Phenomic and Genomic Evaluation of Lentils for Aluminium Toxicity Tolerance Trait

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SUMMARY

Lentil (*Lens culinaris* Medikus) is a self-pollinating, diploid cool season food legume that is cultivated across the world, with Australia being one of the biggest exporters of red lentils. Aluminium (Al) toxicity is a major soil constraint for lentil production as an increased concentration of toxic Al^{3+} ions in acid soil causes poor root growth and development. The development of Al toxicity tolerant varieties is an economical solution to overcome this limitation in comparison to farm management solutions such as application of lime. Efficient phenotyping and deployment of molecular markers could greatly accelerate the lentil breeding programmes towards the development of Al toxicity tolerant varieties. Hence this thesis work aimed to establish an efficient screening method, identification of tolerant accessions and linked markers. In the present study, a high throughput hydroponics system was established to screen 386 lentil accessions at the seedling stage for Al toxicity tolerance. The tolerant accessions were identified based on relative root growth measurements during three-day screening at optimised Al treatment. Results were further confirmed through soil screening, histochemical and biochemical analyses. Evaluation of the tolerant and sensitive accessions for toxicity symptoms, stain accumulation and Al content in root and shoot tissue gave insight into the tolerance mechanisms of lentil. All the lentil accessions were genotyped using a Genotyping by Sequencing (GBS-t) approach that identified 65,874 high quality Single Nucleotide Polymorphism (SNP) markers. Genetic diversity and population structure analysis identified highly diverse, Al toxicity tolerant subpopulations and divergent landraces, which are useful for lentil breeding. The identified subpopulation's specific selection signatures could be used as molecular keys to further characterise genebank accessions. Genomic regions associated with Al toxicity tolerance were identified by Genome Wide Association Study (GWAS) study. These markers can be deployed into breeding programmes to make informed selections for Al toxicity tolerant germplasm.

STATEMENT OF AUTHORSHIP

Except where reference is made, the content of this thesis has not been published elsewhere or extracted in whole or in part from a thesis for the award of any other degree or diploma. No other person's work has been used without due acknowledgement in the main text of the thesis. This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

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DEDICATION

To my family for all the support, motivation and encouragement.

CHAPTER 1: Introduction

Abbreviation: AB, ascochyta blight; AFLP, marker Amplified Fragment Length Polymorphism; Al, aluminium; *ALMT1*, aluminium activated malate transporter; BC, backcrosses; BSA, bulk segregant analysis; BWA, burrows wheeler alignment tools; CMLM, compressed mixed linear model; EC, electrical conductivity; FarmCPU, fixed and random model circulating probability unification; FIGS, Focused Identification of Germplasm Strategy; GBS, genotyping-by-sequencing; GWAS, genome wide association studies; *HvMATE*, Hordeum vulgare aluminium activated malate transporter; ICARDA, International Centre for Agricultural Research in Dry Areas; LG, linkage group; LD, linkage disequilibrium; LOD, logarithm of the odds ratio; MAS, marker assisted selection; *MATE*, Malate Transporter; MLM, mixed linear model; MLMM, Multi-Locus Mixed-Model; NGS, next generation sequencing; PCR, Polymerase Chain reaction; Vp, phenotypic variation; pH_{Wa}, pH in water; pH_{Ca}, pH in calcium chloride; QNT, quantitative trait nucleotides; QTL, quantitative trait loci; RAPD, random amplified polymorphic DNA; RIL, recombinant inbred lines; RRE, relative root elongation; RRG, root regrowth; SNP, Single nucleotide polymorphism; *ScALMTA*, Secale cereal aluminium activated malate transporter; SSR, Simple Sequence Repeats; STAR, spliced transcripts alignment to a reference; WANA, West Asia and North Africa;

1.1 Origin of lentil, evolution and global distribution

The genus *Lens* phylogenetically groups within the tribe Vicieae which contains cool season legumes belonging to the subfamily Papilionoideae, of the family Fabaceae (Schaefer et al., 2012). The most recent classification shows seven taxa grouped into the four species *L. culinaris* Medik. *ssp. culinaris*, *L. culinaris ssp. orientalis*, *L. culinaris ssp. tomentosus, L. culinaris ssp. odemensis*, *L. ervoides*, *L. lamottei,* and *L. nigricans* (Ferguson et al., 2000; Gupta et al., 2011). It is generally considered that *L. culinaris ssp. orientalis*, is the wild progenitor of the ssp. *culinaris* and *L. nigricans* is the most distant relative (Wong et al., 2015). The Middle East is the primary centre of diversity for both the domestic *L. culinaris* and *L. culinaris ssp. orientalis* (Zohary, 1972; Sandhu & Singh, 2007). The International Centre for Agricultural Research in Dry Areas (ICARDA) has the world collection of *Lens* germplasm and a global mandate for research on lentil improvement. The ICARDA lentil genetic resources collection is comprised of 10,578 accessions, including 8,858 landraces and cultivars from 69 different countries, 1,137 ICARDA breeding lines, and 583 accessions of six wild *Lens* taxa (Redden et al., 2007). Cultivated lentil (*L. culinaris ssp. culinaris)* is a diploid ($2n=2x=14$) annual crop that is a good source of dietary protein (22-35%) (Kumar et al., 2015). Lentil seeds are particularly low in fat but high in protein (Iqbal et al., 2006), and are an excellent source of both soluble and insoluble fibre, complex carbohydrates, vitamins (B vitamins) and minerals (such as potassium, phosphorus, calcium, magnesium, copper, iron and zinc) (Yadav et al., 2007).

