airspaces or aerenchyma between them, were counted on all of the 82 sections that were analysed for root diameter.

3.2.10 Damaged sections

Some of the roots were damaged during sectioning, while I did try to refine the technique for example changing the method to use low melt agarose to reduce heat damage, there is still scope for it to be improved further. While many of the more damaged section images had to be discarded, some of the damaged sections were still usable in my analysis. In order to use these sections in I estimated the fraction of the root that had been lost and used the data from the rest of the section to estimate the number of cells that would have been in the damaged section. An example is shown in figure 22, the undamaged sections were mostly symmetrical apart from the vascular system. Therefore, with sections like the damaged one in image 22 where the damage was to the outer layers an estimate of the proportion of missing cells was taken. In this example there is about 25% of the epidermis, cortex and endodermis missing from the section. Therefore, when the section was analysed using CellSeT the results for these layers were approximately 75% of what would have been measured from an undamaged section. The cell number and area results for the epidermis, cortex and endodermis were therefore recalculated using the estimated loss of cells to get an approximate value for what the results undamaged section would have been.

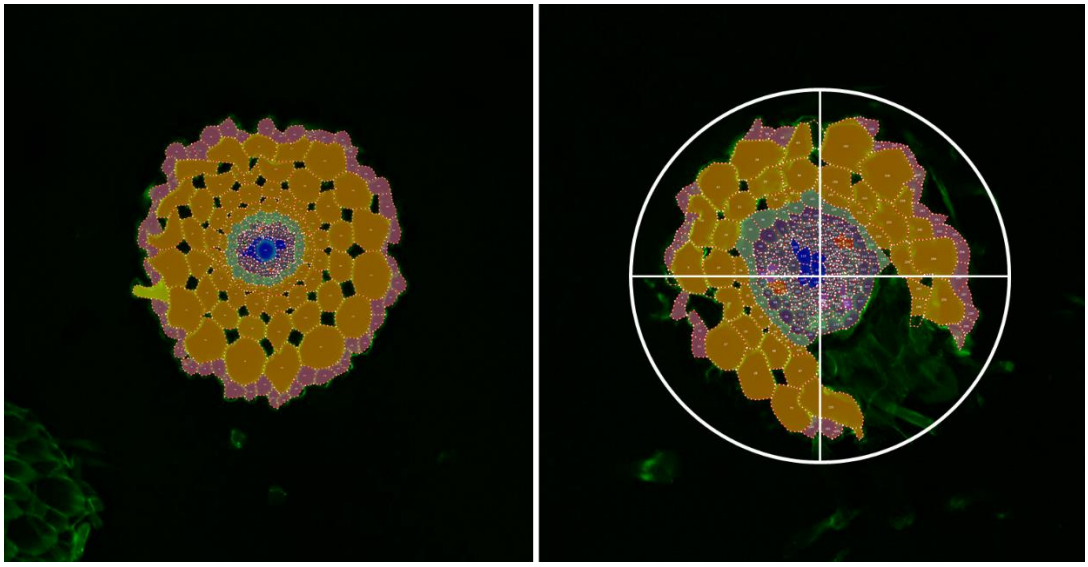

Figure 22: shows a comparison between the CellSeT analysis of a relatively undamaged section and that of a damaged section. The overlay on the damaged section is to show that an estimated 25% of the epidermis, cortex and endodermis have been lost.

3.2.11 Statistical analysis

In the root architecture experiment a total of 351 images were analysed using RootNav, 188 in the low group (Prince n=76, Caramba n=61, and Gefion n=51) and 163 in the high group (Pacific n=69, Montego n=64, Musette n=30).

In the root anatomy experiment 44 images were able to be analysed for cell number and cell area using CellSeT. A further 38 images were too damaged for CellSeT analysis of individual cell types but were able to be included in the analysis of root diameter giving a total of 82 images analysed for root diameter.

Table 4 - a breakdown of the number of samples analysed in the anatomy experiment. 44 sectioning images were analysed using CellSeT to gather data on the cell number and cell area of the sections. The same 44 images were analysed in ImageJ along with an additional 38 section images to gather root diameter data.

Anatomical trait	Total number of samples	Prince	Caramba	Gefion	Pacific	Montego	Musette
Cell number	44	6	7	8	10	7	6
Cell area	44	6	7	8	10	7	6
Root diameter	82	8	12	12	20	16	14

Initially a simple t-test was run in excel to determine if there was a significant difference between the two groups of varieties, high and low phosphorus. A p value below 0.05 was considered to be significant. To gain a clearer understanding of the relationships between the individual varieties the results from the 6 varieties were analysed in GenStat using a 1-way ANOVA. A p value below 0.05 was considered to be significant.

Additional post hoc analysis was carried out by comparing the mean of each variety to the mean of every other variety. If the means of the varieties varied from each other by more than the mean standard error of difference, then they were considered to be significantly different from each other. This same method was used to compare the means of the treatment groups to establish if the results of different treatments were significantly different from each other. This additional analysis shows which of the varieties or treatments are significantly different from each other and therefore shows if there are trends between the high and low phosphorus groups or between particular individual varieties.

3.3 Results

3.3.1 Comparison of root architecture traits between the high and low phosphorus accumulating *Brassica napus* lines

3.3.1.1 Selecting root architecture traits to measure.

Nine root architectural traits that may affect nutrient foraging were selected for study: Primary root length, lateral root length, total root length, lateral root number, convex hull area, maximum width of root system, maximum depth of root system, width/depth ratio and the y coordinate of the centroid. These traits were chosen because they are linked to the changes seen in roots experiencing low phosphorus conditions with plants increasing their root biomass to increase foraging and altering growth to a shallower root system that explores the topsoil where phosphorus is most abundant(Duan et al., 2020).

In Arabidopsis plants grown under low phosphorus conditions primary root growth is reduced in favour of lateral production to explore the soil (Abel, 2017). Therefore, measuring changes in primary root length, lateral length, total length and lateral number gives information about changes to the growth of different root types that may indicate a switch to topsoil exploration.

Not only is the length of the roots important but the area that the roots occupy. Depth, width, convex hull and y- coordinate of the centroid all describe the shape of the root system in the soil. The greater the area explored by the root architecture the more soil the plant can forage for nutrients. Particularly in the case of immobile nutrients such as phosphorus which the roots have to seek out the area explored is key to acquiring the phosphorus. The depth and width measurements can be used to produce a width/depth ratio which can be compared between varieties and phosphorus treatments to establish if the plants are changing their root arrangement, potentially a wider shallower root system may be seen as an adaptation in the high phosphorus varieties.

Convex hull is the area of the smallest convex polygon that covers the boundaries of the root system (see figure 23). Plants may increase the area that is foraged as an adaptation to improve phosphorus uptake (Adeleke, Millas, Mcneal, Faris, & Taheri, 2019). The centroid is the geometric centre of the convex hull, and its location relative to the seed is measured by coordinates on the x and y axis (Atkinson et al., 2015).

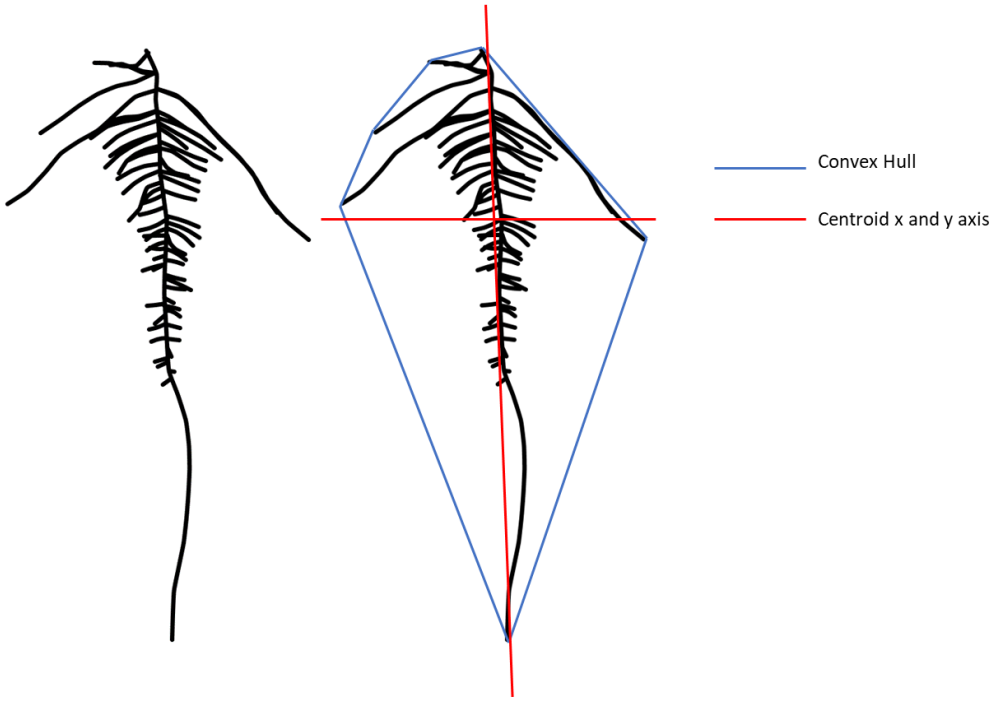

Figure 23: this diagram shows one of the root-nav root maps with the convex hull and centroid x and y axis overlaid. The convex hull is the area that the root architecture occupies. The centroid is the geometric centre of that area.

Since phosphorus is mostly found in the topsoil, plants with a greater volume of roots near the surface of the soil will be more efficient at taking up phosphorus. The centroid is a measure of where the geometric centre of the convex hull is, by looking at specifically the y-axis we get a measure of how far down the paper that is. Plants with a lower y-axis measurement have the centre point of their root mass at a shallower depth than those with a higher y-axis measurement (Atkinson et al., 2015).

3.3.1.2 Root length was significantly different between the groups of varieties.

Root length was analysed in three ways, primary root length, lateral root length, and the total length of all roots. Longer roots could indicate increased soil foraging, particularly when the lateral roots are longer. By including the total root length, it made it possible to establish if differences in primary and lateral length are due to an overall larger root system or if the different varieties have the same total amount of root but have prioritised different root types.

All of the root length measurements, primary root length ($p=0.01$), lateral root length ($p=0.01$) and total root length ($p=0.009$) were significantly different between the low (Prince, Caramba and Gefion) and high (Pacific, Montego and Musette) groups of accessions.

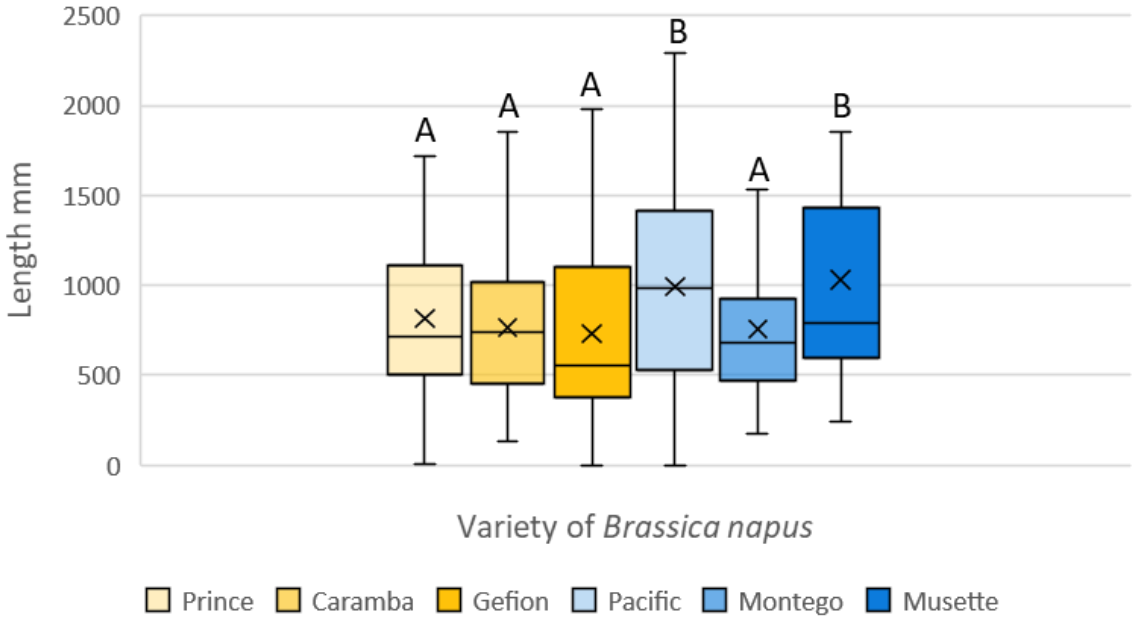

Figure 24 - figure showing the primary root length of *Brassica napus* varieties grown on ¼ strength MS media (9.68mg/L phosphorus). There was a significant difference between the primary root lengths of the high lines Pacific, Montego and Musette (blue) and low lines Prince, Caramba and Gefion (yellow) when the results of the varieties were combined (t-test p=0.012). The high group had significantly longer primary roots than the low group.

Further one-way ANOVA analysis showed that there was also a significant difference between individual varieties (p=0.003). Post-hoc analysis comparing the means showed that Pacific and Musette (B) were significantly different from the other varieties (A) but not significantly different from each other.

(Total:n=351 Low (yellow) - Prince:n=76 Caramba:n=61 Gefion:n=51 High (blue) - Pacific:n=69 Montego:n=64 Musette:n=30)

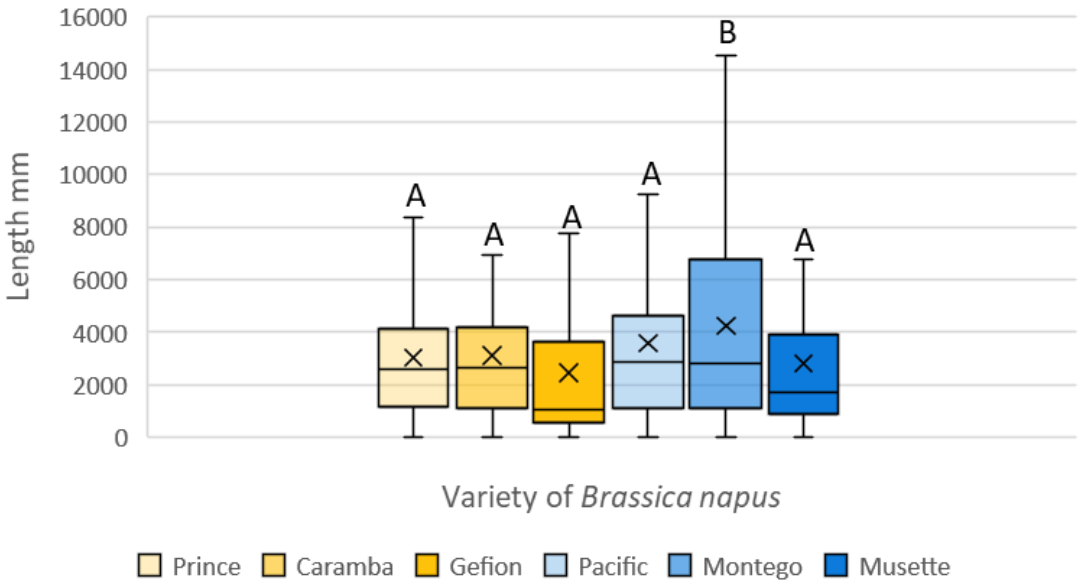

Figure 25 - Figure showing the lateral root length of *Brassica napus* varieties grown on ¼ strength MS media. A T-test showed that the high lines Pacific, Montego and Musette (blue) had significantly total longer lateral root length than the low lines Prince, Caramba and Gefion (yellow)(t-test p=0.012).

Additional one-way ANOVA analysis indicated that there was a significant difference between some of the individual varieties. Post hoc analysis showed that Montego (B) was significantly different from the other varieties (A) but that there were no other significant differences between individual varieties.

(Total:n=351 Low - Prince:n=76 Caramba:n=61 Gefion:n=51 High - Pacific:n=69 Montego:n=64 Musette:n=30)

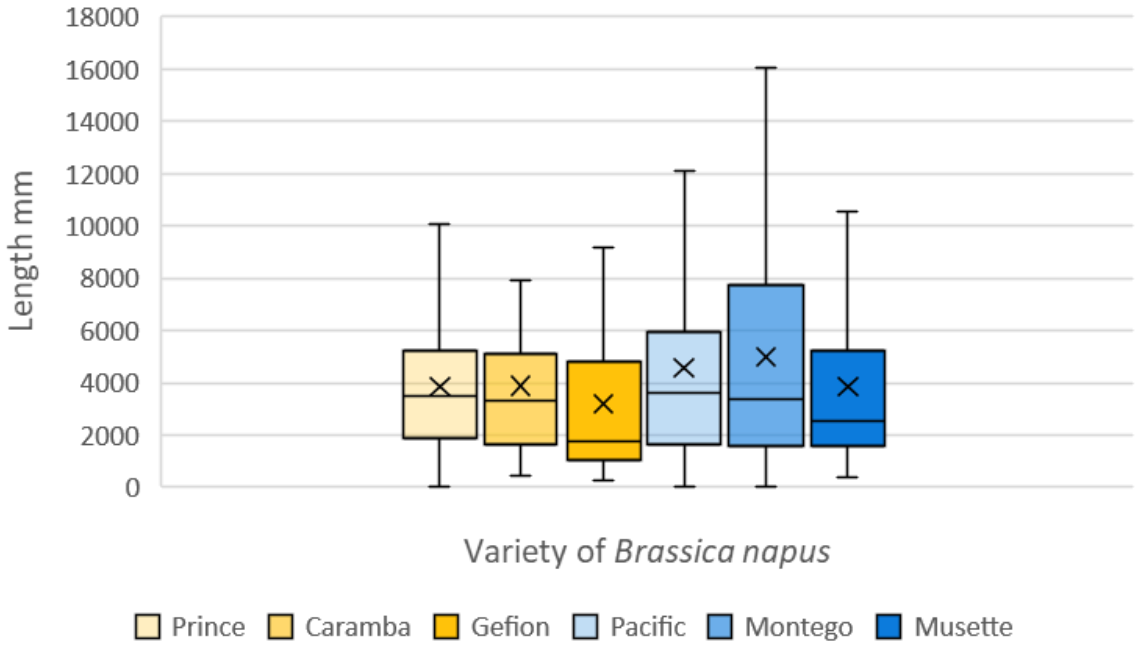

Figure 26 - Figure showing the total root length of *Brassica napus* varieties grown on ¼ strength MS media. The t-test showed that there was a significant difference in total root length between the low (yellow) and high (blue) varieties (p=0.009) with the high phosphorus varieties having significantly longer roots than the low varieties

There was however no significant difference between any of the individual varieties of *Brassica napus*

(Total:n=351 Low - Prince:n=76 Caramba:n=61 Gefion:n=51 High - Pacific:n=69 Montego:n=64 Musette:n=30)

There were also significant differences between the varieties in primary root length (figure 24) (p=0.003) and lateral root length (figure 25) (p=0.031) however the total root length (figure 26) was not significantly different (p=0.06) between the 6 varieties of *Brassica napus*. The Primary root lengths of the varieties Pacific and Musette were significantly different from the other varieties but not significantly different from each other (figure 24). The lateral root length of Montego was significantly different from that of all of the other varieties (figure 25).

3.3.1.3 Lateral root count was not significantly different between the groups.

As lateral roots are key to soil exploration for nutrients both the lateral length and the number of laterals that the plants produced was of interest. The RootNav program produced a count of the laterals of each plant which could then be analysed to see if there was any difference in the number of laterals each variety produced. There was no significant difference between the number of laterals produced by the plants of the low and high phosphorus groups of varieties ($p=0.147$) The number of lateral roots was also not significantly affected by the variety of *Brassica napus* being studied ($p=0.109$).

3.3.1.4 Root area correlates to phosphorus acquisition

As both the area the roots explored and the arrangements of the roots within that area were important several characteristics were measured. The convex hull was used to measure the maximum area that the roots were covering. The maximum width, maximum depth, width depth ratio and the y coordinate of the centroid were measured to show if the arrangement of the roots varied within the area that they covered. A change to the width and depth may indicate if a variety has a shallower root system which may be advantageous for foraging the topsoil. If a variety has changes to the width and depth but not to the ratio, then that would indicate that the root system has increased in size but that there has been no change in the arrangement of the roots.