In agriculture lentil is used as a rotation crop due to its ability to fix biological nitrogen and to break cycles of disease, insect pest and weeds. Cultivated lentil has two varietal types; small seeded (microsperma) and large seeded (macrosperma). Microsperma mostly have red cotyledons with seeds typically 2-6 mm in diameter and 1000-seed weight of approximately 25 g (Sandhu & Singh, 2007) and are cultivated mainly in South Asia and Sub-Saharan Africa. In contrast, macrosperma seeds are approximately 6-9 mm in diameter and have a

1000-seed weight of up to 70 g. They mostly have yellow cotyledons and are native to West Asia and North Africa (WANA) and Southern Europe (Barulina, 1930; Kumar et al., 2016). The major geographical regions of lentil production are South Asia and China (44.3%), North America (41%), Central and West Asia and North Africa (WANA) (6.7%), Sub-Saharan Africa (3.5%) and Australia (2.5%) (Kumar et al., 2016). It is cultivated globally as a rainfed crop in more than 52 countries, with world production equating to approximately 7.59 Mt from 6.58 Mha with Canada, India, Turkey, USA, Kazakhstan, Nepal, Australia and Russian Federation being the main contributors (FAOSTAT, 2017) (Figure 1.1). Of these, the major exporting countries are Canada, Australia and USA whereas Middle East and North Africa are major importers (Muehlbauer et al., 2006).

Top 10 Producers in 2017

Country	MT Production
Canada	3, 732, 900
India	1, 220, 000
Turkey	430, 000
USA	339, 380
Kazakhstan	313, 156
Nepal	254, 308
Australia	221, 436
Russian Federation	197, 858
China Mainland	171, 888
Bangladesh	168, 837

Figure 1.1. Major lentil producing countries in the world (countries with blue circle).

Source: Figure created from the data (Food and Agriculture Organization of the United Nations STAT 2017).

In Australia, lentil is considered a high-value pulse crop, mainly grown in the semi-arid regions of Victoria and South Australia which have winter dominant rainfall patterns, with annual average rainfall of 350-500 mm. Australia is a significant producer of red lentil with gradually increasing areas under green lentils as speciality types (GRDC, 2017). About 95% of Australian grown lentils are exported to the Middle East and South Asia, with a small domestic market (PulseAustralia, 2015). The estimated production of lentil in Australia is 485 kt from an area of 353,000 ha which mainly comes from Victoria (200 kt) and South Australia (250 kt) from an average area of 163,000 ha (ABARES, 2018). Recent total lentil exports earned $230,761,625 to Australian economy (personal communication with Arun Shunmugam Pulse Breeder, AgVic, Horsham).

1.2 Constraints to lentil production

Lentil is affected by several biotic and abiotic stresses around the world, which causes nearly 52% and 28% losses respectively in lentil growing area leading to huge economic losses (Kumar et al., 2013). Among the biotic stresses, fungal diseases such as ascochyta blight (AB) (*Ascochyta lentis* Vassilievsky) (Erskine et al., 1993; Ye et al., 2002; Chen et al., 2011), Fusarium wilt (*Fusarium oxysporum* f.sp. *lentis*) (Hamdi & Hassanein, 1996), anthracnose (*Colletotrichum truncatum*), stemphylium blight (*Stemphylium botryosum*), rust (*Uromyces viciae-fabae*), botrytis gray mold (*Botrytis cinerea* and *B. fabae*), and white mold (*Sclerotinia sclerotiorum*) (Sharpe et al., 2013; Kumar et al., 2015), cause substantial yield losses. Abiotic stresses such as drought, frost/winter hardiness, heat and soil toxicities (boron and acid soil aluminium), all have major impact on lentil production worldwide, but, when compared to biotic stresses, research and progress has generally been more limited (Materne et al., 2007). However, a greater understanding about the general adaptation of lentil and the impacts of abiotic stresses, has aided in the better design of resistance/tolerance breeding for abiotic stresses in lentil.

The major soil toxicity is acid soil, resulting in aluminium toxicity which is a wider concern in all parts of the world (Bojórquez-Quintal et al., 2017). Acid soils present several physical and chemical challenges to plants and the primary reason most plants grow poorly on acid soils is due to increased concentrations of the soluble aluminium cation (Al^{3+}). To date very little work has been reported in lentil for aluminium toxicity tolerance breeding. Hence the work described in this thesis aims to address this information gap with a more efficient high throughput screening method established at the seedling stage, examining variability among diverse lentil accessions and identification of the genomic regions for the marker trait association. This will hopefully enable the expansion of lentil production to other soils conditions mainly in Australia, where acid soil reduces the yield in Western Australia and New South Wales.

Acid soils – limitations for agriculture production:

Soil acidity is determined by the amount of hydrogen (H^+) activity in the soil solution which is influenced by edaphic, climatic and biological factors. However, soils derived from granite tend to be more acidic than soils derived from basalt or sedimentary rocks. Theoretically any pH < 7.0 is acidic, however pH_{Ca} (pH in calcium chloride) ≤ 5.5 in agricultural soil are generally considered acidic as they begin to affect the sensitive crop species (NLWR-Audit, 2001; Ryan, 2018). Based on the pH_{Ca} value soils are classified as moderately acidic (4.8 to 5.5), highly or strong acidic (4.3 and 4.8) and extremely acidic (< 4.3) (NLWR-Audit, 2001; Lockwood et al., 2003). Soil acidification at the soil surface limits surface root development. As acidity is leached into deeper layers, subsurface acidity occurs when surface pH_{Ca} drops below 5 with all root growth is restricted (Upjohn et al., 2005). Soil acidity and its associated mineral toxicities are major constraints to agricultural production in several parts of the world (Pariasca-Tanaka et al., 2009). They affect approximately 30% of the total land area and up to half of the potentially arable land mainly in tropical and sub-tropical regions (Von Uexküll & Mutert, 1995; Kochian et al., 2004).

1.2.1 Distribution of acid soils in Australia

Australia has some of the oldest soils in the world and, due to being highly weathered, these are typically shallow and relatively infertile. Most of the landscape is arid or semi-arid, with only ~10% being suitable for cropping after improvement. Naturally acidic soils in Australia span most of the productive landscape. When looking at acid soil distribution across Australia (Figure 1.2), strong acidic soils generally occur in permanent pastures where returns from grazing have been too low to support sustained amelioration efforts (Scott et al., 2000). These soils typically occur in central and southern New South Wales (NSW) and north-eastern Victoria where rainfall exceeds 600 mm year–1 (Scott, 2003).

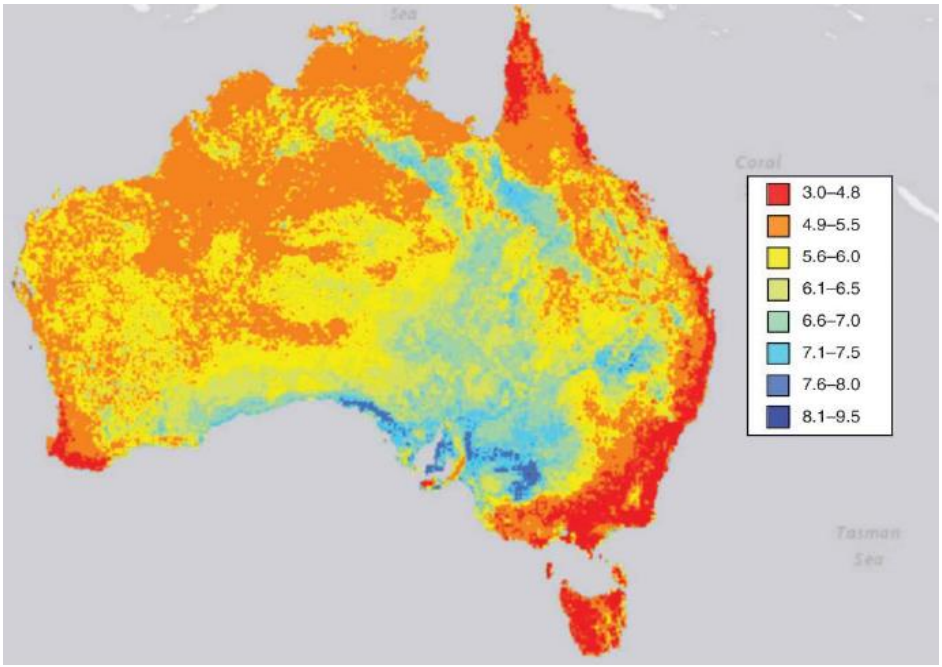

Figure 1.2. Distribution of acid soils in Australia.
Data show the estimated value for soil pH at 5-15 cm depth. Source is the Soil and Landscape Grid of Australia (www.asris.csiro.au/viewer/TERN/).

In Australia, 50 million hectares of surface soil and 23 million hectares of subsurface soil is affected by soil acidity (NLWRA, 2001) (Table 1.1). Western Australia is seriously affected by soil acidity, with 72% of surface soil and 45% of subsurface soil being below 5.5 and 4.8 pH_{Ca} respectively, resulting in predominant losses in production (DPIRD, 2018).