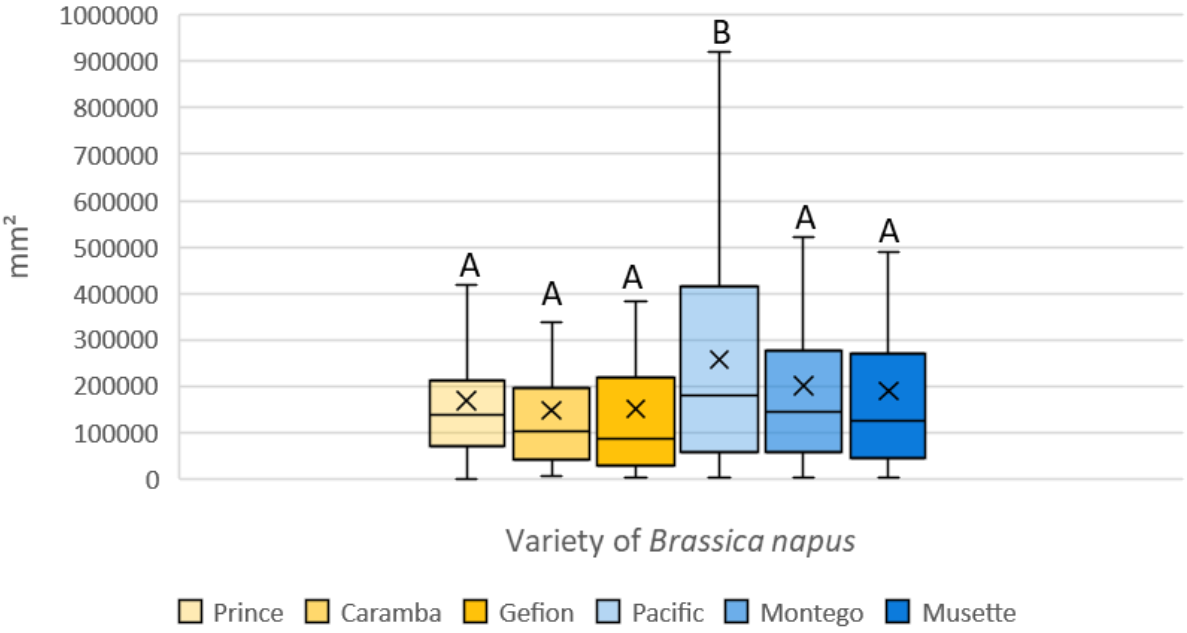

Figure 27- Shows the Convex hull area (mm²) of 6 varieties of *Brassica napus* when grown with a ¼ strength MS media treatment. A t-test showed that the convex hull of the low (yellow) varieties had a significantly lower area than that of the high (blue) varieties (t-test p=0.001).

Post-hoc analysis using an ANOVA showed that there were significant differences between some of the individual varieties (p=0.009). Pacific (B) had a significantly larger convex hull area compared to the other varieties.

(**Total**:n=351 **Low** - Prince:n=76 Caramba:n=61 Gefion:n=51 **High** - Pacific:n=69 Montego:n=64 Musette:n=30)

Convex hull area was significantly different between the two groups of varieties (p=0.001). The low phosphorus group, Prince, Caramba and Gefion have a smaller convex hull area than the high group Pacific, Montego and Musette.

There were also differences in the area of the convex hull (figure 27) depending on which individual variety was being grown (p=0.009). Pacific had a significantly different convex hull area compared to the other varieties. The convex hull area of the Pacific plants varied more than the plants of any other accession (figure 27)

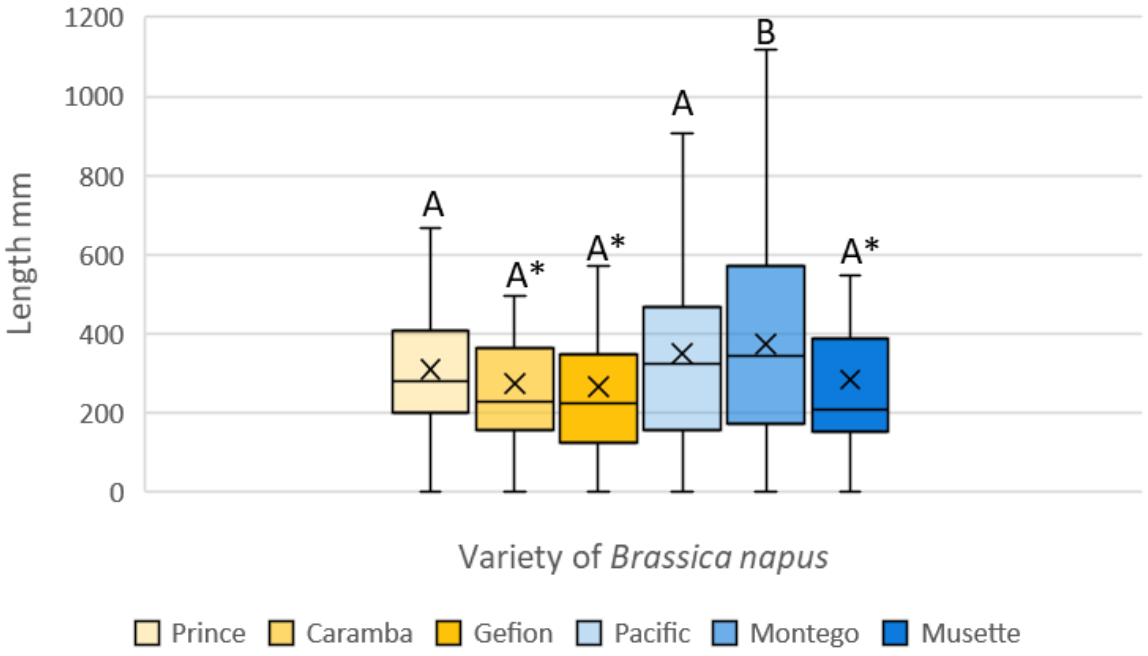

Figure 28- shows the maximum width of the roots of 6 varieties of *Brassica napus* when grown with a ¼ strength MS media treatment. The two groups, low (yellow) and high (blue) were significantly different when compared using a t-test (p=0.004) with the high group having woots that spread significantly wider than the low group.

Further analysis with a one-way ANOVA showed that there was a significant difference in the width of root spread between the individual varieties (p= 0.017). Post hoc tests revealed that Montego (B) has significantly wider roots than Caramba, Gefion and Musette (A*). The width of the roots wan not, however, significantly different from Prince or Pacific (A). None of the other varieties are significantly different from each other

(**Total**:n=351 **Low** - Prince:n=76 Caramba:n=61 Gefion:n=51 **High** - Pacific:n=69 Montego:n=64 Musette:n=30)

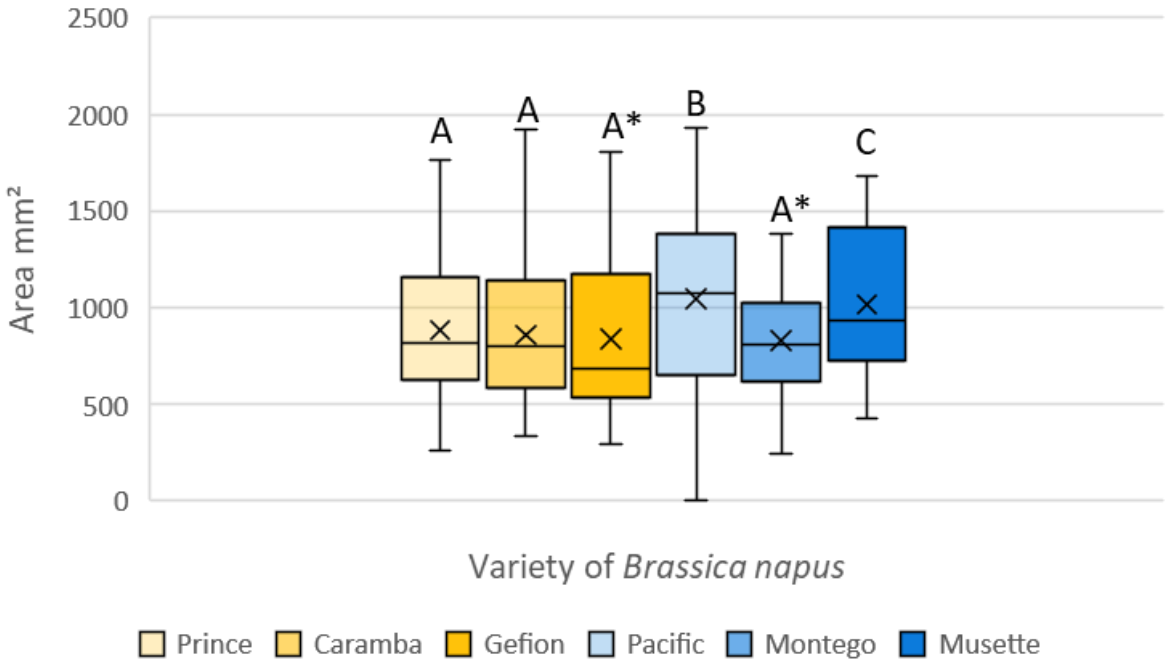

Figure 29 - shows the maximum depth of the roots of 6 varieties of *Brassica napus* when grown with a ¼ strength MS media treatment. the low group varieties (yellow) had a significantly shallower root depth compared to the varieties in the high group (blue)(t-test p=0.011).

A one-way ANOVA also indicated significant differences between individual varieites (0.002).

This was confirmed with post-hoc analysis which showed that Pacific (B) had significantly deeper root depth to all other varieties except for Musette (C). Additionally, Musette (C) had significantly deeper roots than Gefion and Montego (A*). Prince, Caramba, Gefion and Montego were not significantly different from each other.

(**Tota**l:n=351 **Low** - Prince:n=76 Caramba:n=61 Gefion:n=51 **High** - Pacific:n=69 Montego:n=64 Musette:n=30)

Both the maximum width (figure 28) and the maximum depth (figure 29) that the roots reached significantly changed between the two groups of accessions (Max width p=0.004, max depth p=0.01) There were also additional differences between the varieties of *Brassica napus.* Post hoc tests revealed that the width of Montego (B) is significantly different from that of Caramba, Gefion and Musette (A*). It is not however significantly different from Prince or Pacific (A). None of the other varieties are significantly different from each other (Width p=0.0017) (figure 28). The root depth of Pacific (figure 29) was significantly different from that of all the

other varieties except for Musette, while Musette was significantly different from both Gefion and Montego (Depth p=0.002). However, despite the changes in both width and depth the width/depth ratio was not significantly different either between the low and high groups (p=0.732) or when examining the individual varieties of *Brassica napus* (p=0.062).

The y coordinate of the centroid was not significantly affected whether the results were considered within the low and high groups (p= 0.116) or as individual varieties of *Brassica napus*(p=0.119), meaning the geometric centre of the convex hull did not significantly move higher or lower.

3.3.2 Comparison of root anatomy traits between the high and low phosphorus *Brassica napus* lines

The sections were from the plants grown for the root architecture experiment using the pouch and wick method as laid out above. These plants were all grown under identical conditions and were treated the same before being sectioned. Of the 103 sectioning images produced, 44 were able to be analysed using the program CellSeT. The other images were either too blurred or too damaged for an accurate CellSeT analysis to be done. The cell set analysis produced data on the number of cells of each tissue type and the area of these cells. Additional analysis was done using ImageJ to measure the diameter of the sections, for this analysis an additional 38 images were able to be analysed resulting in a total of 82 sections being analysed for root diameter.

3.3.2.1 Cell number does not correlate to phosphorus acquisition.

Cell number was investigated for each cell type to establish if there was an increase or decrease in any of the cell types across the different varieties. 44 sections were analysed using CellSeT. None of the cell types showed any significant difference between the two groups of accessions when analysed with a t-test. Additional ANOVA analysis showed that the only cell type that showed a significant difference between the individual varieties was the epidermal cell layer (figure 30) (p=0.028). The Low phosphate group variety Caramba was significantly different from Gefion,

Pacific and Montego but not to Prince and Musette. Additionally, Musette was significantly different from Pacific. Prince, Gefion, Pacific and Montego are not significantly different from each other. Caramba generally had a higher number of epidermal cells than the other varieties while Musette had the greatest range in epidermal cell numbers. All of the other cell layers showed no significant difference in number between the six varieties (see supplemental file in Appendix 7)

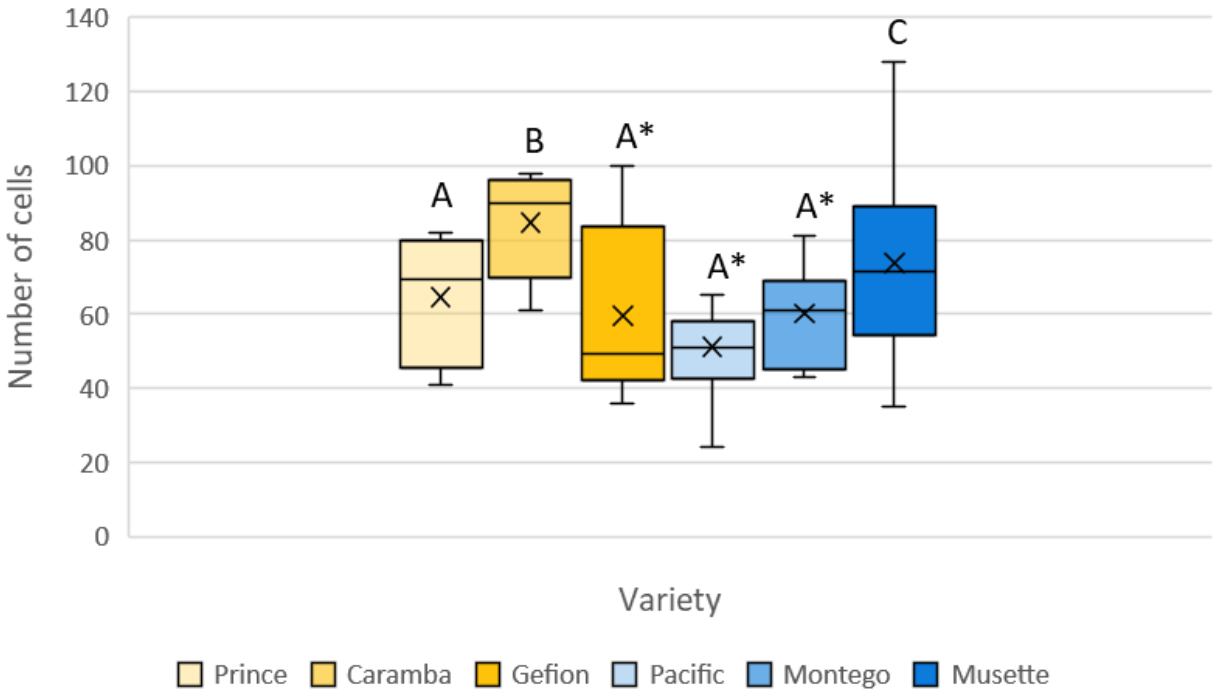

Figure 30 - shows the total number of epidermal cells in root cross-sections of 6 varieties of *Brassica napus* when grown with a ¼ strength MS media treatment. There is no significant difference in the total number of epidermal cells between the low (yellow) and high (blue) groups of accessions (t-test p=0.142). There was, however, a significant difference between individual varieties number of epidermal cells (p=0.028)

Caramba (B) had significantly more epidermal cells than Gefion, Pacific and Montego (A*) but was not significantly different from Prince (A) and Musette (C). Additionally, Musette (C) had significantly more epidermal cells than Pacific.

Prince, Gefion, Pacific and Montego are not significantly different from each other.

(Total:n=44 Low - Prince:n=6 Caramba:n=7 Gefion:n=8 High - Pacific:n=10 Montego:n=7 Musette:n=6)

3.3.2.2 Root diameter is not correlated to phosphorus acquisition.

Root diameter was measured in ImageJ taking the measurement in micrometres across the widest point to get the diameter of the root section. As almost all sections showed some damage to the outer cell layers the diameter of the "core", comprising the endodermis, steele and vascular tissues, was also measured. 82 of the sectioning images were able to be analysed for diameter. A comparison of the low (Prince, Caramba and Gefion) and high (Pacific, Montego and Musette) accessions showed that there was no different in the diameter of either the whole root or the core of the root.

3.3.2.3 Cortical cell layers are not correlated to phosphorus acquisition.

A T-test showed that there was no difference in the number of cortical cell layers between the low and high groups of accessions (p=0.09). Further statistical analysis showed that there was also no significant difference in the number of cortical cell layers that the different varieties of *Brassica napus* had (p=0.068)

3.3.2.4 Cell area is not correlated to phosphorus acquisition.

The area of each tissue type was measured on the 44 images that were analysed using CellSeT. Two area measurements were analysed, the area in μm^2 of the tissue and the percentage of the total area of the root taken up by the tissue. The two groups showed no difference in the area of different tissues, total area or the percentage of the root made up by any one tissue. There was one measurement where there was a significant difference between some of the individual varieties, the percentage of the roots made up by cortical cells (figure 31) (p=0.023). The variety Pacific is significantly different from all other varieties except for Gefion. None of the other varieties are significantly different from each other. Pacific has a higher percentage of its total root area made up by cortical cells.

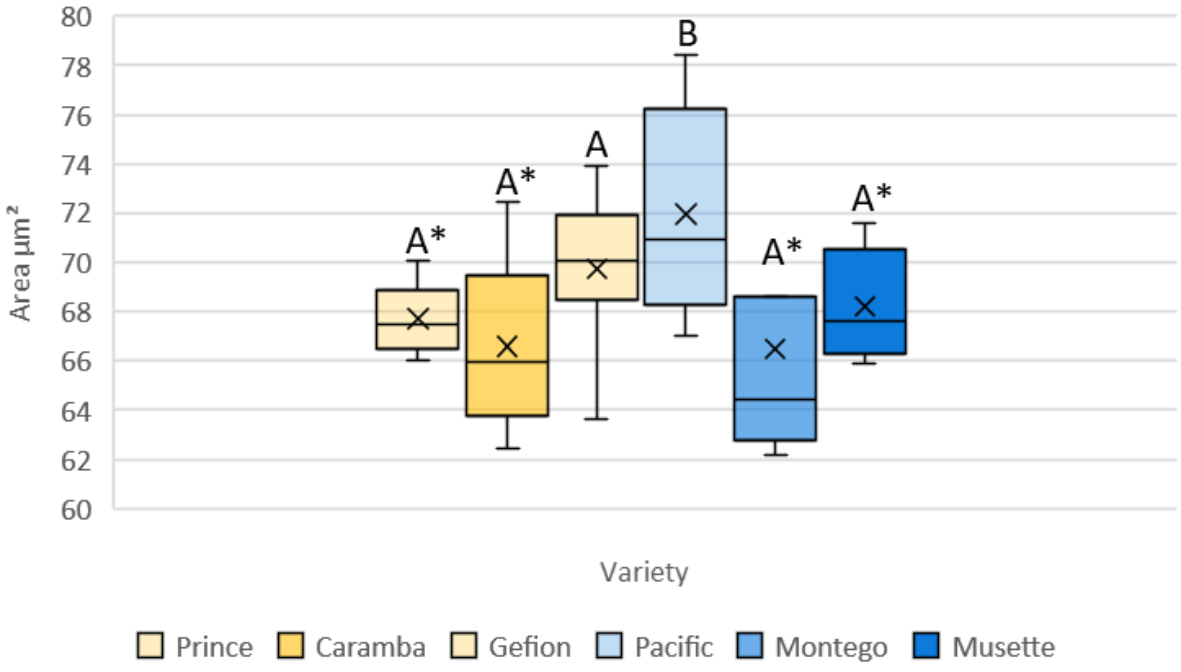

Figure 31 shows the percentage of the root that was taken up by cortical cells in 6 varieties of *Brassica napus* when grown with a ¼ strength MS media treatment. A T-test showed that there were no significant differences between the high (blue) and low (yellow) groups of varieties t-test (p=0.315)

There was, however, a difference between the individual varieties in the percentage of the root taken up by cortical cells (p=0.023).

Pacific (B) had significantly more of its area taken up by cortical cells than all the other varieties (A*) except Gefion (A). None of the other varieties are significantly different from each other.

(Total:n=44 Low - Prince:n=6 Caramba:n=7 Gefion:n=8 High - Pacific:n=10 Montego:n=7 Musette:n=6)

3.4 Discussion

3.4.1 Root architecture

The root architecture experiment was testing for any correlation between the leaf phosphorus content of the two groups of accessions chosen from the 80-winter oilseed rape modern accessions in the RIPR population and their root architectural traits. The two groups were made up of low (Prince, Caramba and Gefion) or high (Pacific, Montego and Musette) phosphorus accessions based on element analysis of the leaves when grown in soil experiments (Alcock et al, 2016). In addition to

comparing the two groups the individual varieties were also compared to identify any additional differences in root architecture traits in order to establish if all accessions within a group were displaying the same traits.

There was a significant difference between the high and low groups for all of the root length measurements; primary root ($p=0.012$), lateral root ($p=0.012$) and total root ($p=0.009$). Which suggests that root length may affect the phosphorus uptake between the two groups.

However, on further examination the individual varieties within the groups do not all show the same results. In particular, it can be seen in figure 24 that in the high phosphorus group Pacific and Musette have significantly longer primary roots than the other varieties while Montego is not significantly different from any of the varieties in the low group. Figure 25 shows that for the lateral root length the inverse is true with Pacific and Musette showing no significant difference to the low group varieties while Montego has significantly longer lateral roots than the low group varieties.

Overall, the total length of the roots is significantly different between the low and high groups ($p=0.009$), with the high group having a greater total root length than the low group. This is consistent with expectation that longer roots increase the root area available for phosphorus uptake. However, the inconsistency between the three accessions in the high group suggests that the differences in phosphorus uptake between these varieties and the other group are not due to simply increasing the length of the roots. Potentially, the differences between the high phosphorus varieties indicate that there may be different strategies for phosphorus acquisition. For example, the results indicate that Montego has increased lateral root growth compared to both the low group varieties and to the other high group varieties. This could increase phosphorus acquisition through increased lateral root area and increased soil exploration. However, Pacific and Musette have significantly increased primary root length compared to both Montego and the low phosphorus varieties. Increasing the primary root length could also increase phosphorus acquisition through greater root area and increased soil foraging. While apparently opposites both strategies would potentially increase the phosphorus acquisition of

the plants. These results fit with the hypothesis that the high phosphorus varieties would display an increase in traits that have been linked to phosphorus acquisition compared to the low group. However, it appears that the high group varieties may have different phosphorus acquisition strategies.