Table 1.1. The estimation of extent and annual loss by soil acidity in Australia

States	Soil affected by acidity (Mha)	Annual loss in production (Million $)	Reference
New South Wales	13 affected and 6 at risk	90-380	(AcidSoilAction, 2001; ENRC, 2004)
Western Australia	15 affected	500	(Dolling, 2001; Gazey, 2013, 2014)
Victoria	~3-5 affected and 2 at risk	470	(Slattery & Hollier, 2002; SoE, 2009)
South Australia	2.5 affected	Not available	(SoilQuality, 2016)

New South Wales is the second most affected state with annual losses of $90-380 million. In South Australia, soil is at risk of acidity and affected in the Lower Eyre Peninsula, Kangaroo Island, the South East and Lofty Ranges. In the Lower and Eastern Eyre Peninsula of South Australia almost all of the surface soils sampled had a pH below the critical pH_{Ca} 5.0 with 23% showing that level below in the subsoil (Masters, 2015). Acid soils also occur in agriculture regions of Tasmania, but information is insufficient to be able to quantify the extent and impact on production. In Victoria, soils have a wide range of pH (4 to 10) and 23% of the state soils are affected by soil acidity. These extremes in acidity and alkalinity cause the loss in production in many agriculturally important crops and affect their symbiotic Rhizobia. Figures 1.3 and 1.4 show the soil pH trend across Victoria. Surface soil pH is acidic in the Eastern and Western Uplands, the Strzelecki and Otway Ranges and in north-eastern Victoria. Farming of crops and pastures such as barley (*Hordeum vulgare* L.), phalaris and lucerne are limited by soil acidity and Al toxicity in north-eastern Victoria and the Central Highlands (VRO, 2014).

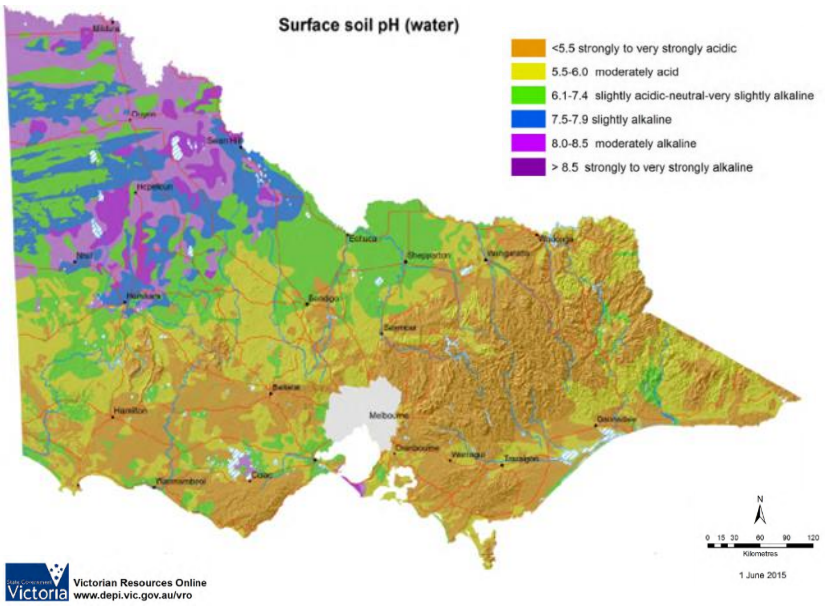

Figure 1.3. Surface pH trend in across Victoria.

Source: Victorian Resource Online

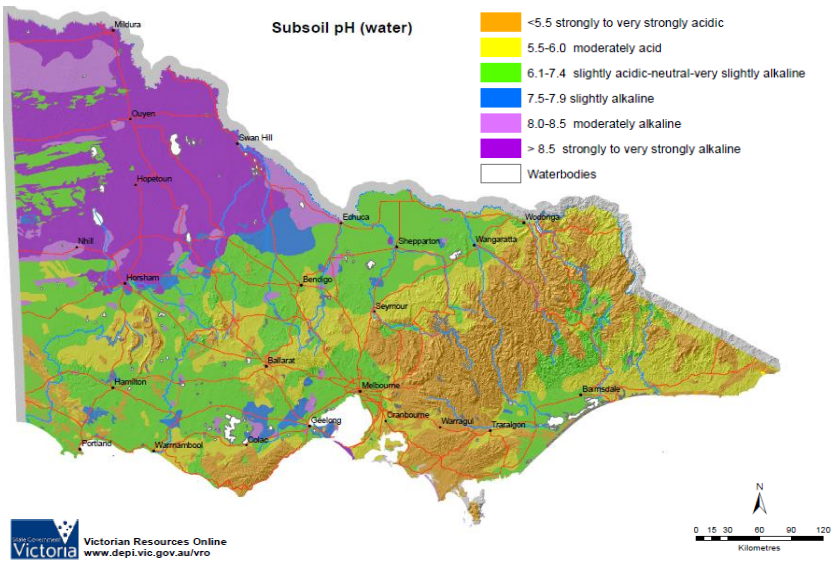

Figure 1.4. Sub-surface pH trend in across Victoria.

Source: Victorian Resource Online.

1.2.2 Soil pH effects on availability of soil nutrients

Most agricultural crops require a soil pH_{Ca} range between 5.2 to 8.0 which provides the optimum availability of the required nutrients for healthy plant growth and development. Nutrient solubility and its availability to plants varies with soil pH (Lake, 2000; Charman & Murphy, 2007) (Figure 1.5). Low pH causes less availability, disturbs the transport and absorption of beneficial elements such as phosphorous (P), magnesium (Mg), molybdenum (Mo) and calcium (Ca), thus limiting plant growth (Poschenrieder et al., 1995; Upjohn et al., 2005; Charman & Murphy, 2007). However other metal elements such as aluminium (Al), iron (Fe), copper (Cu), manganese (Mn), and zinc (Zn) may become more readily available, and can reach toxic levels and inhibit plant growth (Foy, 1988; Upjohn et al., 2005; Charman & Roper, 2007).