The number of laterals does not significantly vary between the groups (p=0.116) or between any of the individual varieties suggesting that the number of laterals is not an important factor in the differences in phosphorus uptake seen between the two groups of varieties. While lateral number has been linked to increased phosphorus acquisition in other plants it does not appear that modulating the lateral number is a strategy that is used by *Brassica napus* to increase phosphorus uptake.

The maximum width and maximum depth both showed a significant difference between the low and high groups (width p=0.004, depth p=0.011). The high group of accessions had roots that spread both wider and deeper than the low group. However, the width to depth ratio was not significantly different between the two groups (p=0.732). Since the width and depth changes significantly between the groups while the width to depth ratio remains the same this implies that the whole root system is covering a larger area in the high group but that the overall shape of the root system remains broadly same and that there is not a switch to deeper roots or to more widely spread roots.

The convex hull of the root systems is also significantly different (p=0.001) between the two groups with the high group varieties having a larger area than the low group. Since the roots of the high group have a wider spread and grew deeper than the roots of the low group plant the total area that the roots occupy is also increased. This would allow the plants to forage a greater volume of soil which could be part of why these varieties had greater phosphorus uptake in the RIPR population element analysis experiment.

The results from the width, depth, ratio and convex hull would seem to suggest that the high phosphorus varieties have a root system that covers a larger area than the low phosphorus varieties but that is broadly the same shape. Figure 32 below

illustrates how increasing the width and depth of a root system could result in changes to the convex hull without changing the width/depth ratio.

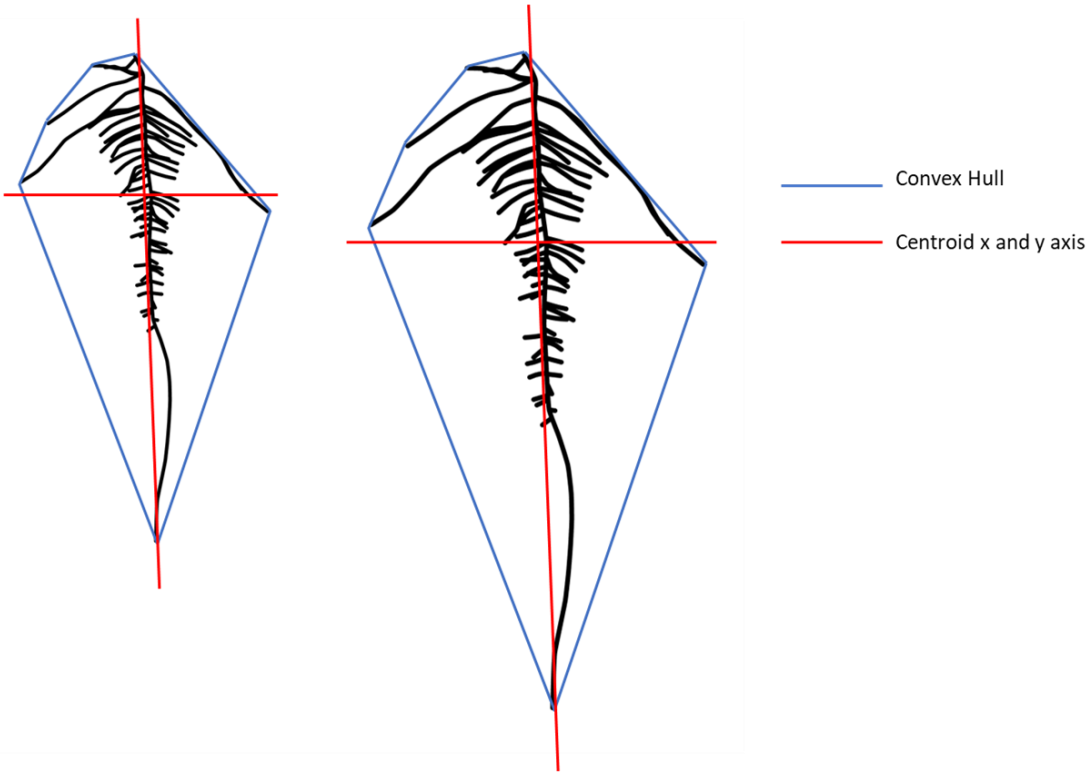

Figure 32: this figure illustrates how a change in the size of a root system could change both the convex hull and the centroid without these changes indicating changes in the shape of the root system.

However, the centroid y axis of the root system was not significantly different between the two groups (p=0.116). This means that while the overall maximum width and depth measurements have increased the centre of mass of the convex hull of the root system has not changed. Therefore, although the root area of the high group is larger than that of the low group the majority of the root area is at the same depth in the soil across all the varieties from both groups. As phosphorus is most abundant in the topsoil it is possible that while the high phosphorus varieties

have an increased root area exploring the soil, as indicated by the width, depth and the convex hull results, the centre of mass of their roots remains located in the topsoil to exploit the higher phosphorus levels found there. This would also link back to the results from the root length measurements where the high phosphorus varieties had a higher total root length than the low phosphorus varieties. The increases to the primary root length of Pacific and Musette correlates with the increase in the maximum depth measurements seen in figure 29 while the increased lateral root length of Montego correlates the to the increased width measurements seen in figure 28.

3.4.2 Confounding factors created by choice of media.

Using the MS media at ¼ strength was intended to mimic low phosphorus conditions that plants may experience when grown in soil, however, this method also reduces the amount of essential nutrients other than phosphorus found in the media. This means that the differences seen here may be linked to phosphorus as hypothesised, or the differences could be due to a deficiency in another nutrient.

Nitrogen deficiency is known to alter root architecture in *Brassica napus*. Plants grown in a hydroponic experiment for 7 days with low nitrogen conditions were found to have alternations in their root architecture (Qin et al, 2019). A Chinese-grown accession of *Brassica napus*, Zhongshuang 11, was used for these experiments. The plants were grown in hydroponics for 7 days at 24°C/20°C day/night temperature with a 14 hour photoperiod and were photographed each day, these photographs were used to measure root traits using RootReader3D which allows 3D reconstruction of plant roots from images (L. Qin et al., 2019). This experiment found that the total root length and maximum depth that roots reached were most sensitive to nitrogen deficiency, although there were also changes in lateral number, width and convex hull volume. Plants experiencing nitrogen deficiency altered root growth to expand the soil volume explored through deeper and wider root systems with a greater root length and convex hull volume. These results are similar to those seen in my experiment, although my experiment

was 2D not 3D, where some varieties increased their root depth or width and had a larger convex hull.

It is therefore possible that the differences between the high and low groups in my experiment are not entirely due to phosphorus foraging adaptations. Potentially the changes seen may be in response to nitrogen deficiency. However, the results appear to fit the hypothesis that the accession with high leaf phosphorus in the RIPR population would have traits that correlate to phosphorus acquisition. Therefore, in order to establish whether these results are due to adaptations to phosphorus acquisition or low levels of essential nutrients other than phosphorus it is necessary to repeat the experiment with an alternative nutrient solution which only alters phosphorus content.

3.4.3 Root Anatomy

Analysis of the anatomical traits showed that there were no differences between the high and low groups when it came to cell number, the number of cortical cell layers, diameter of either the core or the whole root. There were however some differences between individual varieties (see supplementary material appendix 7)

There was no significant difference between the high or low groups. There was also no significant difference between the individual varieties in the number of cells for most of the cell types. The only exception to this was the epidermal cell layer which showed some significant differences between the 6 varieties of *Brassica napus* ($p=0.028$). As is shown in figure 30 Caramba was significantly different from Gefion, Pacific and Montego but not to Prince and Musette. Additionally, Musette is significantly different from Pacific. Prince, Gefion, Pacific and Montego are not significantly different from each other.

There is however a possibility that this significant result is due to the method used rather than to a biological cause. The epidermis was the most frequently damaged layer during the creation of the sections. The roots were damaged by heat, drying out, mechanical stress from being removed from the paper sheets and as the

surface layer the epidermis was the most severely damaged when damage occurred. Therefore, the significant difference in cell number could be due to the damage suffered by the sections affecting the results. Furthermore, it is likely that any mistake on one root, for example allowing the samples to dry out too much, would also affect all the samples in the same agarose block. As I usually section multiple plants from a single variety at a time this means that any mistakes on my part could have caused the data on epidermal cell number for that variety to become skewed. Caramba and Musette had the most heavily damaged sections of the varieties, 2/6 of Caramba and 3/7 of musette sections analysed for cell type and area were seriously damaged. This supports the idea that the significant differences seen in the cell number were caused by damage to the samples during sectioning.

There is no significant difference between either the groups or the individual varieties when it comes to the area each cell type takes up. There was also no difference between the high and low groups in the percentage of the root made up by each cell type. However, there is a difference in the percentage of the total area of the root that is taken up by the cortex cells depending on the variety of *Brassica napus*. As with the significant difference between the epidermal cell numbers it is possible that this is due to damage sustained during the sectioning process. The cortical cells were sometimes distorted by heat damage which caused the cells to become shrivelled, and this could have affected the data on the area of the root occupied by cortical cells.

3.4.4 Primary and Secondary root growth

The roots of dicotyledonous species expand through secondary growth. During secondary growth the epidermis, cortex and endodermis of the root are lost and replaced with secondary xylem, parenchyma and periderm. In cross sections this is visible as the loss of the epidermis, cortex and endodermis, the expansion of the stele and an increase in the size and number of metaxylem vessels (Strock et al, 2018).

Changes to the secondary growth of roots have been seen in a variety of dicotyledonous species undergoing phosphorus stress. These changes include a decrease in the diameter of both the whole root and the stele, a reduction in the number and size of epidermal cells and metaxylem vessels, a reduction in the percentage of root area taken up by the stele and a reduction in cortical cells and xylem vessels (Sarker et al, 2015)(Strock et al, 2018). Phosphorus stress has also been shown to reduce secondary growth in common bean, *Phaseolus vulgaris,* in a genotype dependant manner, where genotypes with a greater reduction in secondary growth had a reduction in their metabolic costs, greater root length, increased shoot biomass and had improved phosphorus capture (Schneider & Lynch, 2020).

However, the plants in the sectioning experiment had not yet begun to have secondary root growth. This can be seen due to the intact epidermis, cortex and endodermis layers visible within the sections. My results suggest that there is no difference between the groups during primary root growth. Since responses to phosphorus availability are known affect the secondary root growth of dicots any differences between the high and low group may become apparent during secondary growth. Therefore, further experiments with older plants that have begun secondary root growth may provide evidence of differences in anatomical characteristics between the groups. This would require an alternative growth method to the pouch and wick system, however, as the pouch and wick only allows the plants to grow for approximately two weeks before the root system becomes too large for the pouches. Potentially hydroponics would be suitable as this would allow the plants to grow for longer than the pouch and wick system accommodates, would allow control of the phosphorus provided to the plant through solution and would allow easy sampling of the roots without the need for washing to remove soil. Alternatively, the plants could be grown on a phosphorus free substrate which could be irrigated with a phosphorus containing solution. These methods would be preferable to a soil based one since the phosphorus could be uniformly provided to the plants whereas soil has heterogenous phosphorus which could affect the results.

2.5 Conclusion

Overall, there are some interesting differences between root architecture traits of the high and low groups which suggest that there may be differences in root structure that are correlated to phosphorus uptake. However, these differences are not universal across all the accessions within the groups, suggesting that the increased phosphorus uptake seen in the RIPR population may be due to different adaptations in different varieties. There is also a possibility that the differences could be due to the low level of nutrients other than phosphorus, for example nitrogen, caused by using the 1/4 strength MS media. Therefore, these varieties need further experiments to be run using different media before the differences in root architecture can be confidently correlated to phosphorus uptake.

There are very limited differences between the anatomy of the six varieties of *Brassica napus* and these differences may be due to mistakes during the sectioning process. Additionally, the plants had not begun to have secondary growth in their roots. Repeating this experiment using samples from older plants might yield different results, however, that would require plants grown via another method since the Pouch and Wick system used here is limited to at most about two weeks for *Brassica napus*. Beyond two weeks there are problems with microbial growth on the paper and with the roots growing too long to be supported by the paper.

Chapter 4 – *Brassica napus* accessions show root architecture and biomass traits that correlate to phosphorus accumulation.

4.1 Introduction

4.1.1 Phosphorus concentration

There are 14 inorganic elements that plants require from soil, or from media solution if grown in a hydroponic system. These are divided into macronutrients and micronutrients based on the amount required by plants. The macronutrients are nitrogen, phosphorus, potassium, calcium, magnesium and sulphur (de Bang et al, 2021). Of these the most important limiting factors for plant growth are nitrogen, phosphorus and potassium (Kulcheski et al, 2015).

In the previous root architecture experiment a ¼ strength solution of MS media was used to provide the plants with a moderately low phosphorus media. This was done with the aim of finding correlations between the low and high phosphorus accumulating varieties selected from the RIPR population. However, by using ¼ strength MS media not only was the phosphorus content reduced but the availability of other nutrients was also reduced, including nitrogen and potassium.

In *Brassica napus* plants grown in hydroponics with low nitrogen content in the solution had a significant increase in their root biomass (Tian et al, 2022). Root length was also increased, with the root length of plants grown under low nitrogen 2.37 times longer than that of plants grown in high nitrogen conditions (Tian et al, 2022). Lateral root number is also affected by low nitrogen, 2 week old *Brassica napus* seedlings grown in hydroponic culture on modified Hoagland nutrient solution had a significant increase in their lateral root number under low nitrogen conditions (Shen et al, 2022).

Potassium deficiency also affects root growth in *Brassica napus*. 22 day old *Brassica napus* seedlings grown in hydroponics with either a low (0.05mM) or optimal (2.0mM) concentration of potassium had a reduced dry root weight under the potassium deficient conditions (Wang et al, 2021). While this experiment only measured the root dry weight rather than specific root architectural traits a change in the root weight is likely to correspond to changes in architecture.

In the ¼ strength MS media experiment, it was not possible to separate the effect of the different phosphorus accumulating groups on root architecture from differences in root architecture caused by a deficiency in a nutrient. Only the possible effect of nitrogen and potassium deficiencies have been highlighted, potentially there could be other effects due to the reduction in the nutrient solution concentration affecting other elements. Therefore, the experiment was repeated using an alternative solution which would specifically alter the phosphorus available to the plant. To do this a recipe was used that was based on Hoagland solution, a hydroponic nutrient solution which has been widely used for *Brassica napus* experiments (Ayyaz et al, 2021)(Seepaul et al, 2019)(Abbaspour & Mousavian Kalat, 2023).

4.1.2 Hydroponics growth systems

The pouch and wick system is useful for high throughput analysis of root architecture traits however it is limited by the size of the paper the roots grow on. This means that for *Brassica napus* the seedlings can be grown to a maximum of 2 weeks old. As mentioned in the introductory chapter other hydroponic growth methods have been used to grow a range of species to maturity including lettuce, herbs, tomatoes, cucumbers, sweet peppers, strawberries and cannabis (Bian et al, 2020) (Chen et al, 2020) (Gomez et al, 2019).

There are several different ways to construct a system for hydroponically growing plants several of which are shown in figure 33 below (Sharma & Singh, 2019). Some of these systems are passive such as the wick system (A) and deep-water culture (D), while the others require a power supply in order to pump the media to the plants.

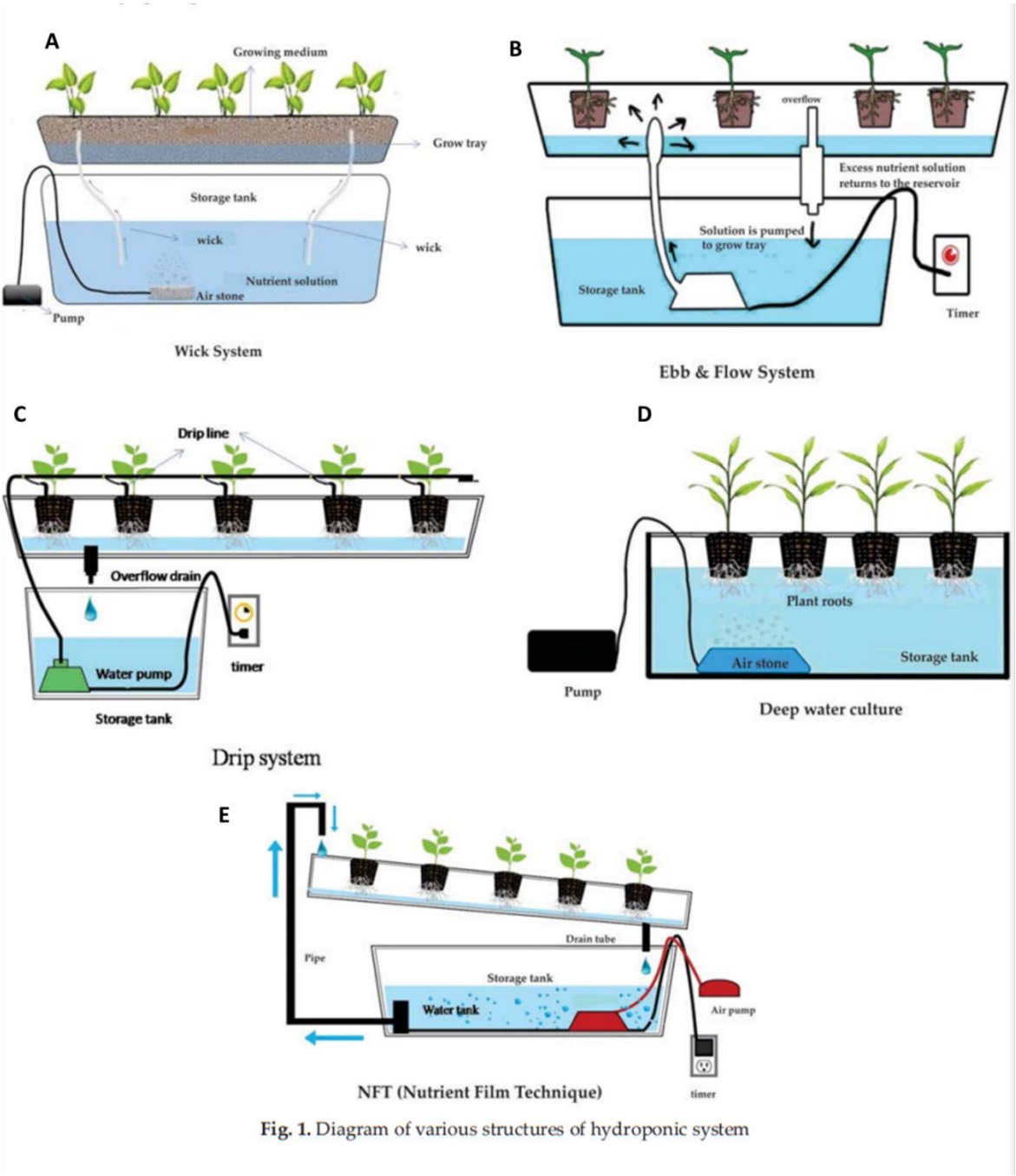

Figure 33: figure showing 5 different hydroponic systems; wick system (A), ebb and flow system (B), drip system (C), deep water culture (D) and nutrient film technique (E). (Sharma & Singh, 2019)

Wick systems (A) use capillary action to supply plants with nutrient media. The plants are placed in a growth tray filled with an absorbent medium such as perlite, vermiculite or bark and a nylon wick runs from the plant roots to a media reservoir. As the plants absorb the nutrient media more media is pulled from the reservoir to the growth tray (Sharma & Singh, 2019). This system benefits from the fact that the

roots are not submerged in the media which can lead to problems with oxygenation and rot. However, due to the use of capillary action it is only suitable for small plants with low water requirements (Sharma and Singh, 2019)

Deep water culture (D) does not require any movement of water from the reservoir, instead the roots of the plants are suspended directly in the nutrient solution reservoir. As the roots are submerged in still water aeration is required to provide the roots with oxygen (Sharma & Singh, 2019). Insufficient oxygen levels around roots can reduce plant productivity (Colombi & Keller, 2019). Additionally, there can be issues with algae and mould growth in the media reservoir which can affect the nutrient concentration, salinity and pH. It is therefore necessary to monitor these in addition to monitoring the oxygen level. This system can be as simple as media in a bucket with plants suspended in a net. However, in a commercial or research setting the bucket is often filled with perlite or another inert substrate for both anchorage and due to the aeration provided (Yang et al, 2023). There have been experiments with *Brassica napus* in which plants were grown in deep water culture without being provided with an anchoring substrate or additional aeration and were grown successfully for 7 weeks after germination (Chen et al, 2023).

Both deep water culture and wick hydroponics benefit from not requiring a power supply as the media is not being pumped between containers. Wick hydroponics has been suggested as an option for agriculture in areas without a consistent power supply (Ferrarezi & Testezlaf, 2016).

Ebb and flow hydroponics (B) works in a tidal manner, nutrient solution from the reservoir is pumped into the plant growth tray and allowed to flood the roots until it reaches a fixed level, the plants remain flooded for a period of time before the nutrient solution is pumped back into the reservoir (Sharma & Singh, 2019). The plants are anchored in the growth tray using a soilless substrate, for example clay gravel. This method allows the water supply to the plants to be controlled through altering the length of time that the plants are flooded and is generally used for the cultivation of aquatic or semi aquatic plants such as watercress (Chidiac, 2017). Drip system hydroponics (C) uses individual tubes to drip-feed nutrient media from a

reservoir to plants growing on a moderately absorbent growth medium (Sharma & Singh, 2019). Both these systems give more control over the nutrients supplied to plants as the flow of media is controlled rather than relying on constant submersion or capillary action to supply the plants. The latter does not allow for easy measurement of the volume of solution provided.