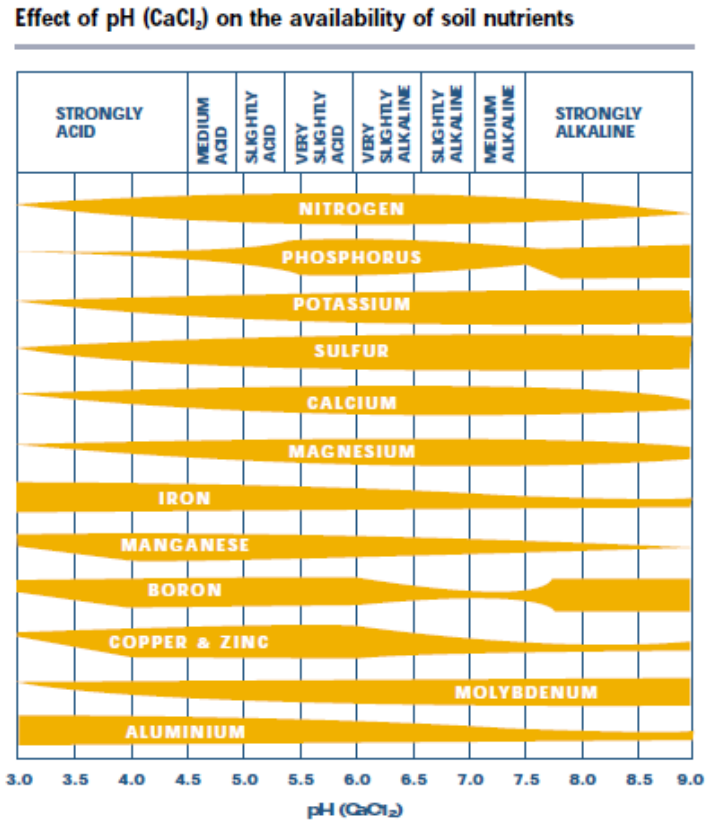

Figure 1.5. Effect of pH_{Ca} on soil nutrient availability.

Source: Understanding soil pH, NSW Agriculture.

1.2.3 Causes of soil acidification

Acidification is a slow natural process and part of normal soil weathering. It occurs because of the disruption in N and C cycle, and imbalance of the H^+ ions in the soil. Many other activities can lead to increased soil acidity including agricultural and industrial, acid rainfall (Krug & Frink, 1983), nitrogen fertilizers (especially acid-forming ones) (Guo et al., 2010) and organic matter decay (Ma & Ryan, 2010). In agriculture system, nitrogen may be fixed from the atmosphere by legumes, decomposed from soil organic matter (the dead remains of plants and animals) by soil organisms, or added in various types of fertilisers. Different nitrogen fertilisers undergo slightly different chemical pathways when they break down in the soil and contribute different amounts of H^+ (acid) to the soil.

The rate of acidification on all soil types, is most commonly exacerbated by the following four agriculture practices (Slattery & Hollier, 2002; Upjohn et al., 2005) during which H^+ ion balance is affected.

1) Inappropriate use of nitrogenous fertilizers

The amount of acidification can depend on the type of nitrogenous fertilisers used for farming. Fertilisers such as ammonium sulphate which contains nitrogen as ammonium, acidifies the soil quicker than calcium nitrate and sodium nitrate which have a neutralising effect on the soil acidity. Urea, aqua ammonia and anhydrous ammonia have low acidification effect. Other fertilisers, such as superphosphate increase the acidification indirectly by building the organic matter which in turn increases soil acidity. In China, acidification was reported as a result of intensive farming and overuse of N fertilizers (Guo et al., 2010).

2) Leaching of nitrogen as nitrate sourced from legume fixation or from ammonium fertilisers

This is the major contributor to agriculturally induced soil acidification. Chemical processes in the soil produce nitrate from fertilisers (ammonium type fertilisers) and from the

breakdown of organic matter, resulting in more acidic soil (Upjohn et al., 2005). Annual plants which are shallow rooted compared to deep rooted perennials increase the risk of leaching the nitrate nitrogen and therefore the level of acidity increases. This acidity can be neutralised by plant discharges such as alkaline substances hence maintaining acidity/alkalinity balance.

3) Build-up of the organic matter

Continuous use of fertilisers improves crop production by increasing the level of organic matter in the soil which improves soil structure but also increases soil acidity. However, acidification caused by this type is not permanent and can be reversed when organic matter breaks down in the soil (Upjohn et al., 2005). However, there will be permanent change to soil acid status if the top layer with organic matter is eroded or removed.

4) Removal of the produce

Farm produce e.g. grains, pasture and animal products are slightly alkaline in nature and removal of these results in the lowering of soil pH over time (Upjohn et al., 2005). When large quantities of produce are removed, for example during hay making, the remaining soil will be more acidic than previously.