The Nutrient film technique (E) hydroponics system was available for my experiments and is most similar to deep water culture hydroponics. Instead of being anchored by a substrate, the roots are floating in the nutrient media. However, unlike deep water culture the roots are not completely submerged and are instead in a stream of media circulating from the reservoir, through a slanted channel containing the plants and back to the reservoir (Velazquez-Gonzalez et al, 2022). The constant presence of water and nutrients can promote fungal infections in the roots in this system (Sharma & Singh, 2019). However, as the media flow is controlled by pumps, more advanced systems can work similarly to an ebb and flow system with media being supplied periodically instead of constantly. NFT systems are used widely for the production of lettuce and other leafy greens, however I had some difficulties growing *Brassica napus*. The lack of an anchoring substrate, while beneficial in harvesting the roots meant that the plants had to be supported to prevent them falling over. Additionally, *Brassica napus* has a larger root system than lettuce, with *Brassica napus* roots reaching depths of over 40cm while lettuce roots reach between 20 and 28cm in depth (Henarejos et al, 2015). This presented a barrier to my original plan to grow the plants to the same growth stage as the plants in the RIPR population as I found that as the *Brassica napus* plants grew the roots clogged the hydroponics channels which caused the media to overflow and also resulted in the roots of different plants becoming inextricably matted together by the end of the experiment. I decided to alter my experiment to be shorter rather than use fewer plants because the reduction in the number of plants would have meant that there were too few results for statistical analysis. This resulted in the 3-week experiments as at that point the roots were beginning to become tangled but were still able to be separated.

While the architecture of the roots cannot be studied in the same way as the pouch and wick system since the roots are free-floating, physiological changes such as changes in biomass can be investigated. In other experiments *Brassica napus* has shown an increase in root biomass and a decrease in shoot biomass in response to low phosphorus availability (Zhang et al, 2011)(Duan et al, 2020). Additionally the changes seen in the growth of primary and lateral roots in response to low phosphorus will affect the biomass of the roots (Abel, 2017). Therefore, the root and shoot biomass were both measured.

In addition to the measurements of biomass measures of plant health were also taken. These were the leaf number and the Soil Plant Analysis Development (SPAD) value, a measure of chlorophyll concentration. The leaf number was measured to see if there was any difference in the speed at which the plants were growing under different phosphorus treatments. The measures of chlorophyll were taken both as a general measure of plant healthy and because phosphorus stress has been linked to reduced chlorophyll concentrations in soybean (Singh et al, 2017)(Kumar & Sharma, 2019). These measurements will help establish if there are any correlations between the high and low phosphorus groups and plant health under the different treatments. Potentially if the high phosphorus group is indeed better adapted to phosphorus uptake, then the health of the high phosphorus plants will be better than that of the low phosphorus plants, particularly under the lowest phosphorus conditions.

These plants were also the source of the leaf samples for the element analysis experiment (see chapter 2).

4.1.3 Aims

The Pouch and Wick experiment described in chapter 3 aimed to compare the root architectural traits of the 6 varieties under low phosphorus conditions and identify correlations between the high and low phosphorus lines. It was predicted that if root architecture was affecting phosphorus acquisition as suggested by the work in chapter 2 the that the high phosphorus group of varieties, Pacific, Montego and Musette would display traits linked to improved phosphorus acquisition when

compared to the low group varieties. The experiment in chapter 3 was flawed as the plants may have been affected by low levels of nutrients other than phosphorus. This experiment was designed to investigate the same traits as the previous experiment but correcting the flaw in nutrient media choice by using custom made media that would only affect phosphorus availability.

As was the case in the experiment in chapter 3, I would expect the high group to have a significantly higher root length, increased number of laterals and increased root area compared to the low group as there are all root architecture traits that have been linked to increased phosphorus acquisition. This improved version of the Pouch and Wick architecture experiment also aimed to compare the root architectural traits of the 6 varieties under 4 different phosphorus conditions as it was thought that differences in the root architecture between the high and low phosphorus acquisition groups may be more apparent and lower phosphorus concentrations.

The hydroponics experiment aimed to compare physiological traits which may be linked to phosphorus uptake using the same lines and treatments but with older plants than it is possible to grow in the pouch and wick system. It also allowed a comparison between measures of plant health; leaf number, biomass and SPAD values, to see if there is any correlation between the high and low phosphorus groups and plant health under the different phosphorus conditions.

4.2 Methods

4.2.1 Plant materials - Pouch and Wick

The same pouch and wick method was used in this experiment as in the first root architecture experiment detailed in chapter 3. The same growth pouches were used, comprising a sheet of A4 blue germination paper (SD7640; Anchor Paper Company, St Paul, MN, USA), clipped together with an A4 sheet of black polythene on either side of the paper (Cransford Polythene Ltd, Woodbridge, UK). The paper and polythene were clipped together on one short edge using bulldog clips which also secure the sheets to an acrylic rod (Acrylic Online, Hull, UK). Once assembled the pouches were suspended in aluminium frames which contain plastic drip trays.

The frames were located in a growth room (2.2 m width, 3.3 m length, 3.0 m height). Photosynthetically Active Radiation (PAR; measured at plant height with a 190 SB quantum sensor; LI-COR Inc., Lincoln, NE, USA) was approximately 207 µmol m−2 s−1, generated by 400 W white, fluorescent lamps (HIT 400w/u/Euro/4 K, Venture Lighting, Rickmansworth, UK). The growth room was set to 20°C days, 18°C nights with a 16hr photo period to mimic conditions that the seed would experience at sowing which for winter oilseed in the UK occurs in late August (Brown et al, 2019).

Each drip tray was filled with 2L of media. One frame was used for each of the 4 treatments detailed below. Before the seeds were sown the pouches were left overnight to absorb the media solution to ensure the paper was fully saturated. Once the paper was fully soaked two seeds were trapped between the paper and black plastic, one on each side of the paper. The roots grow down the paper and can later be photographed. The water in the drip trays was replenished with 1L of deionised water every 4 days. As the experiment was repeated the treatments were rotated through the positions in the room, shown in figure 34, to compensate for any differences is the light intensity or temperature within the growth room.

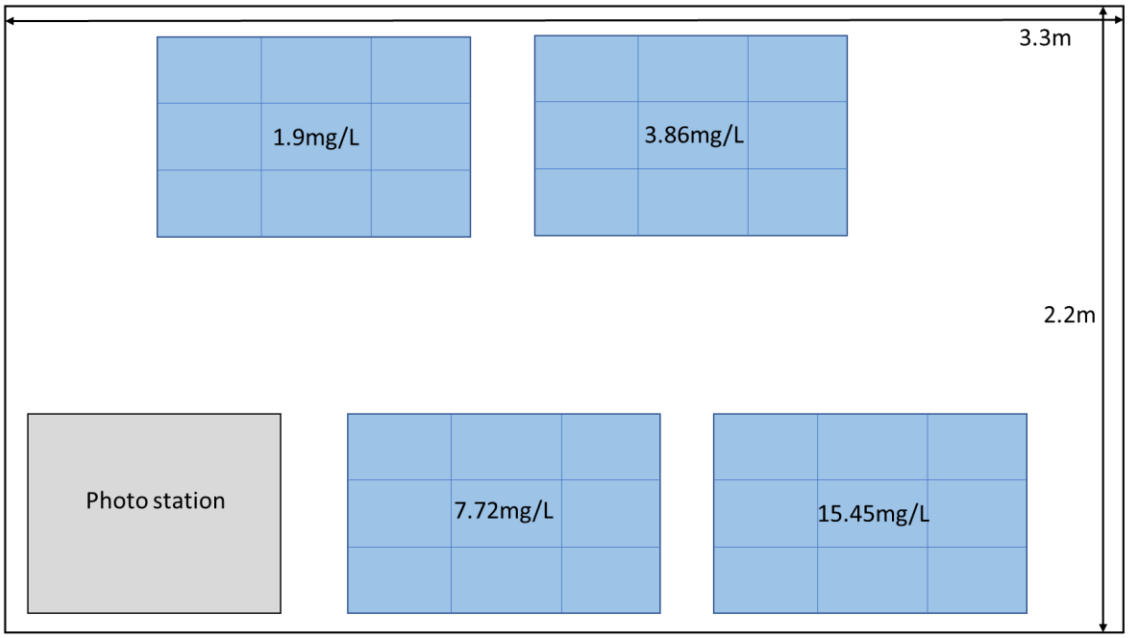

Figure 34 - shows the arrangement of the frames within the growth room. With each repeat of the experiment the treatments were rotated through the positions in the room to compensate for any differences is the light intensity or temperature within the growth room

4.2.2 Plant positioning – Pouch and wick

The placement of seeds in the pouches was randomised and the seeds were sown by trapping them in between the plastic and paper approximately 1cm down from the top edge of the sheet. Figure 35 shows an example plan of the randomisation of the accession placement within the rack. Each pouch had two seeds, one on each side of the sheet of paper to maximise the data collected from each experiment. Both seeds in a pouch were the same accession.

	1	E		31	D		61	B
	2	D		32	C		62	E
	3	D		33	F		63	D
	4	C		34	F		64	B
Tray 1	5	A	Tray 4	35	D	Tray 7	65	D
	6	E		36	C		66	C
	7	F		37	C		67	A
	8	E		38	B		68	C
	9	D		39	E		69	A
	10	D		40	F		70	F
	11	C		41	D		71	D
	12	F		42	A		72	A
	13	D		43	A		73	B
	14	D		44	D		74	B
Tray 2	15	A	Tray 5	45	F	Tray 8	75	F
	16	B		46	C		76	B
	17	E		47	E		77	C
	18	F		48	E		78	F
	19	E		49	A		79	F
	20	E		50	E		80	B
	21	E		51	A		81	A
	22	F		52	C		82	A
	23	E		53	D		83	F
	24	B		54	E		84	B
Tray 3	25	A	Tray 6	55	B	Tray 9	85	D
	26	A		56	A		86	C
	27	C		57	B		87	F
	28	B		58	B		88	B
	29	E		59	C		89	A
	30	C		60	C		90	F

Figure 35 - This figure shows an example of one of the randomized plans of accession placement within the rack. Each of the trays contains 2L of media, in this experiment all of the trays contained 2L of ¼ strength MS media (9.68mg/L phosphorus). Each tray has 10 pouches absorbing media from it, each of which has a seed sown on each side of the paper. The letters in the plan correspond to the 6 accessions Prince (A), Caramba (B), Gefion (C), Pacific (D), Montego (E) and Musette (F). A new randomized plan was created each time the experiment was repeated.

4.2.3 Root architecture photographs.

As in the first root architecture experiment, the plants were grown for 2 weeks after sowing and were then photographed using a stand to maintain a set distance of 55cm from the sheets images were taken using a Digital Single Lens Reflex (DSLR) camera (Canon EOS 1100D, Canon Inc., Tokyo, Japan) once the camera had been attached to the stand and automatically focused for the first image the auto focusing was turned off to ensure the camera settings did not change for subsequent photos. The photographs were then analysed with a program called RootNav to obtain a map of the roots and to get quantitative analysis of root traits

(Pound et al, 2013). The traits analysed were total root length, primary root length, lateral root length, number of lateral roots, convex hull, maximum width, maximum depth, width/depth ratio and the y coordinate of the centroid. Analysis of the photos was carried out using RootNav as detailed in chapter 3.

4.2.3 Solution preparation

This method was used to make the variable phosphorus treatment solutions for both the pouch and wick experiments and the hydroponics experiments. This method was based on that of a previous PhD student Josefina Lozano (Josefina Lozano, 2021). The four treatment levels were 1.9, 3.86, 7.72 and 15.45 mg/L when the solution was made up to its final concentration. The treatments were named 1, 2, 3 and 4 respectively.

The nutrient solution is a combination of seven stock solutions. These stock solutions were prepared to the concentrations detailed in tables 5 and 6 below.

To prepare the four treatments for the variable phosphorus experiments solutions 2-5 were combined according to table 2. These solutions provide the essential nutrients for the plants. Solutions 1 and 7 are added in accordance with the amounts detailed in table 3. These two solutions provide the variable phosphorus levels across the treatments.

When preparing the treatments solutions 1, 2 and 7 should be added to the water first. The pH should then be adjusted to about 6. The other solutions can then be added. Finally, the pH should be checked again and adjusted to a final pH of 6.4. This prevents precipitation which could affect the nutrient levels in the media.

Table 5: Seven stock solutions are required to create the variable phosphorus media. The chemical content and g/L required to make the solution are shown in the table below. The amounts of solutions 1 and 7 are not given in this table as these are variable depending on the treatment.

Solution number	Chemical content	Mol Wt	Molarity (M)	g/L in stock solution	ml/L in treatment	Final solution molarity
1	KH2PO4 KOH	136.09 56.1	0.2667 0.5333	36.30 29.92	**Variable**	
2	MgSO4.7H2O CaCl2.2H2O	246.47 147.02	0.375 0.0125	92.43 1.84	**2**	0.75 0.03
3	FeNaEDTA	367.05	0.05	18.35	**2**	0.10
4	Ca (NO3)2.4H2O	236.15	1	236.15	**2**	2
5	NH4NO3	80	1	80	**2**	2
6	 H3BO3 MnSO4.4H2O ZnSO4.7H2O CuSO4.5H2O Na2MoO4.2H2O	 61.83 223.06 287.55 249.68 241.95	(mM) 30 10 1 3 0.5	 1.85 2.23 0.29 0.75 0.12	 **1**	(uM) 30.00 10.00 1.00 3.00 0.50
7	K2SO4 KOH	174.26 56.11	0.1333 0.5333	23.227 29.62	**Variable**	

Table 6: Amount of solutions 1 and 7 required for the variable phosphorus treatments

Treatment	ml/L solution 1	ml/L solution 7
1 = 1.9mg/L (0.0625mM KH2PO4)	0.23	1.64
2 = 3.86mg/L (0.125mM KH2PO4)	0.47	1.41
3 = 7.72mg/L (0.25mM KH2PO4)	0.94	0.94
4 = 15.45mg/L (0.5mM KH2PO4)	1.88	-

4.2.4 Plant materials - hydroponics

The plants were grown in glasshouses at the Sutton Bonington campus of the University of Nottingham. Glasshouses had additional heating to ensure the temperature did not fall below 20°C and had additional lighting to ensure a minimum day length of 16h.

Seeds were sown on rock wool in propagation trays in the glasshouse to allow germination. The seeds were watered but not provided with any nutrients. After 7 days seedlings were selected to be sown in a hydroponics set up and were then grown for a further 3 weeks or 8 weeks depending on the experimental run. The hydroponic system used the nutrient film technique (NFT) and was built by technician Rory Hayden. The set up consists of a 5m long bench with 4 guttering pipes set on a slope with a 10cm difference from beginning to end. Each of these gutters is covered to prevent media evaporation but has the option for up to 10 spaces to be left open for the rockwool blocks containing the plants to be immersed in the media. The low end of the gutters are connected to 4 black water tanks with a capacity of 84L each. The tanks are black to prevent the growth of algae. Figure 36 below shows a diagram of this set up.

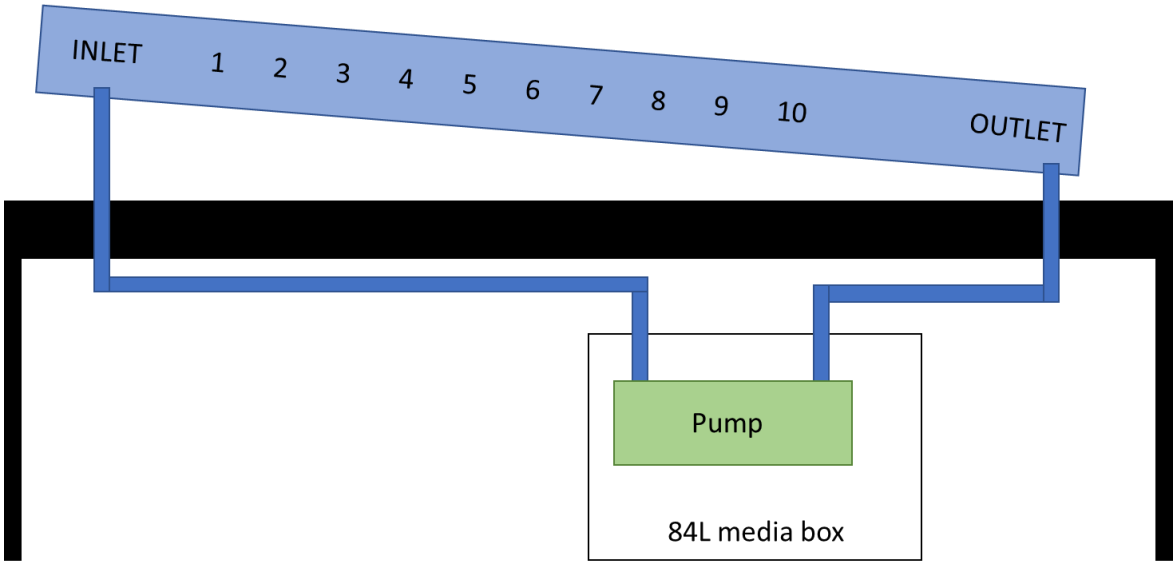

Figure 36 : Side view of hydroponics setup. The bench is showing in black and the guttering and its connected pipes are shown in blue
Media is pumped from the box to the inlet at the higher end of the guttering.
Media flows down to the outlet at the lower end of the guttering and returns to the media box.
Note only one media box is shown here but each bench has four boxes so each gutter has an individual media supply, this allowed up to four different treatments to be used on each bench.
Seedlings are placed at regular intervals down the gutter in rock wool blocks which provide an anchor for the seedlings in place of soil and absorb the media.

The Hydroponic system components on each bench were 4 100mm square white gutters of 4m length and two stop ends for each, 100mm square white gutter all supplied by John A. Stephens LTD (Nottingham, UK). All pipes and pipe fixtures and fittings were supplied by Hortech Systems LTD: ¾ inch. Polyethylene tank connector outlet bulkhead fitting, ¾ inch. Polypropylene back nut threaded fitting, 20mm nutlock elbow for low density polyethylene (LDPE) pipe, 20mm x ¾ inch. Nutlock valve for LDPE pipe, 16mm x 20mm nutlock connector joiner for LDPE pipe, 20mm LDPE pipe. In each 84L tank there was an Aquarius Universal Classic 1500L/hr max flow rate pump also supplied by Hortech Systems LTD (Spalding,UK).

Some experiments took place over the summer, and while steps were taken to reduce the temperature in the glasshouse (opening doors and roof panels during the day) the first 3-week experiment did have a week-long heatwave during its run where external temperatures reached 36°C and temperatures in the glasshouse exceeded 40°C. This took place in the final week of the experiment and while plants were being harvested.

4.2.5 Plant positioning in the hydroponics

Each bench had 4 gutters with 10 spaces for plants in each. For the element analysis experiments 3 benches were used. This gave 12 gutters which had the 4 phosphorus treatments randomly assigned to them. Since there were 3 gutters for each treatment each treatment had a total of 30 spaces for plants, 5 per variety, and the plants were randomly assigned to these 30 spaces. This was repeated for each treatment so that there were 5 plants of each variety receiving each of the 4 phosphorus treatments. Table 7 shows the treatments and varieties present in each gutter.

Table 7 this table shows the randomised placement of the treatments and *Brassica napus* varieties across the 3 benches. First the 4 treatments were randomly assigned to the gutters the 5 of each variety of *Brassica napus* were randomly assigned positions within each treatment.

Bench 1					**Bench 2**					**Bench 3**			
Gutter 1	**Gutter 2**	**Gutter 3**	**Gutter 4**		**Gutter 5**	**Gutter 6**	**Gutter 7**	**Gutter 8**		**Gutter 9**	**Gutter 10**	**Gutter 11**	**Gutter 12**
3.86mg/L	**7.72mg/L**	**3.86mg/L**	**15.45mg/L**		**1.9mg/L**	**3.86mg/L**	**7.72mg/L**	**15.45mg/L**		**15.45mg/L**	**1.9mg/L**	**7.72mg/L**	**1.9mg/L**
Gefion	Pacific	Prince	Prince		Gefion	Caramba	Caramba	Musette		Gefion	Pacific	Caramba	Musette
Caramba	Musette	Prince	Gefion		Montego	Musette	Gefion	Prince		Pacific	Pacific	Gefion	Prince
Pacific	Prince	Pacific	Caramba		Caramba	Musette	Montego	Musette		Prince	Caramba	Prince	Prince
Gefion	Musette	Gefion	Montego		Gefion	Musette	Gefion	Caramba		Caramba	Gefion	Pacific	Pacific
Caramba	Prince	Pacific	Gefion		Musette	Montego	Prince	Pacific		Caramba	Caramba	Caramba	Musette
Montego	Gefion	Musette	Pacific		Pacific	Montego	Montego	Montego		Caramba	Gefion	Musette	Prince
Caramba	Pacific	Gefion	Gefion		Pacific	Montego	Gefion	Pacific		Montego	Prince	Prince	Musette
Pacific	Pacific	Prince	Prince		Prince	Montego	Caramba	Musette		Gefion	Montego	Montego	Caramba
Pacific	Montego	Prince	Musette		Montego	Gefion	Pacific	Montego		Pacific	Gefion	Caramba	Montego
Prince	Montego	Musette	Montego		Caramba	Caramba	Musette	Prince		Musette	Musette	Musette	Montego

4.2.6 Chlorophyll measurements

Measurements of chlorophyll concentrations were carried out immediately before harvesting the plants, 3 weeks or 8 weeks after transfer to the hydroponics system. Three mature leaves from each plant were selected, any leaves which showed discolouration or damage were discounted. Measurements were taken using a SPAD-502 meter (Minolta), a handheld device that allows rapid and non-destructive measurements of chlorophyll concentrations to be taken. The device uses two light emitting diodes and a photodiode receptor to measure the leaf transmittance of red light, and infrared light (Ling et al, 2011). The measurements produce a relative SPAD value that is proportional to the chlorophyll concentration. It is possible to convert the values to chlorophyll per unit leaf area using calibration equations

however the calibration equation has to be calculated for the particular species of plants being studied (Ling et al, 2011). For the purposes of these experiments the SPAD values were considered to be sufficient to show any change in the chlorophyll levels in the leaves. While taking the SPAD measurements the number of leaves of each plant were also counted.

4.2.7 Weight measurements

After SPAD values and leaf number data was collects the plants were then harvested for drying. The leaves and stems were harvested and bagged separately from the roots. Some of the root mass was lost as the initial root growth was through the rock-wool block the seeds were sown into and therefore the roots nearest to the base of the stem could not be harvested. Once harvested the plant material was placed into labelled paper sacks and dried for 1 week at 50°C. After drying the plant material was then weighed in the paper sacks and the average weight of one of the sacks was removed from the weight.

4.2.8 Statistical analysis

In the root architecture experiment a total of 400 images were analysed using RootNav, 242 in the low group (Pacific n=125, Caramba n=71, Gefion n=46) and 158 in the high group (Pacific n=24, Montego n=49, Musette n=85). Initially a simple t-test was run to determine if there was a significant difference between the two groups of varieties, high and low phosphorus. A p value below 0.05 was considered to be significant. Then additional t-tests were run for the results of each treatment separately. To gain a clearer understanding of the relationships between the individual varieties the results from all of the 6 varieties and 4 treatments were also analysed using a 2-way ANOVA. A p value below 0.05 was considered to be significant.

Additional post hoc analysis was carried out after the ANOVA by comparing the mean of each variety to the mean of every other variety. If the means of the varieties varied from each other by more than the mean standard error of difference, then they were considered to be significantly different from each other. This same method was used to compare the means of the treatment groups to

establish if the results of different treatments were significantly different from each other. This additional analysis shows which of the varieties or treatments are significantly different from each other and therefore shows if there are trends between the high and low phosphorus groups or between particular individual varieties.

The same analysis was carried out for the hydroponic experiments. These experiments were separated into 3 individual sets of data. This was done because while both of the three-week experiments were done on the same varieties of *Brassica napus* and had the same phosphorus treatments applied the conditions within the greenhouse were different for each experiment. The first three-week experiment took place in the summer and experienced an extreme heat wave while the other took place in the autumn when temperatures in the greenhouse were under control. The additional heat stress may have affected the results of the first 3-week experiment and therefore the two experiments were analysed separately.

In the first of the three-week experiments four measurements were taken, leaf number, leaf and stem weight, root weight and whole plant weight. 103 of the possible 120 plants grew and were measured. (Low group Total n=46, Prince n=13, Caramba n=20, Gefion n=13) (High group Total n=57 Pacific n=17, Montego n=21, Musette n=19)

In the second of the 3-week experiments 5 parameters were measured, leaf number, stem and leaf weight, root weight, whole plant weight and chlorophyll concentration measured in SPAD units. 213 plants out of the total 240 plants grew in this experiment. (Low group Total n=96, Prince n=40, Caramba n=37, Gefion n=19) (High group Total n=117 Pacific n=38, Montego n=40, Musette n=39)

The same measurements were going to be taken from the plants in the 8-week experiment, however after 8 weeks the roots of the plants had become matted together to the point that the roots of individual plants could not be separated. As a result, it was not possible to analyse root weight or whole plant weight in this experiment. The weight of the leaves and stems, the number of leaves and the SPAD measurements of chlorophyll were still analysed as in the other experiments.

108 plants grew in this experiment. (Low group Total n=58, Prince n=21, Caramba n=19, Gefion n=18) (High group Total n=56 Pacific n=16, Montego n=21, Musette n=19)

Table 8 - a break-down of the number of samples analysed in the hydroponics by accession and treatment.

Experiment	Treatment	Total number	Prince	Caramba	Gefion	Pacific	Montego	Musette
1st 3-week	1.9mg/L	23	2	5	2	4	5	5
	3.86mg/L	25	3	5	3	4	5	5
	7.72mg/L	25	2	5	4	5	5	4
	15.45mg/L	28	5	5	4	4	5	5
2nd 3-week	1.9mg/L	54	10	10	4	10	10	10
	3.86mg/L	55	10	10	5	10	10	10
	7.72mg/L	55	10	10	5	10	10	10
	15.45mg/L	47	9	7	5	8	9	9
8-week	1.9mg/L	29	5	5	5	4	5	5
	3.86mg/L	27	5	4	4	4	5	5
	7.72mg/L	28	5	5	5	3	5	5
	15.45mg/L	28	5	5	4	5	5	4

4.3 Results

4.3.1 Identifying correlations in root architecture traits using the pouch and wick system.

In the Pouch and Wick experiment the same set of nine architectural traits were analysed as in the Murashige and Skoog media experiment: primary root length, lateral root length, total root length, lateral root number, convex hull area, maximum width of root system, maximum depth of root system, width/depth ratio and the y coordinate of the centroid. These traits have been linked to phosphorus uptake. As with the first root architecture experiment the aim was to identify correlations between the high phosphorus accumulating varieties and the low phosphorus accumulating varieties which may be linked to the difference in phosphorus acquisition by these accessions.

4.3.1.1 Primary root length correlates to phosphorus accumulation under low phosphorus.

As with the non-variable experiment root length measurements were separated into primary root length, lateral root length and total root length. In this experiment only the primary root length showed any difference between the groups and then only under treatment 3.86mg/L ($p=0.0498$) where the low phosphorus group of varieties (Prince, Caramba and Gefion) had significantly longer primary roots than the high phosphorus group (Pacific, Montego and Musette). Further testing using ANOVAs and additional post-hoc tests showed that the primary root of Prince (A) was significantly longer than Gefion and Pacific (B*) but not the other varieties and that Montego (C) was significantly longer than Pacific but not the other varieties. Caramba and Musette (B) were not significantly different from any of the other varieties.

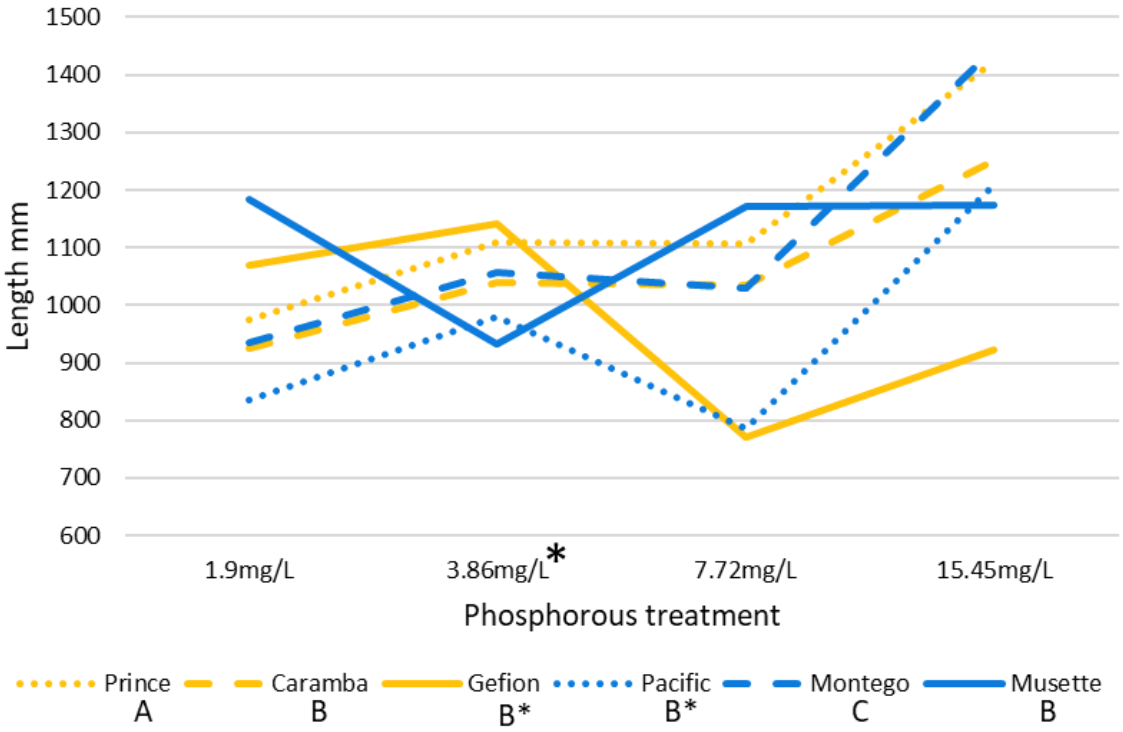

Figure 37 - shows the primary root length of the six *Brassica napus* accessions under four different phosphorus treatments. Treatment 3.86mg/L showed a significant difference (p=0.0498) between the high (blue) and low (yellow) accession groups when compared using a t-test with the low group having significantly longer primary roots than the high group.

There were also differences between the individual accessions (p=0.018). Prince's (A) primary root was significantly longer than both Gefion and Pacific (B*) but not the other varieties. Also Montego (C) was significantly longer than Pacific but not the other varieties. Caramba and Musette (B) were not significantly different from any of the other varieties.

None of the individual treatments showed a significant difference between the groups for either total root length or lateral root length. Further ANOVA analysis showed that the individual varieties had some significant differences to each other (Lateral p= < 0.001, Total p= < 0.001). Both the lateral and total root length of Pacific and Montego were significantly different from that of all of the other varieties including each other. Musette was significantly different from Pacific, Montego and Gefion but not from Prince or Caramba.

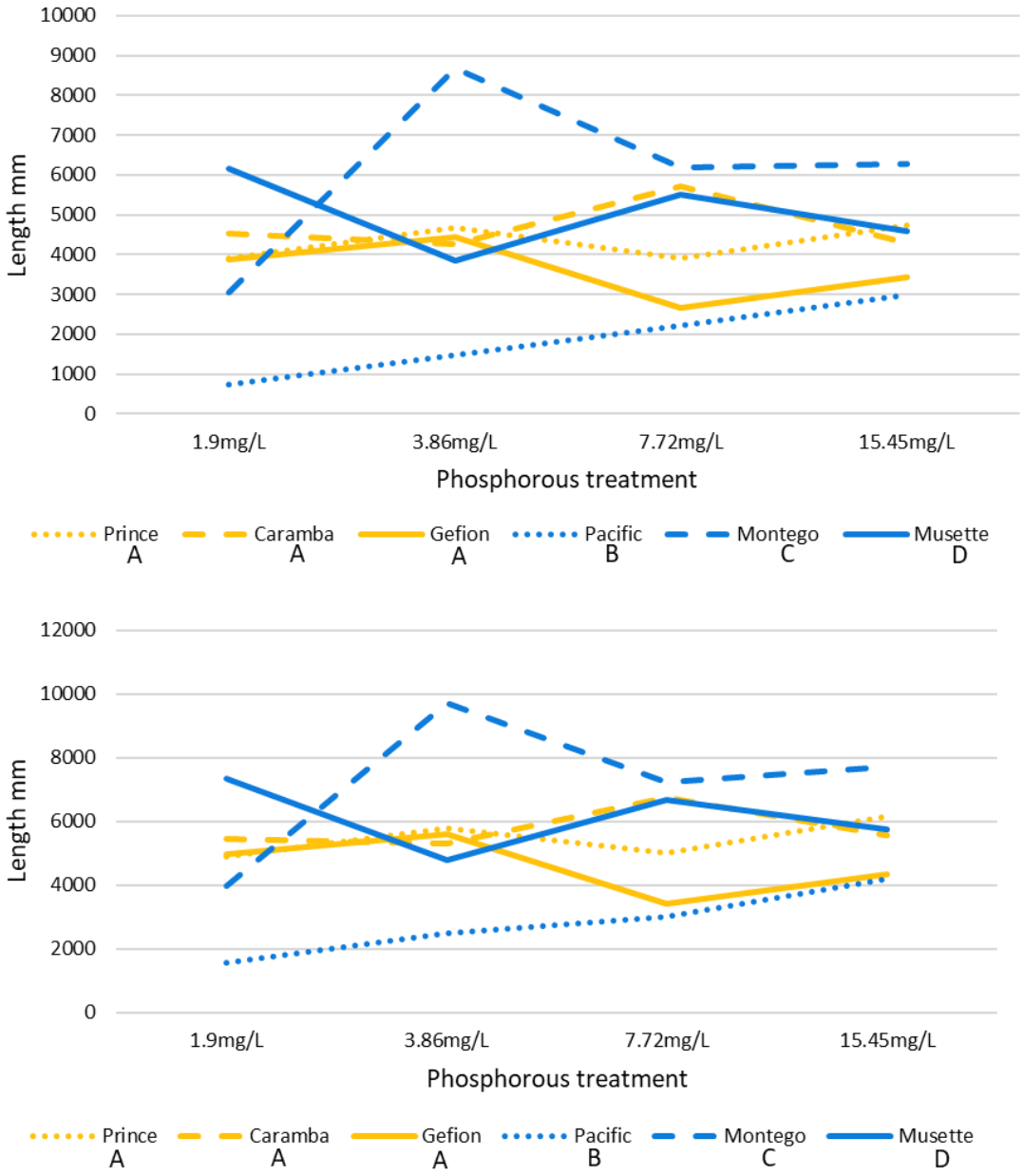

Figure 38 - shows the Lateral Root Length (top) and Total Root Length (bottom) for the six accessions under the four phosphorus concentrations. Both the lateral root length and the total root length showed no significant difference between the high and low groups. Further ANOVA analysis showed that the individual varieties had some significant differences to each other (Lateral p= < 0.001, Total p= < 0.001). Both the lateral and total root length of Pacific (B) and Montego (C) were significantly different from that of all of the other varieties including each other. Pacific was significantly lower and Montego significantly higher for both the lateral and total root lengths. Musette (D) was significantly different from Pacific, Montego and Gefion but not from Prince or Caramba.

4.3.1.2 The high phosphorus accumulating varieties have higher numbers of laterals.

The number of lateral roots could affect the uptake of phosphorus as lateral root development is one of the key processes by which plants increase the size, and therefore surface area, of the root system(Waidmann, Sarkel, & Kleine-Vehn, 2020). Interestingly in the non-variable experiment the groups of *Brassica napus* varieties had no significant difference in lateral number, however, as can be seen in figure 39, in this experiment the groups had significantly different numbers of laterals in 3 of the 4 treatments with the high phosphorus accumulating group having a greater number of laterals (1.9mg/L p= 0.0023, 7.72mg/L p=<0.001 and 15.45mgL p=0.035). There were also significant differences between some of the individual varieties when compared with an ANOVA (p=<0.001). The low group, Prince, Caramba and Gefion were not significantly different from each other however there were some significant differences in the high group. Pacific and Musette were both significantly different from all of the other varieties including each other. Pacific had significantly lower numbers of laterals and Musette significantly higher compared to the other varieties. Montego also had significantly higher numbers of laterals than all other varieties except for Prince and Musette.

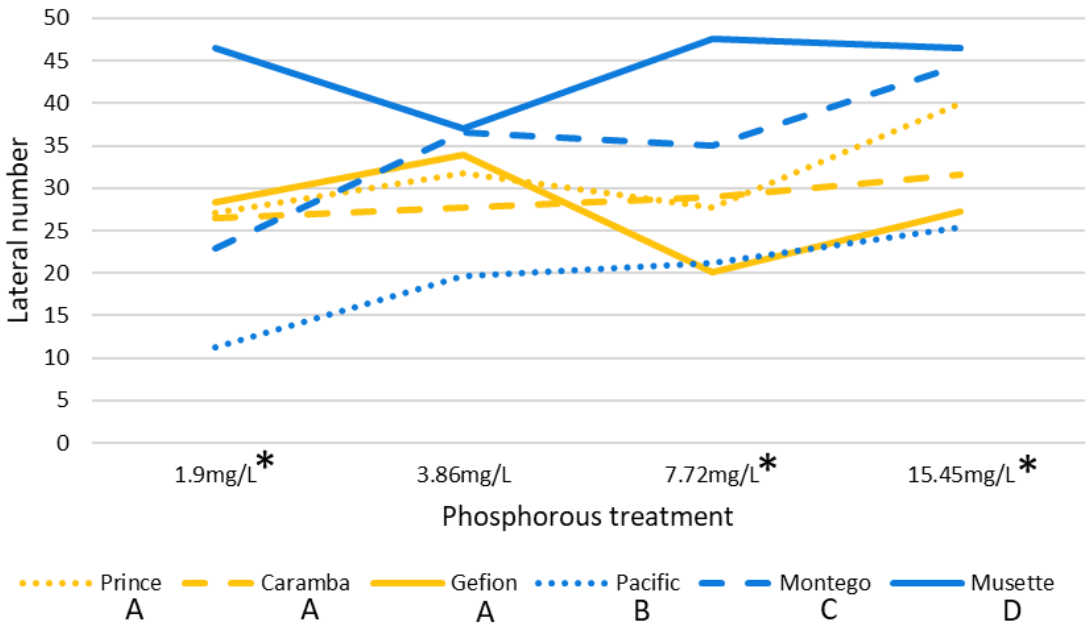

Figure 39 - shows the lateral root number across the six varieties under the four treatments. There was a significant difference between the low (yellow) and high (blue) groups for three of the treatments (1.9mg/L p= 0.0023, 7.72mg/L p=<0.001 and 15.45mgL p=0.035) with the high group having significantly more lateral roots than the low group.

Additionally, there were some significant differences between the individual varieties (p=<0.001) The low group varieties, Prince, Caramba and Gefion (A) were not significantly different from each other however there were some significant differences in the high group. Pacific (B) had significantly more lateral roots than all of the other varieties except for Musette. and Musette (D) had significantly higher numbers of lateral roots than all of the other varieties. Montego (C) had significantly fewer lateral roots than every variety except for Prince.

4.3.1.3 The high phosphorus accumulating group has a smaller and shallower root system.

There was only a significant difference between the two groups of varieties in the 3.86mg/L treatment (p=0.031). As in the non-variable experiment the convex hull was significantly affected by the variety of *Brassica napus* (>0.001) with the high accumulating group having a lower convex hull area than the low accumulating group.

Some individual variation was seen between the accessions. There was no significant difference between Prince, Caramba and Montego (A). Gefion and Musette (B) were significantly different from the other varieties but not to each

other, having a lower area than Prince, Caramba and Montego and a higher convex hull area than Pacific. Pacific (C) had a significantly lower convex hull area than all 5 of the other varieties.

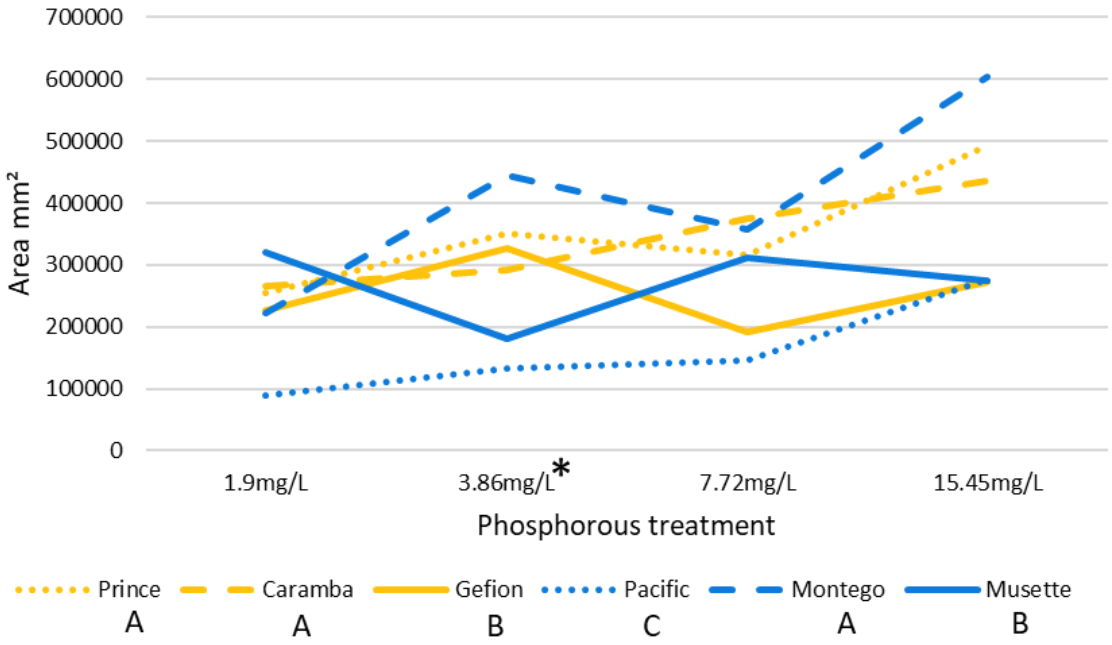

Figure 40 - shows the convex hull area of the six varieties under four different phosphorus treatments. Only the 3.86mg/L phosphorus treatment showed a significant difference between the high and low groups (p=0.031). The high group had significantly smaller convex hull area than the low group varieties.