1.2.4 Management of soil acidity

The practical way of neutralising soil acidity is by applying limestone or other liming materials such as calcium carbonate, dolomite, magnesite or hydrated lime, which will raise the pH_{Ca} above 5.5 when applied in enough quantity (NLWRA, 2002 ; Upjohn et al., 2005; Robson, 2012). It can take many years for the lime effect to move into deeper layers (20 cm), especially in heavy clay soils. When acidity affects the soil subsurface, limestone will only be effective if the surface soil is maintained with the pH_{Ca} above 5.5. However, liming to increase the pH_{Ca} above 6.0 should be avoided as it may induce deficiencies of Zn, B and Mn in well weathered soils (Upjohn et al., 2005). Soils that are acidic in both the surface and subsurface soils are hard to manage, but the most economical way to reduce the rate of

acidification in the subsoil layers is by growing acid tolerant crops. The rate of soil acidification can be minimised by minimising leaching of nitrate nitrogen, use of less acidifying fertilisers, avoiding surface soil erosion and minimising the removal of the farm products after harvest (Upjohn et al., 2005).

1.3 Aluminium toxicity in acid soils

Aluminium (Al) is the third most common element in the earth's crust (Ryan & Delhaize, 2010). It is insoluble at high pH and starts to show deleterious effects at lower pH as it becomes soluble (Delhaize & Ryan, 1995). Al toxicity is severe below pH 5.0 but is also problematic at pH 5.5 in kaolinite soils (Foy, 1984). Thus the critical soil pH at which Al becomes soluble or exchangeable in toxic concentrations depends on many soil factors such as pre-dominant clay minerals, organic matter levels, concentrations of other cations, anions and total salts as well as plant species or cultivars (Foy, 1984). These complexities make it difficult to devise a soil Al test to accurately predict toxicity under all these conditions. Release of soluble Al in soil solution also depends on the soil, where highly weathered soil (other than soils with high iron and aluminium oxides) tend to release large amounts of aluminium compared to weakly weathered soils (Upjohn et al., 2005). In some cases extremely weathered soils with siliceous sands (Mallee sands of western Victoria and parts of Western Australia), will not release Al and Mn even when the pH measured in water (pH_{Wa}) <5.5 (Slattery et al., 1999). Soils such as podzolic and krasnozems which have abundant minerals, have a large store of Al in the crystalline structures, and hence, will release more Al (Fenton & Helyar, 2007). Many soils which are undergoing acidification are highly weathered in nature and contains sufficient clay and amorphous minerals which can release Al and Mn in very acidic pH (< 4.8) (Murphy, 2015). Soils near Merredin and Katanning in the central agricultural region have an Al concentration >5 mg/kg in the upper 70 cm in six of nine profiles tested, with some reaching 20 mg/kg (Ryan, 2018). This concentration will limit the production of barley, canola and even wheat production,

especially as access to subsurface moisture is required for grain filling (Ryan, 2018). Acidity and Al toxicity stress are difficult to differentiate as Al is only soluble in acid solutions (Foy, 1984) and acid soil toxicity is caused by a combination of heavy metal toxicity, lack of essential nutrients and acidity itself (Foy et al., 1978).

Hydrogen toxicity and Al toxicity occur together under acid soil conditions, but these stresses are distinct, and tolerance to each is governed by multiple physiological processes (Nakano et al., 2020). Generally, hydrogen ion toxicity dominate the top soil layer of the acid soil where organic matter is concentrated, as opposed to Al toxicity that is prominent in the subsoil layer (George et al., 2012). Hence, the direct impact of Al can be estimated by comparing two treatments: one with an acidic pH with Al and another without Al.

1.3.1 Measurement of soil aluminium

There are two methods commonly used to measure soil aluminium (Upjohn et al., 2005; DJPR, 2017).

1) Calcium chloride extractable aluminium (Al_{Ca}), where 0.01M $CaCl_2$ is used to extract aluminium and to determine pH_{Ca}. This extracts most of the Al dissolved in the soil and gives the best estimate of aluminium that will be encountered by the plant roots. It is expressed as mg/kg.

2) Exchangeable aluminium as a percent of the cation exchange capacity (Al_{ex}%). The cation exchange capacity of the soil is the sum of Ca, Mg, sodium (Na), K and Al. This percentage varies with the electrical conductivity (EC, dS/m) of the soil hence Al% must be interpreted with the known salinity levels.

Critical Al concentration is defined by the level that will reduce the plant growth by 10%. Table 1.2 shows the critical Al concentrations for plant growth within the highly sensitive to highly tolerant to Al range for the different tests based on Al concentration (Upjohn et al., 2005).

Table 1.2. Critical Al concentrations for plant growth from different Al tests

Aluminium tolerance of plants	**Al_{ex}% Low salinity (<0.07 ds/m)**	**Al_{ex}% Med salinity (0.07-0.23 ds/m)**	**Al_{ex}% High salinity (>0.23 ds/m)**	**Al_{Ca} (mg/kg)**
Highly sensitive	9-16	2-8	0.5-2	0.5-2
Sensitive	16-21	8-12	2-6	2-4
Tolerant	21-32	12-21	6-10	4-8
Highly tolerant	32-43	21-30	10-16	8-13

Al_{ex}% = Exchangeable aluminium as a percent of the cation exchange capacity, Al_{Ca} = Calcium chloride extractable aluminium