There was no significant difference between Prince, Caramba and Montego (A) however the ANOVA and post hoc testing did show differences between other varieties. Gefion and Musette (B) were significantly different from the other varieties but not to each other, while Pacific (C) had a significantly lower area than all 5 other varieties.

There were no significant differences between the width of the roots in the high and low groups for any of the treatments. However, the depth the roots reached was significantly different between the two groups for the 1.9mg/L treatment only (p=0.001) where the high phosphorus accumulating group had significantly deeper roots than the low group. The width to depth ratio was also significantly different between the high and low groups for three of the four treatments (1.9mg/L p=0.0018, 7.72mg/L p=0.005, 15.45mg/L p=0.02) with the high phosphorus accumulating group having a lower width to depth ratio than the low group. Both measurements also had differences between the individual varieties when compared with an ANOVA (p=<0.001). There was no significant difference between

the maximum depth measurements of Prince, Caramba, Gefion or Montego. However, Pacific was significantly shallower than both Prince and Musette, while Musette had significantly deeper roots than all other varieties except for Prince. The results of the width to depth ratio analysis were similar with Prince, Caramba, Gefion and Montego having no significant differences between them. However, Pacific had a significantly lower width do depth ratio than Prince, Caramba and Gefion, but not Montego while Musette's width to depth ratio was significantly lower than all varieties except Pacific.

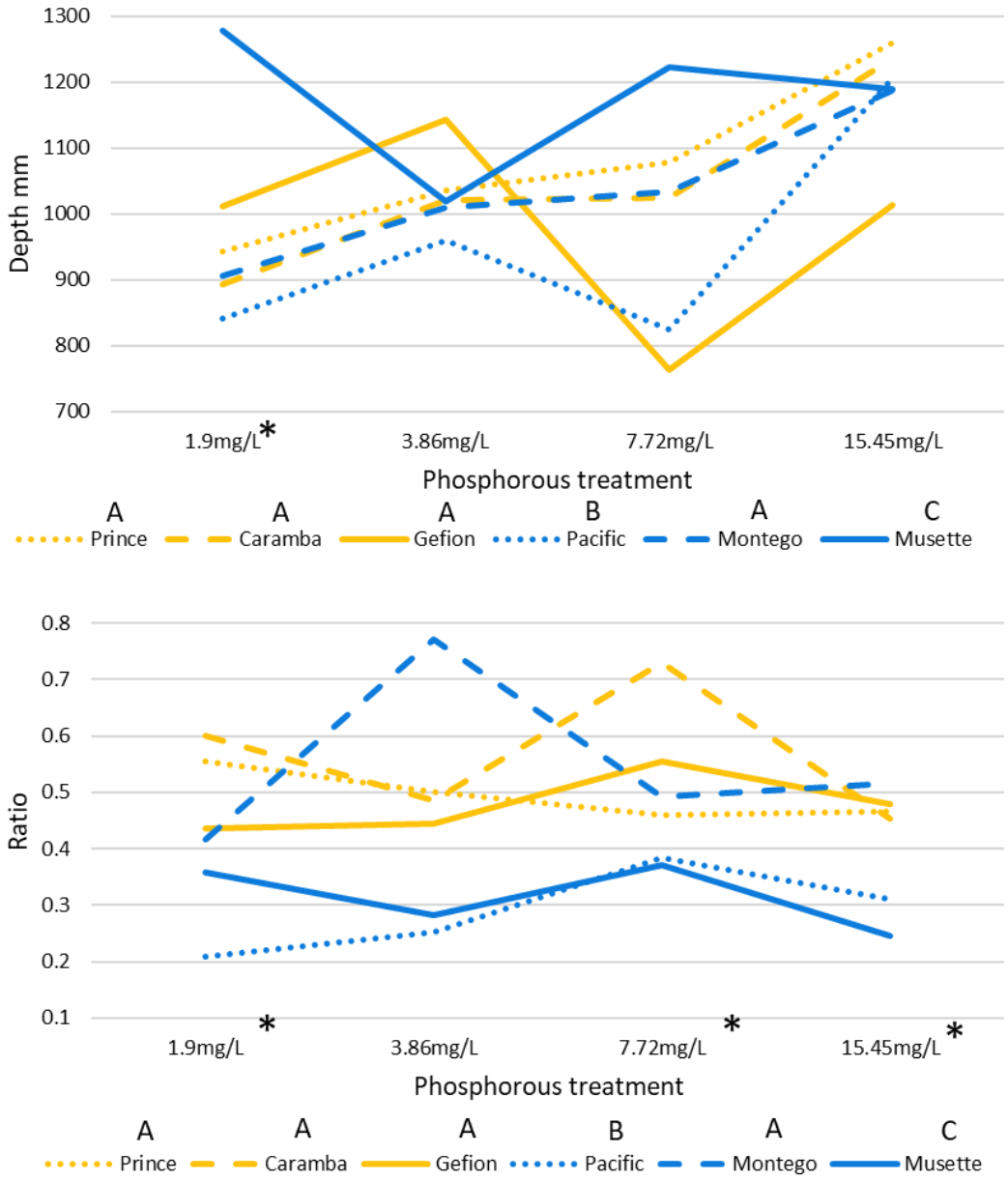

Figure 41 - Shows the maximum root depth (top) and the width to depth ratio (bottom) of the varieties under the four treatments. The depth of the roots was only significantly different between the two groups for the lowest phosphorus treatment (1.9mg/L p=0.001) where the high group had significantly deeper roots than the low group. The ANOVA of the depth measurements showed that Pacific (B) was significantly lower than Prince and that Musette (C) was significantly higher than all varieties except for Prince (p=<0.001).

In the width to depth ratio measurements there were significant differences between the high and low groups for 1.9mg/L, 7.72mg/L and 15.45mg/L (p=0.0018, 0.005 and 0.02 respectively). Further analysis showed that Pacific (B) was significantly lower than to Prince, Caramba and Gefion and that Musette (C) was significantly lower than all other varieties except for Pacific which it was not significantly different from (p=<0.001).

The y co-ordinate of the centroid was significantly shallower in the high phosphorus accumulating group under the 1.9mg/L treatment only (p=0.002) with the high group having significantly lower y co-ordinates than the low group. There were also some significant differences between individual varieties with the y-coordinate of Pacific being significantly shallower compared to that of Prince, Montego and Musette, and Musette being significantly deeper compared to Gefion and Pacific.

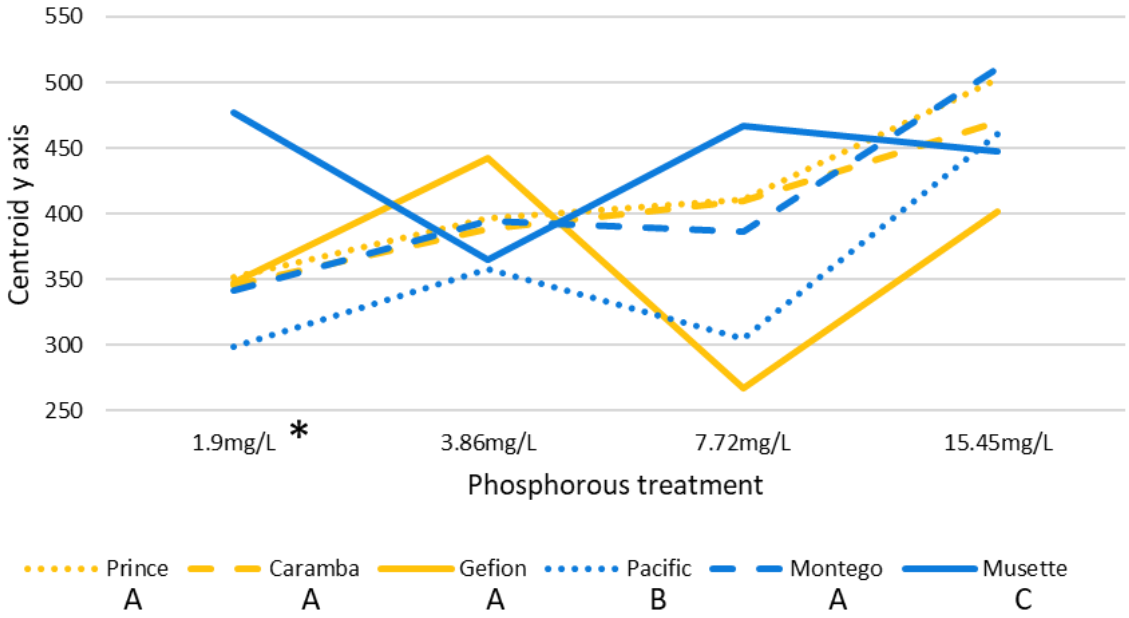

Figure 42 - Shows the centroid y co-ordinate for the six varieties under different phosphorus treatments. Only the 1.9mg/L phosphorus treatment showed significant differences between the high and low groups (p=0.002) with the low group having a significantly deeper y centroid co-ordinate than the high group. Further analysis with ANOVA and post hoc testing showed that Pacific (B) had a significantly shallower y co-ordinate than Prince, Montego and Musette and that Musette's (C) y co-ordinate was significantly higher than different from Gefion and Pacific (p=>0.001)

4.3.2 Results of the hydroponics experiments

4.3.2.1 The high phosphorus accumulating varieties had increased shoot biomass in the 8-week experiment.

The first hydroponics experiment ran for 8 weeks and was designed to test plant biomass accumulation, both roots and shoots, to compare the speed of plant growth based on leaf count and also to compare plant health through SPAD measurements. The time was selected so that the plants could reach the same growth stage, the rosette stage, as the plants in the RIPR experiment reached when they were sampled for element analysis. Four measurements were to be taken, leaf count, stem and leaf dry weight, SPAD measurements and root dry weight and the end of the experiment. However, by 8 weeks the roots were inextricably matted together preventing root weight from being measured.

There were no significant differences between the high and low phosphorus accumulating groups of accessions in either the rate of growth, measured by leaf number, or in the health of the plants based on the SPAD measurements.

Only the stem and leaf dry weight showed any significant difference between the high and low groups, and then only in the 7.72mg/L treatment where the high phosphorus accumulating group had a higher leaf biomass compared to the low group ($p=0.039$). As in the root architecture experiments ANOVA analysis showed that there were significant differences between individual varieties. The low group had no significant differences between them however Pacific was significantly lower than both of the other high group varieties, Montego and Musette ($p=<0.001$). This can be seen in figure 43 where Pacific has a significantly lower stem and leaf dry weight than the other high phosphorus varieties.

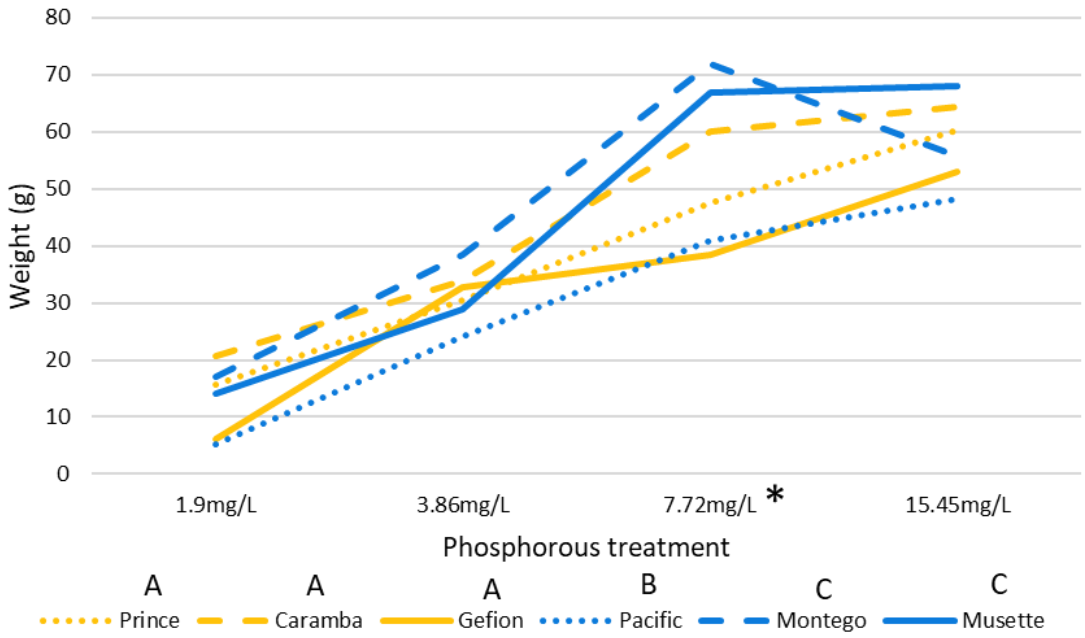

Figure 43 - shows the stem and leaf dry weight for the 8 week experiment under the four different treatments. T-tests showed that was a significant difference between the high and low groups of varieties under the 7.72mg/L treatment (p=0.039). The high group had significantly greater biomass than the low group under the 7.72mg/L treatment.

Additional analysis with ANOVA and post-hoc testing showed that the weight of Pacific (B) was significantly lower than that of Caramba (A), Montego and Musette (C). Montego and Musette (C) were also significantly higher than to Gefion (A). There were no significant differences in weight between the three low group varieties Prince, Caramba and Gefion (A)

It should also be noted that as the concentration of phosphorus increased across the treatments the biomass of the leaves increased for all of the varieties. The two-way ANOVA showed that there was a significant difference between the treatments as well as between the varieties (p=<0.001). This suggests that the growth of the plants was limited by the concentration of phosphorus under the low treatments.

4.3.2.2 The high phosphorus accumulating accessions had increased root and shoot biomass in the first 3-week experiment.

As the 8-week experiment resulted in matted roots it was decided to repeat the experiment with a shorter growth time to allow the root biomass to be studied. In addition to the root biomass, it was intended that the shoot biomass, leaf number and SPAD measurements would also be collected. The aim was to identify correlations between biomass, plant growth speed or plant health and the groups of accessions.

The plants in this experiment were harvested during a heatwave, this caused extensive wilting of the leaves which prevented accurate SPAD measurements from being taken, therefore, only measurements of weight and leaf count were taken. There was no significant difference in the number of leaves between the high and low groups for any of the treatments, however, there were differences in both the root dry weight and the leaf dry weight.

The root dry weight was significantly different between the high and low groups under two of the four phosphorus treatments 1.9mg/L and 15.45mg/L ($p=0.003$ and $p=0.019$ respectively) with the high phosphorus accumulating group having a greater root biomass. Pacific continued to show different responses to that of both Montego and Musette, having a significantly lower root weight than either of the other high group varieties. Post-hoc testing also showed that Montego and Musette were significantly higher than all other varieties except for each other. Also, that Caramba was significantly higher than Prince ($p=<0.001$).

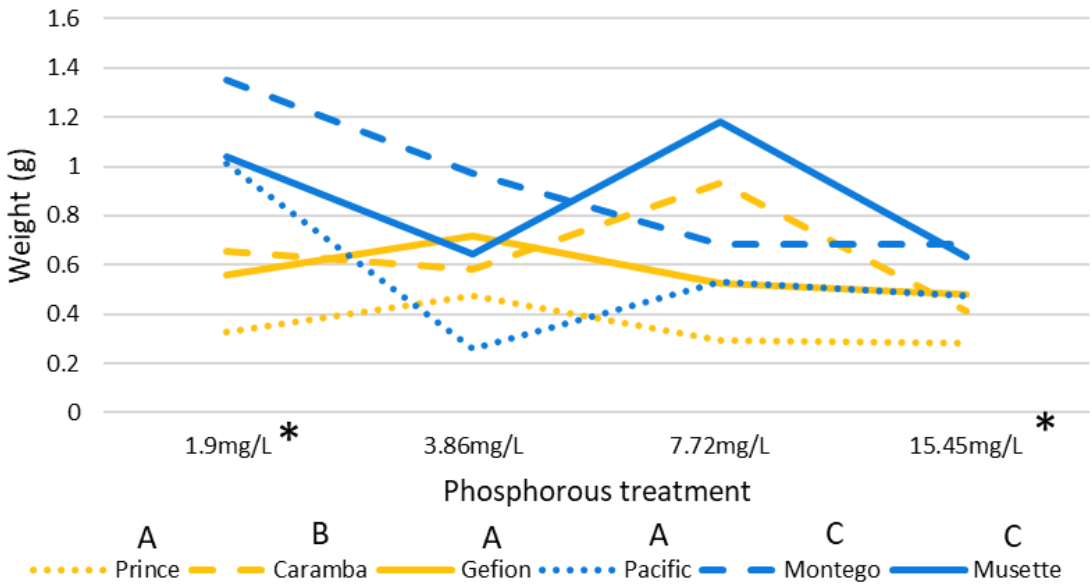

Figure 44 shows the root dry weight results of the first 3-week hydroponics experiment under four different treatments. There were significant differences between the high and low groups for the 1.9mg/L and 15.45mg/L phosphorus treatments with the high group having significantly greater biomass than the low group in both treatments (p=0.003 and p=0.019 respectively).

The additional analysis with ANOVA and post-hoc testing showed that Caramba (B) was significantly higher than Prince (A). Montego and Musette (C) had significantly greater biomass than all other varieties except for each other (p=<0.001)

The stem and leaf dry weight also had significant differences between the high and low groups of varieties under the 1.9mg/L and 15.45mg/L treatments, with the high accumulating group having significantly higher leaf and stem biomass (p=0.02 and p=0.026 respectively). Post-hoc analysis showed that Prince was significantly lower than all other varieties. Additionally, Pacific was significantly lower than Musette, but neither was significantly different from the other varieties, excluding Prince (p=<0.001).

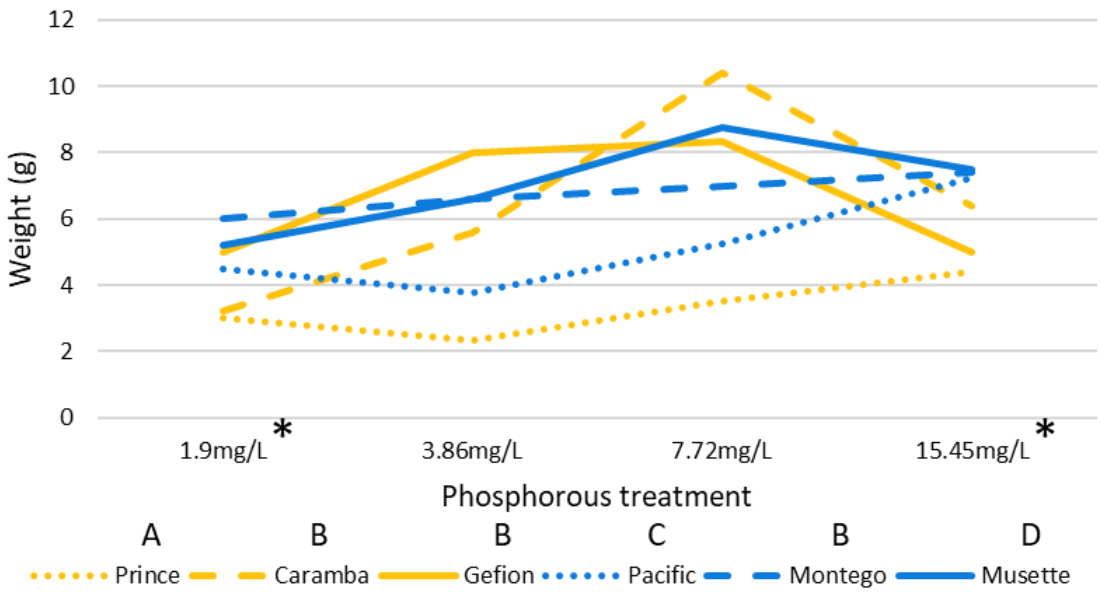

Figure 45 shows the results of the stem and leaf biomass measurements from the first 3 week hydroponics experiment. T-tests showed that there were significant differences between the high and low groups of varieties for the 1.9mg/L and 15.45mg/L phosphorus treatments (p=0.02 and p=0.026 respectively). The high group had significantly greater stem and leaf biomass than the low group under the 1.9 and 15.45mg/L treatments.

There were also some differences between individual varieties when tested with an ANOVA (p=<0.001). Additional post-hoc testing showed that Prince had significantly lower biomass than all other varieties. Musette and Pacific were also significantly different from each other, with Musette having significantly higher biomass than Pacific, but neither variety was significantly different from the other varieties, excluding Prince.

Both the biomass of the leaves and the biomass of the roots were affected by the treatment the plants received. The two-way ANOVA showed that there was a significant difference between the biomass of plants grown under different treatments (First 3-week experiment stem and leaf biomass p=<0.001, root biomass p=0.001) The stem and leaf biomass increased with higher phosphorus concentrations. The root biomass may decrease as the concentration of phosphorus increases however the variation in how individual varieties react to different treatments makes this unclear.

4.3.2.3 The high accumulating varieties had higher biomass measurements but lower SPAD values in the second 3-Week experiment.