A plant's response to different levels of Al toxicity is dependent on its tolerance. Table 1.3 highlights some of the crop and pasture plants along with their sensitivity and tolerance to toxic levels of Al (Upjohn et al., 2005; DJPR, 2017). Plants which are sensitive and highly sensitive to toxic Al concentrations need to have control measures in place in order to reduce yield losses. As a general rule, soil Al concentration between 2-5 ppm is toxic to sensitive species, whereas tolerant species can tolerate above 5 ppm (Figure 1.6) (SoilQuality, 2013). Generally, the Al concentration from the topsoil analysis are not very useful, as although Al is higher than subsoil, its effect is reduced if sufficient organic matter is found in the topsoil layer. When the subsurface pH_{Ca} is above 4.5, the Al concentration is usually less than 2 ppm, a level that is generally tolerated by most crops, but when pH_{Ca} is below 4.5, the Al concentration can increase quickly to toxic levels for many plants (DPIRD, 2018).

Table 1.3. Aluminium sensitivity and tolerance of some crop plants

Aluminium tolerance	**Cereals and pulses**
Highly sensitive	Durum wheat, barleys, lucerne, most annual medics, faba beans, lentils, chickpeas, berseem and Persian clovers and tall wheat grass
Sensitive	Some wheats (hybrids, Vulcan, Rosella, Janz), canola, most phalaris cultivars (Sirosa, Sirolan), albus lupins, white clover (Kenya), caucasian, red and balansa clovers
Tolerant	Most subterranean clover, annual and perennial rye grasses, tall fescue, white clover (Haifa), some wheats (Diamondbird, Swift, Sunstate, Whistler, Dollarbird, Hartog), fodder rape, woolly pod vetch and rose clover
Highly tolerant	Some cocksfoots, some subterranean clovers (Karridale, Trikkala, Woogenellup), lovegrass, Kikuyu, maku lotus, narrowleaf lupins, slender serradella, most oats, most triticale (Tahara, Empat, Muir), yellow serradella and Cereal rye

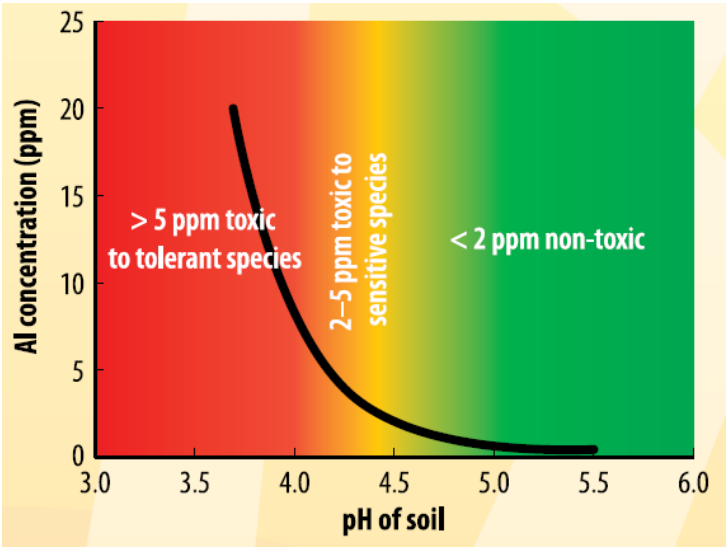

Figure 1.6. The relation between pH_{Ca} and the aluminium concentration in subsurface soils.

Source: (SoilQuality, 2013).

1.3.2 Nature of Al speciation

Aluminium is mainly present as aluminosilicate which produces Al oxides and hydroxides as a result of weathering. In addition, it can be found as precipitates or conjugated organic and inorganic forms, and molecular ions depending on the soil pH (Figure 1.7). As soils acidify, Al dissolves from oxides and hydroxides to become toxic (Horst et al., 1992). However, not all Al in the soil solution is toxic: Inorganic Al monomers (Al^{3+}, $Al(OH)^{2+}$, $Al(OH)_2^{+}$) are more toxic than the inorganic polycations $AlO_4Al_{12}(OH)_{24}(H_2O)_{24}^{7+}$ (known as Al_{13}), while the organic Al complexes such as Al-citric, Al-malic acids, Al-fulvate and Al-humate, are less toxic to plants. Changes to Al solubility depend on soil pH, with the inorganic Al monomer Al^{3+} more available and toxic at pH <5.0, compared with the hydroxyl Al species ($Al(OH)_2^{+}$, $Al(OH)^{2+}$) which increases with an increase in pH. At the neutral pH, $Al(OH)_3$ or gibbsite occurs; however, it is non-toxic and relatively insoluble. Aluminate, $Al(OH)_4$, is the dominant specie when the pH is alkaline (pH > 7) (Kinraide & Parker, 1989b; Kinraide, 1991; Delhaize & Ryan, 1995; Brautigan et al., 2012). Different plant species have variable responses to toxicity of inorganic Al monomers. The Tyler wheat variety (*Triticum aestivum* L, cv, Tyler) showed sensitivity to Al^{3+} but not to hydroxyl-Al ($Al(OH)^{2+}$, $Al(OH)_2^{+}$) (Kinraide & Parker, 1989a), whereas dicotyledonous species (Red clover, lettuce and turnip) appeared to be sensitive to hydroxyl-Al and unaffected by Al^{3+} (Kinraide & Parker, 1989b). The authors suggest that determining the relative toxicities of Al^{3+} and mononuclear hydroxyl-Al may be an intractable problem because hydroxyl-Al monomers can be expressed as a function of the activities of Al^{3+} and H^{+}. Therefore, toxicity attributed to mononuclear hydroxyl-Al may be Al^{3+} toxicity influenced by pH (Kinraide & Parker, 1989b).