As with the previous 3 week experiment the goal of this experiment was to identify if the results of the root biomass, shoot biomass, leaf number and SPAD measurements correlated to the high or low phosphorus accumulation groups. This experiment also aimed to eliminate the problems of the previous experiments had with root matting and SPAD measurement collection.

As was the case with the two previous hydroponics experiments there was no difference between the number of leaves produced by the high and low varieties regardless of the treatment applied. However, there were differences between the groups in both the weight measurements and the SPAD measurements.

There was a very clear difference between the dry root weight of the high and low groups in this experiment. The high group had significantly heavier roots than the low group in all four treatments (1.9mg/L $p=<0.001$, 3.86mg/L $p=<0.001$, 7.72mg/L $p=0.002$, 15.45mg/L $p=<0.001$). There were some differences between the individual varieties as well. The low accumulation group varieties were all significantly lower than the high group varieties but otherwise were not significantly different form each other. However, within the high group Pacific had a significantly lower root biomass than both Montego and Musette as can be seen in figure 46 ($p=<0.001$).

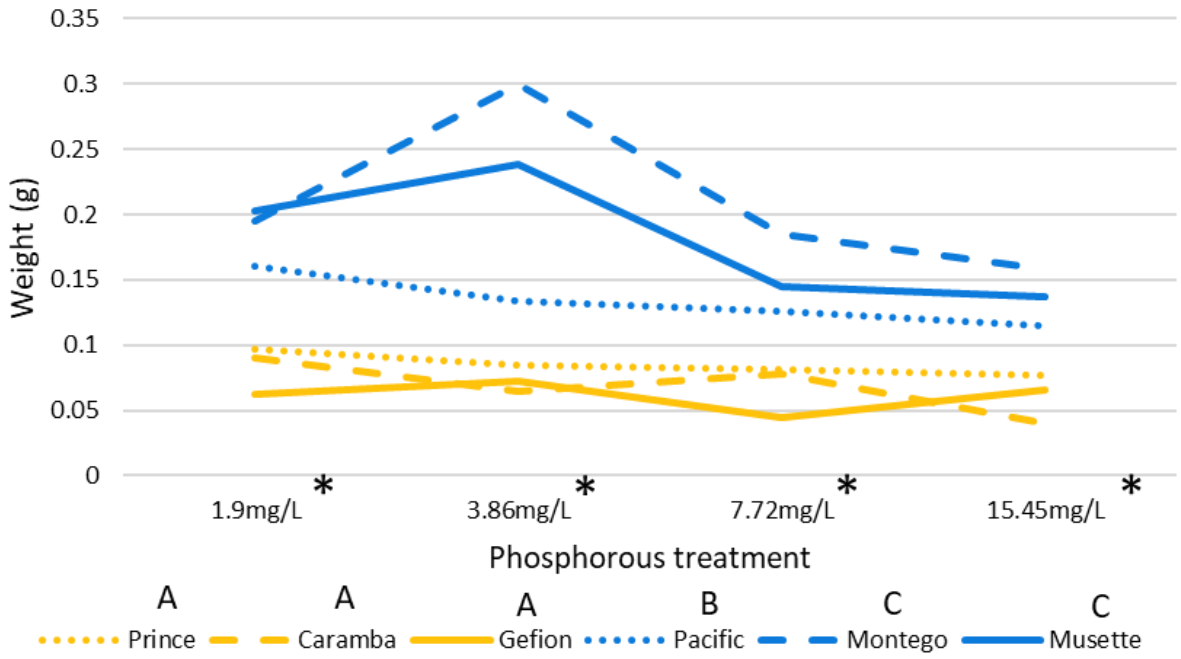

Figure 46 - shows the second 3-week hydroponic experiment root dry weight results. The two groups of varieties had significantly different root weights for all of the treatments (1.9mg/L p=<0.001, 3.86mg/L p=<0.001, 7.72mg/L p=0.002, 15.45mg/L p=<0.001). The high phosphorus varieties had a larger dry biomass than the low group varieties.

The additional testing with ANOVA and post hoc analysis showed that there were also some differences between the high group varieties, Pacific had a significantly lower root biomass than either Montego or Musette but was still significantly higher than the low group (p=<0.001).

The difference between leaf dry weight of the high and low varieties was less clearly defined than that of the dry root weight, however, there was a significant difference between the two groups for all of the treatment concentrations (1.9mg/L p=<0.001, 3.86mg/L p=0.042, 7.72mg/L p=0.027, 15.45mg/L p=0.003) with the high phosphorus accumulating group having significantly higher shoot biomass. Post hoc analysis also showed differences between individual varieties within the groups. In the low group Gefions biomass was significantly higher than that of Prince although not significantly different from that of Caramba. In the high group Pacific had significantly lower biomass than Musette while Montego was not significantly different from either of them.

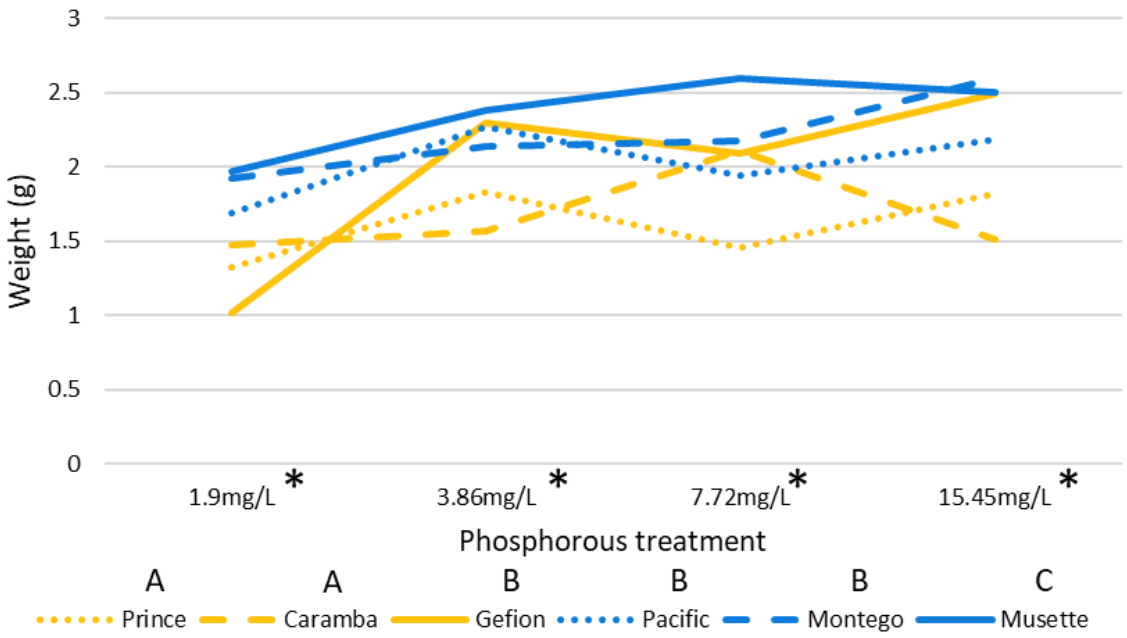

Figure 47 - Shows the leaf dry weight of the second 3-week hydroponics experiment. T-tests showed that there were significant differences between the high and low groups for all of the phosphorus concentrations (1.9mg/L p=<0.001, 3.86mg/L p=0.042, 7.72mg/L p=0.027, 15.45mg/L p=0.003). The high group had significantly greater leaf biomass than the low group in all of the treatments.

ANOVA and post-hoc analysis also showed differences between the individual varieties (p=<0.001) Prince (A) was significantly different from all except Caramba (A) and Musette (C). Gefion (B) was significantly different from Prince (A) and Musette (C). Pacific (B) was significantly different from Prince and Caramba (A) and Musette (C). Montego was significantly different from Prince and Caramba (A).

Although the difference isn't as clear as in the eight-week experiment the biomass of both the roots and the shoots was affected by the treatment that the plant received. The two-way ANOVA showed that as well as variety affecting biomass the treatment also affected the biomass of the plants (Second 3-week experiment stem and leaf biomass p=<0.001, root biomass p=0.011). The shoot biomass increased as the phosphorus concentration increased while the root biomass decreased.

Only one of the four treatments, 15.45mg/L in the second 3-week experiment showed a significant difference between the SPAD measurements of the two groups (p=0.045) with the low phosphorus accumulating group having higher SPAD values. There were some interesting results from the post-hoc analysis however, which showed that while there were no significant differences between any of the individual varieties in the high group the low group had much more variation in

SPAD measurements (p=<0.001). Both Prince and Gefion were significantly different from all other varieties with Prince significantly higher and Gefion significantly lower. While Caramba was significantly different from Prince, Gefion and Pacific but was not significantly different from Montego and Musette.

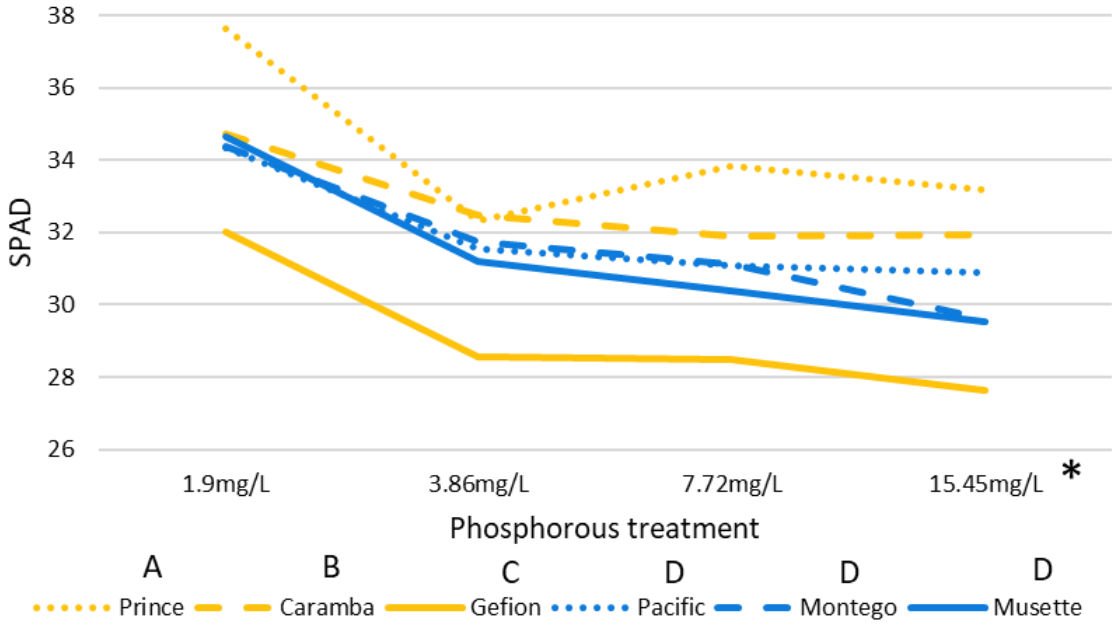

Figure 48 – shows the SPAD values from the second 3-week experiment. t-tests showed that the SPAD values were only significantly different between the two groups in the 15.45mg/L treatment (p=0.045). The high group had significantly lower SPAD values than the low group.

There were also differences between the individual varieties within the groups (p=<0.001). Both Prince (A) and Gefion (C) were significantly different from all other varieties with Prince significantly higher and Gefion significantly lower. Caramba (B) was significantly different from all other varieties except for Montego and Musette. There was no significant difference between any of the high group varieties when considered individually.

4.4 Discussion

4.4.1 Root architecture

The aim of this experiment was to examine the root architecture of the varieties and look for correlations between the high and low groups under different phosphorus concentrations. It was thought that adaptations in root architecture that increase phosphorus acquisition would be more prominent under lower phosphorus conditions. This was similar to the first root architecture experiment, but with an improved design, as it removed the confounding variables caused by

using the ¼ strength MS media. By using custom media solutions based on the Hoaglands nutrient solution only the level of phosphorus was changed. As expected, there were some differences between the results of the first root architecture experiment and this experiment, possibly caused by only the phosphorus concentration being altered rather than the concentration of other nutrients. For example, in the ¼ MS media experiment there were significant differences in primary, lateral and total root length between the high and low phosphorus accumulating groups. However, in this experiment only the primary root was significantly different between the groups with the low phosphorus group having longer primary roots. This suggests that the root length results from chapter 3 may have been due to a lack of another nutrient. Potentially nitrogen, as low nitrogen conditions have been linked to increased root length as the plant forages for nitrogen (Tian et al, 2022).

The aim of the four treatments was to examine whether the plants had the same traits under different phosphorus concentrations. However, in retrospect having four phosphorus treatments makes understanding the data more difficult as there are several instances, for example convex hull, where there are only significant differences between the groups in one treatment out of the four. Given the time constraints it may have been better to have a single treatment or perhaps a low phosphorus treatment and a high phosphorus treatment. This would have increased the replicates and allowed a stronger correlation to be made between the high and low groups. Narrowing the focus to two treatments with a larger difference in phosphorus concentration between them would have still fulfilled the aim of examining how the root traits responded to phosphorus concentration while increasing the number of replicates.

Previous research has shown that under low phosphorus conditions *Brassica napus* alters root architecture to increase the length of roots (Lyu et al, 2016). Therefore, it was thought that the accessions with a high phosphorus content in the RIPR population may display increased root length as a possible adaptation accounting for their increased phosphorus uptake. In the first experiment using the ¼ strength MS there were indeed significant differences between the high and low groups for

all three root length measurements, primary, lateral and total root length (p=0.012, p=0.012 and p=0.009 respectively). With longer roots in the high phosphorus accumulation group of accessions. However, in this experiment only the primary root length showed any significant differences between the high and low groups of varieties and then only under the 3.86mg/L phosphorus treatment where the low accumulation group had significantly longer primary roots (p=0.0498). This suggests that the results in chapter 3 were due to the effect of another nutrient or nutrients and that root length is not an important trait for phosphorus acquisition in plants of this age.

Increasing the area of soil accessed by the roots has been seen as an adaptation to low phosphorus conditions (Adeleke et al, 2019). Based on this it was thought that the high phosphorus acquisition varieties would have had a greater convex hull area than the low group. The convex hull area was indeed significantly different between the two groups in the MS media experiment (p=0.001) with the high group having a larger convex hull area than the low phosphorus group. This experiment also had a significant difference between the high and low groups, although only in one of the treatments, the 3.86mg/L treatment (p=0.031). However, in this experiment the high phosphorus accumulating group had a lower convex hull area than the low group. This indicates that the strong correlation from the previous experiment in chapter 3 may have been due to the low levels of a nutrient other than phosphorus. Part of the difference in convex hull area in this experiment may be due to the differences in primary root length. As convex hull is calculated by drawing around the furthest spread points of the root system a significant increase in the primary root length could create a corresponding increase in convex hull area. These results indicate that the prediction that the high phosphorus accumulating accession would have a larger convex hull area is incorrect for these varieties at this stage of growth.

In addition to root length and the area of soil explored, the shape of the root system within the soil has also been linked to phosphorus uptake. A shallower root system allows plants to forage more effectively in the topsoil where phosphorus is most abundant (Duan et al, 2020). It was therefore thought that the high phosphorus accumulating plants may have a shallower root system where the bulk

of the roots could exploit the topsoil. In this experiment both the maximum depth and the Y centroid co-ordinate were significantly different between the high and low groups under the 1.9mg/L treatment with the high acquisition group having a shallower root system than the low group. This would suggest that the high acquisition group is showing the expected shallower root system that improved topsoil foraging. However, further work should be carried out to solidify this result. There was only a significant difference between the groups in one of the four treatments and the plants in the pouch and wick experiment are very young. Furter experiments with older plants would show if these plants continue to prioritise topsoil foraging.

The number of lateral roots produced by the plant has been linked to phosphorus foraging with increased numbers of laterals improving the acquisition of phosphorus in low phosphorus conditions (Duan et al, 2020). Based on this it was predicted that the high phosphorus acquisition group of varieties would have a higher number of laterals than the low group. It was therefore surprising in the MS media root architecture experiment that the lateral root number was not significantly different between the high and low groups ($p=0.147$). In this experiment using custom media, however, the lateral root number was significantly higher in the high acquisition group for three of the four treatments, the 1.9mg/L, 7.72mg/L and 15.45mg/L treatments ($p=0.002$, $p=<0.001$ and $p=0.035$ respectively). This strongly suggests that the prediction was correct and there is a correlation between the greater number of laterals and increased acquisition of phosphorus.

This is particularly interesting as while there was a significant increase in the number of laterals the high phosphorus group have there was no significant difference in the total lateral root length or the maximum width that the roots reached between the two groups. Therefore, it appears that while some of the lateral roots of the high group are reaching the same maximum width as the low group, the rest of the high group laterals are comparatively shorter. This suggests that any lateral root-based increase in acquisition relies on the number of laterals, not on an increase in lateral root length or distance from the plant.

Potentially having a greater number of short laterals could allow the high phosphorus acquisition plants to explore the soil in more directions at once than the lower numbers of long laterals that the low group have. This hypothesis could be investigated further using an alternative growth system. Due to the 2D nature of the pouch and wick system it is impossible to accurately measure the direction and angle at which laterals are emerging.

4.4.2 Biomass is significantly affected by both accession and treatment.

As it was not possible to make more detailed measurements of root architecture the biomass of the roots was measured instead. An increase in root biomass has been seen as an adaptation to phosphorus stress (Duan et al, 2020). And across the hydroponics experiments the root biomass was affected by the phosphorus concentration. As expected, root biomass decreased as phosphorus concentration increased. Correlates to what is already know about phosphorus stress increasing root biomass.

Because root biomass increases are linked to improved phosphorus foraging it was thought that the high phosphorus accumulating group would have a greater root biomass than the low group. Although root weight could not be measured for the 8-week plants both of the 3-week experiments had measurements of root biomass taken. In the first 3 week experiment the dry root weight was significantly different between the high and low groups for the 1.9mg/L and 15.45mg/L treatments ($p=0.003$ and $p=0.019$ respectively). Under these treatments the high group of varieties had significantly higher root biomass. In the second 3-week experiment the dry root biomass was significantly different for all four treatments (1.9mg/L $p=<0.001$, 3.86mg/L $p=<0.001$, 7.72mg/L $p=0.002$, 15.45mg/L $p=<0.001$). Again, the high group had significantly heavier root biomass than the low group.

From these results it appears that the high phosphorus varieties from the RIPR population have a higher root biomass than the low phosphorus varieties. This is in line with the expected results, a larger root system would potentially increase the phosphorus foraging of the plants. The difference between the results, with the first 3-week experiment only having significant results for the 1.9 and 15.45mg/L

treatments while the second 3 week experiment had all treatments showing a significant difference, could be due to the low sample size of the first 3-week experiment. Ideally the results would have been collated together however the drastic differences in greenhouse temperature due to the heatwave prevented this. Further repeats of the experiment may confirm the results seen here.

These results do contrast somewhat with the results of the custom media Pouch and Wick experiment. In the Pouch and Wick experiment the results of root length measurements showed that the low phosphorus accumulating group had longer primary roots, although there was no difference in the lateral or total root length. However, the higher root biomass in the hydroponics experiment suggests that the high phosphorus accumulating group has more roots than the low group by this stage of growth.

These experiments only give a snapshot of the root growth, and the hydroponics in particular, gives a very limited measure of the roots in the form of biomass. The next step could be to run an experiment using CT scanning. The plants could be grown on an inert substrate to maintain control of phosphorus provision through irrigation with nutrient media. Since CT scanning is non-destructive it would be possible to take multiple measurements of the roots and develop an understanding of how the roots grow over time. It would also allow testing of the hypothesis that the increased number of laterals in the high phosphorus group allow the plant to forage for phosphorus in more directions than the low group.

4.4.3 Plant health and growth speed are not significantly affected by accession.

The number of leaves did not vary significantly between the high and low groups of varieties in any of the hydroponic experiments. This suggests that there is no difference in growth speed between the high and low varieties as leaf number indicates the growth stage that the plant has reached. However, the results of the leaf and stem biomass measurements indicate that under some treatments the high phosphorus varieties had a greater biomass than the low phosphorus varieties. This could indicate that while the plants are reaching the growth stages at the same speed the high phosphorus varieties are growing leaves and stems that are larger

than those of the low phosphorus varieties. This could potentially be confirmed by further experiments where leaf area is measured.

It could also be interesting to repeat the hydroponic experiment and grow the plants to senescence and compare the branching of the stem and the seed yield between the high and low varieties. Potentially there may be differences in the branching of the stem at the inflorescence stage of growth. Phosphorus deficiency has been linked to decreased seed yield in *Brassica napus* as it reduces the branching of the stem and the formation of seeds (Wang et al, 2021).

As well as growth speed the health of the plants was also measured. SPAD gives a measurement of relative chlorophyll levels within the leaves. Chlorophyll concentration is often used as an indicator of general plant health (Kumar & Sharma, 2019). The SPAD value was only significantly different between the two groups in the second three-week experiment and then only under the 15.45mg/L treatment ($p=0.045$).

If there are differences in phosphorus uptake ability as predicted, then potentially the low group are benefiting from the higher phosphorus concentration in the media. However, it was also thought that if the high phosphorus varieties are adapted to take up phosphorus more efficiently that they would be significantly healthier under the low phosphorus treatments, and this is not supported by these results. Also, there were no significant differences in the 8-week spad measurements suggesting that if there is a difference in the plant health at 3 weeks then this does not continue as the plants age. Therefore, it does not appear that there is a strong difference in the SPAD levels between the two groups of accessions, and no indication that the health of the plants varies between the two groups of accessions.

4.4.3 Variation of results within groups of accessions

The variability in these results makes drawing strong conclusions about correlations between the groups of varieties and particular traits difficult. While there are significant differences between the high and low groups in many of the traits, which suggests that the groups are responding to phosphorus differently, there is also a deal of variation between the individual varieties, particularly in the high phosphorus group. This variability suggests that the individual varieties within the group may not have the same adaptations to phosphorus uptake.

In both the root architecture and hydroponics experiments detailed in this chapter the high group of varieties had greater variation in their results than the low group. Across the measurements where there was a significant difference between the groups further post-hoc analysis showed that Pacific was significantly different from both Montego and Musette in most measures. The exceptions to this were the width to depth ratio in the architecture results where pacific and Montego were significantly different from Musette, the SPAD measurements from the second 3-week experiment where there was no significant difference between the high group varieties, and the stem and leaf biomass measurements from the second 3-week experiment where Pacific was significantly different from Musette but not from Montego. In contrast while there were some differences between the low group varieties these were fewer and there was no single variety that was consistently different from the others.

This suggests that while the high group has some significant differences from the low group; greater root biomass, increased lateral number an altered width to depth ratio; there are also significant differences between the individual varieties within the high phosphorus group. This raises the possibility that the high phosphorus varieties could have developed different adaptations to increase phosphorus uptake.

Expanding the number of varieties investigated would therefore be of interest for several reasons. Firstly, to see if the significant differences in root architecture and biomass seen in these experiments apply to larger groups of high and low

phosphorus varieties. Secondly, to investigate whether the high phosphorus varieties to have a high level of variability between individual varieties across a wider group or if Pacific is an outlier.

4.5 Conclusion

These results show a correlation between the phosphorus content of the plants in the RIPR population and physiological differences in both the root architecture and the biomass of the roots. The high phosphorus acquisition plants show an increase in the number of lateral roots and a decrease in the depth of the y-centroid co-ordinate at two weeks growth and an increase in the root biomass at three weeks growth in the hydroponics. Further experiments which expand on the number of accessions studied and increase the number of replicates may strengthen the evidence for these correlations. It would be particularly beneficial to expand the number of accessions as variation within the varieties in the high group suggest that there may be more than one physiological “strategy” for phosphorus acquisition being employed by *Brassica napus*.

Chapter 5: Discussion

5.1 General conclusions

The key conclusions that can be drawn from the research presented here:

Root architecture impacts phosphorus acquisition in *Brassica napus* (Chapter 2).

There are no significant differences in the root anatomy of the accessions studied during the early growth of the *Brassica napus* seedlings (Chapter 3).

The accessions that accumulated more phosphorus in the RIPR population had increased root and shoot biomass compared to the low phosphorus accessions (Chapter 4).

There are significant differences in root architecture between the high and low phosphorus groups of accessions which should be investigated further by expanding the number of accessions experimented on (Chapter 4).

There were significant variations in root architecture and biomass of individual accessions within the groups, particularly within the high phosphorus accumulating genotypes (Chapter 4).

5.2 General discussion

5.2.1 Variation in phosphorus acquisition linked to root architecture.

This work aimed to identify correlations between root traits and increased phosphorus acquisition in *Brassica napus*. This was done with the aim of finding traits that may improve phosphorus uptake. To do this six *Brassica napus* lines were selected, three which had low phosphorus levels in their leaves and three with high phosphorus in their leaves.

The selection of the varieties was based on the Renewable Industrial Products from Rapeseed (RIPR) population a subset of the ERANET-ASSYST consortium diversity population. In 2016 the RIPR population had element analysis of their leaf tissues carried out (Alcock et al, 2016). These plants were grown in soil and had their phosphorus level sampled at the rosette stage.

The RIPR population is made up of a variety of brassicas, spring oilseed rape, winter modern oilseed rape, winter oilseed rape, swede and fodder. I focused on the

winter modern oilseed rape varieties as winter is the most common growth period for oilseed rape grown in Europe (Gourrion et al, 2020). There are 80 winter OSR modern varieties in the RIPR population and the phosphorus content of the leaves of these varieties varies from 3290mg/kg to 5698mg/kg. A t-test was carried out on the highest and lowest 20 accessions leaf phosphorus data to establish if the variation in phosphorus was significant. This t-test showed that there was a significant difference between the highest and lowest phosphorus accessions ($p=3.51226E-17$). This suggests that there is significant variation in phosphorus acquisition between the highest and lowest of the winter oilseed rape modern varieties. Based on these 6 varieties were chosen from the highest and lowest phosphorus groups (3 from each group). These varieties were Prince (3290mg/kg), Caramba (3675mg/kg) and Gefion (3720mg/kg) as the low phosphorus content accessions and Pacific (5307mg/kg) Montego (5698mg/kg) and Musette (5231mg/kg) as the high phosphorus accessions.

Phosphorus uptake is influenced by more factors than the root architecture and anatomy. Other factors that affect phosphorus uptake include root exudates and mycorrhizal interactions. Plants can also alter phosphorus use efficiency under phosphorus stress. Therefore, it was necessary to establish if there was any indication of a correlation between root architecture and phosphorus uptake, before continuing to investigate specific root architectural traits.

In order to do this, an experiment was carried out in a hydroponic set up. Using nutrient film technique hydroponics, where the roots are freely floating in a constant stream of media, removed root architecture as a factor in phosphorus uptake. This method also removes the effect of exudates and mycorrhizal interactions.

In the soil exudates are concentrated around the root where they assist with nutrient foraging by converting phosphorus compounds in the soil into soluble phosphorus that the plant can take up (Redel et al, 2019). In the hydroponics any exudates from the roots will be washed away and circulate in the solution and therefore will not be directly beside the roots to assist with phosphorus uptake.

Mycorrhizal colonisation of roots will also be prevented in the hydroponics. In soil mycorrhizal interactions increase phosphorus foraging through increasing the soil volume explored. The hyphae of the fungi can reach several centimetres into the soil surrounding the root, far beyond the phosphorus depletion zone around roots, increasing the area from which phosphorus is absorbed by as much as two orders of magnitude (Püschel et al, 2021).

However, in the hydroponics the loss of these adaptations may be mitigated by the phosphorus being in a constantly circulating liquid media. The phosphorus is not bound to other elements and therefore does not need exudates to convert it into a soluble form, nor does it require the foraging at a distance from the roots that mycorrhizal interactions provide. In this way it was thought that any reduction in the role of the exudates and mycorrhizal interactions could be compensated for in the hydroponics. There are root architecture traits, for example root length and number of laterals that are less likely to be affected by growth in hydroponics. A larger root surface area from a larger root system will still provide greater uptake of phosphorus whether grown in soil or in the hydroponics.

However, the root architecture was thought to be likely to be more strongly affected by growth in the hydroponics as there are a number of root architecture adaptations that relate to the roots positioning within the soil relative to other roots. For example, the measure of the convex hull area of the roots indicated the total area of soil the roots forage in. When roots are floating in media adaptations that rely on relative soil position like this would be removed. If in the hydroponics there was a significant difference in the phosphorus levels in the plants compared to the in-soil experiments in the RIPR population then this would suggest that root architecture may play a significant role in phosphorus acquisition in these *Brassica napus* varieties. While the changes to the exudates and the mycorrhizal interactions may have also been factors in changing phosphorus uptake, as stated it was thought that these may be less strongly affected than root architecture.

The experiment was carried out twice, once on three-week-old *Brassica napus* seedlings and once on eight-week-old *Brassica napus* plants. In both experiments four phosphorus concentrations were used 1.9mg/L, 3.86mg/L, 7.72mg/L and

15.45mg/L. When the plants were sampled, there were no significant differences in phosphorus concentration between the two groups of accessions in either experiment regardless of the treatment used. This suggests that removing root architecture as a factor may have affected phosphorus acquisition by the plants. These results made me confident that these accessions where candidates for further study to investigate the role of root architecture further.

5.2.2 Root anatomy does not vary between accessions during early growth stages.

The sectioning experiment in chapter 3 showed that there was no significant difference between the accessions for any of the root anatomy traits examined. The sections were taken from plants grown in the pouch and wick system and were therefore only 2 weeks old when sectioned. The roots had not yet begun to undergo secondary root growth as evidenced by the intact epidermis, cortex and endodermis layers visible in the sections. In dicots such as *Brassica napus* the roots expand through secondary growth were the epidermis, cortex and endodermis are lost and replaced with parenchyma, periderm and secondary xylem tissues.

Based on my results there are no significant differences in the anatomy of roots during the primary growth stage that correlates to phosphorus uptake. However, differences in secondary root growth have been linked to phosphorus acquisition in other dicotyledonous plants including Lentil (*Lens culinaris*) and Chinese fir (*Cunninghamia lanceolata*) (Sarker et al, 2015)(Haroon et al, 2023)

5.2.3 Increased biomass in high phosphorus group.

In both of the 3-week hydroponics experiments the high phosphorus group of accessions had a higher root and shoot biomass than the low group. While root biomass could not be measured in the 8-week experiment the shoot biomass was significantly higher for the high phosphorus acquisition group. An increased root biomass may be an adaptation to phosphorus acquisition as an increase in the number of roots could potentially improve the phosphorus foraging ability of the plants. This is further supported by the results of the analysis comparing root

biomass under the different phosphorus treatments, where the root biomass decreased as the phosphorus concentration in the treatments increased, suggesting that under lower phosphorus concentrations the plants are investing more resources into root production to increase phosphorus foraging.

If, as the results suggest, the high phosphorus plants are more able to take up phosphorus then this could be promoting the increased shoot biomass seen in the high group compared to the low phosphorus group. Increased phosphorus foraging would allow the plants to invest more resources into the shoot, therefore increasing the shoot biomass.

5.2.4 Differences in root architecture between the high and low phosphorus groups.

There were significant differences between the root architecture traits of the high and low phosphorus groups in both chapter 3 and chapter 4 which suggests that these traits correlate to phosphorus uptake. The methodology for chapter 3 means that there may have been confounding variables introduced due to the choice of media.

In chapter 4 there were several root architecture variables correlated with phosphorus acquisition. The high phosphorus group had longer primary roots and an increased numbers of lateral roots compared to the low phosphorus group. There was also a significant difference in the convex hull area with the high group having a significantly smaller area than the low group.

Increasing the primary root length and the number of laterals may allow the high phosphorus plants a greater root surface area for phosphorus uptake. However, the fact that the root area is decreased in the high phosphorus varieties is surprising and may be linked to the other key finding, that the high phosphorus group had considerable variation between the individual accessions within the group.

5.2.5 Significant variations in root architecture and biomass of individual accessions within the groups.

Two sets of statistical analysis were carried out, t-tests to compare the two groups of accessions and ANOVA and post hoc analysis to compare the individual accessions to each other. This second analysis showed that in many of the experiments whether the t-test had a significant result or not there were significant differences between the individual accessions within the groups. In particular, Pacific (high P-accumulating line) often had the opposite result to the other high P-accumulating lines (Montego and Musette). The low phosphorus varieties had some variation but less than the high group.

This was consistent across both the root architecture experiment in the pouch and wick system and the hydroponics experiment using the nutrient film technique. The variability in the results raises the possibility that there are different adaptations being used by the varieties and that phosphorus acquisition may have a more complicated interplay of factors than the ones examined in this thesis.

My work has contributed to quantifying a selection of root traits and their correlation with phosphorus uptake within these 6 varieties of *Brassica napus*. There is evidence that some of these root architecture traits are correlated to the increased phosphorus acquisition seen in the high group, Pacific, Montego and Musette. The increased root biomass, lateral root number and the shallower y centroid coordinate seen in the experiments in chapter 4 are in line with existing knowledge on adaptations to phosphorus scarcity seen in *Brassica napus* (Duan et al, 2020).

However, the variability in the results of this group have raised further questions about how these traits influence the differences in phosphorus acquisition seen in the RIPR population. Due to the differences in the results of Pacific compared to Montego and Musette it appears that there may be different strategies for phosphorus acquisition in plants. These strategies may be related to other root architecture traits which have not been studied here. Potentially I have overestimated the importance of root architecture and the differences are due to a

lack of mycorrhizal interactions caused by the use of hydroponic rather than soil-based growth systems.

5.3 Further work

5.3.1 Increase the number of accessions studied.

This work has produced some interesting results linking specific root traits, increased root biomass, reduced convex hull area and increased lateral root number, to the higher phosphorus acquisition of Pacific, Montego and Musette. However as stated above, within the groups of accessions, particularly in the high phosphorus accessions, there was a lot of variation in the root traits measured. Pacific often had the opposite reaction to Montego and Musette, for example, Pacific had a lower number of laterals than the low phosphorus varieties while Montego and Musette had an increased number of laterals. This variability was seen in both the root architecture and the biomass measurements. An expanded set of accessions could clarify whether Pacific is an outlier. Alternatively, an increased number of accessions may show that there are different strategies for phosphorus acquisition used by different accessions.

5.3.2 Investigate root architecture traits in older plants.

The pouch and wick system is an effective tool as a high throughput method for establishing if there is any evidence for differences between the high and low phosphorus accessions. However, A key limitation of this system is that plants only be grown for 2 weeks before the roots grew off the paper and could no longer be accurately photographed. The short growth time may affect the results of the architecture experiments due to the seed nutrient content. The anatomy experiment was also affected by the short growth time as the roots had not yet entered secondary growth.

An alternative to the pouch and wick system would be to use CT scanning and grow the plants in sand filled tubes. This method would allow for a longer growth time and would also allow for repeat measurements to be carried out over that growth

time. Additionally, as the images taken would be 3D the range of root architecture variables could be expanded. I would propose the use of sand, or another inert growth medium, as this would allow for greater control of the nutrients provided to the plant than using soil. Furthermore, extracting the roots from sand is likely to be significantly easier than extraction from soil which would allow root samples to be taken for sectioning. This would enable further anatomy experiments to be carried out to establish if there are any differences between the high and low accessions during secondary growth.

5.3.3 Examine the effect of seed nutrients on early growth.

An additional concern with the results from the pouch and wick experiments is that due to the age of the plants the root growth could be influenced by the nutrient content of the seeds. As the plants grown in this system can only be grown for about two weeks the plants had not got past the cotyledon stage. In dicotyledonous plants like *Brassica napus* the embryo of the seed, which consists mostly of proteins and triacylglycerols, and the endosperm are used to fuel post-germination growth of seedlings until photosynthesis can be established (Shi et al, 2020). The cotyledons are also components of the seed and are capable of storing nutrients until the first true leaves appear (Shi et al, 2020). Therefore, in these very young plants the effect of nutrients from the seed may be high. During sowing efforts were made to use seeds of a uniform size to minimise the effect of seed nutrient differences on the growth of the seedlings. The plants in my experiments appear to respond to the changes in phosphorus treatment as seen in the second pouch and wick experiment, however, differences in the existing nutrient stores within the seeds could potentially affect the results.

While this effect would be less of a factor if an alternative growth method and longer growth period were used as suggested. Older plants will have grown beyond the stage where they are drawing nutrients from the seed and cotyledons and would be reliant on the nutrients that could be obtained from the media.

However, an experiment to compare the nutrient content could be carried out as a more quantitative measure of seed nutrients than using seeds that visually appear

the same. By carrying out element analysis of the seeds, as was done on the plants in chapter 2, would show if the plants have any significant difference in the nutrients that are available during the initial growth of the seedlings. If there are no significant differences in the nutrient content of the seeds, then it is unlikely that the results of the pouch and wick experiment would have been affected by seed nutrition. However, if there are significant differences in the nutrients stored in the seeds of different varieties then this could be factored in future research.

5.3.4 Repeat sectioning in older plants to examine secondary growth of roots.

As well as the potential effects of seed nutrients on the results the use of the pouch and wick system also limited the scope of the anatomy experiment. When the roots of the plants grown in the pouch and wick system were sectioned it was found that the roots were still in the primary growth phase. To examine the secondary growth of the roots the growth method would need to be changed to allow the plants to grow for longer before being sectioned. The CT scanning method outlined above would allow for a longer growth period. The growth substrate would need to be selected carefully to allow for easy excavation of the roots to minimise mechanical stress that could affect the sections, although older roots may be more robust than the roots that were sectioned in this work. Sectioning older roots should allow a comparison of the secondary root growth of the different varieties. Similar traits such as diameter, cell number and cell area should be examined although the tissue types will be different than those seen in the sectioning in this work as the epidermis, cortex and endodermis will have been replaced.

5.3.5 Deep water hydroponics

It was unfortunate that the hydroponics experiment was impeded by both the weather and the hydroponics system used. The experiments done in this work cannot be collated due to the heatwave and therefore had to be treated as separate experiments with smaller sample sizes. Further repeats of the experiment would allow for a larger sample size if the results of repeats could be collated which would improve the strength of the statistical analysis.

However, an alternative to further repeats would be to use deep-water hydroponics instead of the nutrient flow technique used in this work. This would require a deep-water system to be built that could accommodate the plants, however, there would be significant benefits to using this system. In the nutrient flow technique, the plants could only be grown for 3 weeks before the roots began to mat together. In a deep-water system there would be significantly more space for root growth which would allow for older plants to be grown. Additionally, in the NFT system there were issues with the volume of roots in the growth channels restricting the flow of media which would not be an issue in a deep-water system as the roots are submerged directly into the nutrient reservoir. Some consideration would need to be made for the fact that once past the rosette stage *Brassica napus* plants become very tall and without soil to anchor them they become very unstable. In the NFT system a few plants which passed the rosette stage and began to bolt had to be supported using a frame. If a similar support were included in a deep-water system, then it would potentially be possible to grow *Brassica napus* from seed to senescence in the hydroponics. This could allow for a range of experiments to be carried out on the roots over the lifetime of the plant. It could also allow for repeated measurements of SPAD to monitor leaf health under different phosphorus conditions over the lifetime of the plants.

5.4 Conclusion

This work has shown that root architecture is a factor in phosphorus acquisition and has identified specific root characteristics that are correlated to increased phosphorus acquisition in six varieties of *Brassica napus*. Increased root length increased lateral number and increased root biomass are all linked to improved phosphorus acquisition. However, as evidenced by the variability in root architecture traits within the high phosphorus group there is more than a single phenotype that is linked to increased phosphorus acquisition, suggesting that phosphorus uptake is a complicated process that is influenced by many factors. This work can therefore form the basis for future research that expands on the results found here.

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Appendices

Appendix 1

The Renewable industrial products of rapeseed (RIPR) element analysis data on which I based my selection of winter oilseed rape varieties for study (Alcock et al., 2018).

The data can be found at the following link:
https://www.frontiersin.org/articles/10.3389/fpls.2018.01487/full

Supplementary table 1

Appendix 2

Element analysis of hydroponic grown plants raw data (chapter 2). This document contains the raw element data from the element analysis done by Lancrop.

The data can be found at the following DOI: 10.17639/nott.7209

Appendix 3

Additional element analysis graphs not included in thesis (Chapter 2)

DOI: 10.17639/nott.7316

Appendix 4

RootNav results from analysis of images of the plants grown in the pouch and wick experiment (Chapters 3 and 4). This Excel document contains the raw data for both plants grown in MS media and the plants grown in the variable phosphorus media.

The data can be found at the following DOI: 10.17639/nott.7206

Appendix 5

Additional MS media graphs not included in thesis (Chapter 3)

DOI: 10.17639/nott.7316

Appendix 6

Macro for combining split confocal channels in ImageJ

```
input = getDirectory("Choose an input directory");
output = getDirectory("Choose an output directory");

processFolder(input);

function processFolder(dir) {
	list = getFileList(dir);
	for (i=0; i<list.length; i++) {
		if(endsWith(list[i], ".ids")) { //add the file ending for your images
			processFile(dir, output, list[i]);
		} else if(endsWith(list[i], "/") && !matches(output, ".*" +
substring(list[i], 0, lengthOf(list[i])-1) + ".*")) {
			//if the file encountered is a subfolder, go inside and run the
whole process in the subfolder
			processFolder(""+dir+list[i]);
		} else {
			//if the file encountered is not an image nor a folder just print the
name in the log window
			print(dir + list[i]);
		}
   }
}
function processFile(inputFolder, output, file) {
	open(inputFolder + file);
	title = getTitle();
	run("Split Channels");
	three = "C3-" +title;
```

```
        two = "C2-" +title;
        one = "C1-" + title;
        run("Merge Channels...", "c1=["+three+"] c2=["+two+"] c3=["+one+"]");
        saveAs("tiff", output + file);
        close(file);
}
```

Appendix 7

The raw data from the anatomy experiments (chapter 3). This data was produced using the program CellSet to measure the cell number and cell size and the program ImageJ to measure the diameter of the roots.

The data can be found at the following DOI: 10.17639/nott.7207

Appendix 8

Additional graphs from sectioning experiment (Chapter 3)

DOI: 10.17639/nott.7316

Appendix 9

Additional graphs from custom media architecture experiment (chapter 4)

DOI: 10.17639/nott.7316

Appendix 10

The raw hydroponics physiology data (chapter 4). This excel document contains the raw data from all 3 of the hydroponics experiments.

The data can be found at the following DOI: 10.17639/nott.7208

Appendix 11

Additional graphs from hydroponics physiology experiment (chapter 4)

DOI: 10.17639/nott.7316