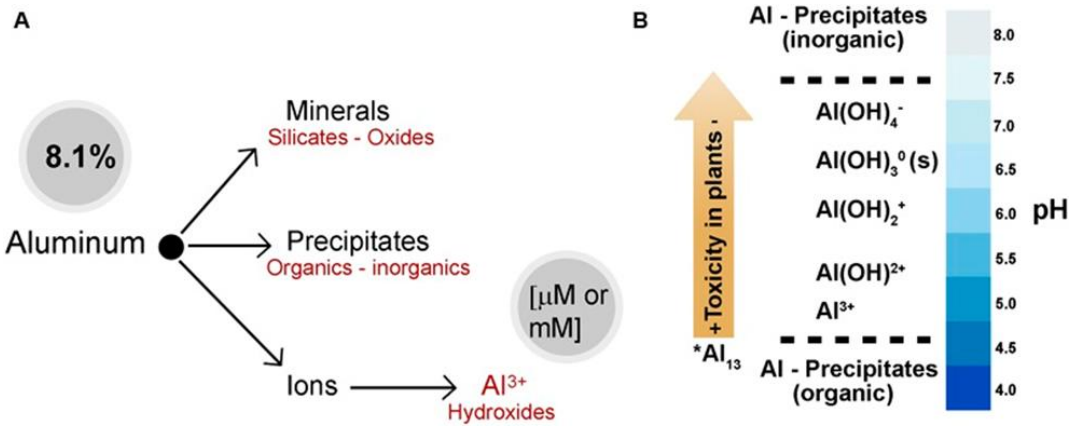

Figure 1.7. Aluminium abundance and speciation in the earth's crust.
Different forms of Al in the soil and water (A) and Al-speciation in soil solution (B). *Al_{13}, speciation of this polynuclear Al species depends on Al concnetration in soil solution.

Source: adapted from (Bojórquez-Quintal et al., 2017).

1.3.3 Phytotoxic Al effects and symptoms

The most obvious and primary symptom of Al toxicity is the inhibition of root growth, which can usually be detected within 30 min to 2 hr of Al exposure, even at low concentrations (Barceló & Poschenrieder, 2002) in sensitive species. The inhibition of root elongation is also accompanied by changes in the architecture and morphology of the roots. A reduction in the formation of lateral roots and root hairs, changes in colour or brown discoloration, thickening, atrophy and curvature of the roots or forming "corraloid" root system are common symptoms (Čiamporová, 2002) which usually makes the roots brittle, especially the root tips (Mossor-Pietraszewska et al., 1997). Depending on the duration of exposure and concentration, Al may increase cell wall rigidity, causing the rupture of the rhizodermis and outer cortex of the meristem, which inhibits elongation of the root tips (Blarney et al., 2004; Jones et al., 2006; Kopittke et al., 2008). In maize, seminal and adventitious root lengths were reduced with high Al content in nutrient solution (Magnavaca et al., 1978). Although Al can affect all growing regions of the root, in pea (Yamamoto et al., 2001), com (Jones et al., 2006; Souza et al., 2016) and bean roots (Kopittke et al., 2008) these ruptures

occurred predominantly in distal part of the transition zone which is the region within approximately 1 – 2 mm of the root tip (Sivaguru & Horst, 1998).

The cells most affected by toxic Al are found in the root cap, the root meristem, root hairs and branching initials (Čiamporová, 2002). The main physiological symptoms includes; inhibition of cell elongation and reduced division, disruption in the cytoskeleton organisation in roots, a reduction in the availability of P, changes in cell wall depositions and increased cell wall rigidity (Čiamporová, 2002).

Aluminium induced inhibition of root growth in an Al-sensitive cultivar of barley caused disruption to both cell division in the meristematic region and cell expansion in the zone of root elongation (Nichol & Oliveira, 1995). In wheat (Scout 66), exposure to 6 hr treatment of Al caused an increase in cell wall polysaccharides, mainly hemicellulose polysaccharides, which made the cell wall thick and rigid (Tabuchi & Matsumoto, 2001). Furthermore, several genotype specific changes in phospholipids and steryl lipids were also observed. For example Al sensitive wheat genotypes showed more accumulation of the phospholipid phosphatidylcholine after 3 days post Al treatment (Zhang et al., 1997) compared to tolerant genotypes.

In lentil, sensitive varieties (BARI Masoor-4, L4147) to toxic Al were used in hydroponic experiments where they were exposed to a high content of Al (148 uM) for 65 days. The plants showed reduction in root and shoot lengths, dry weights of roots and shoots and pods/plant in compared to Al tolerant varieties (ILL-6002, L-7903, L-4602), with a more prominent effect observed on root growth than shoot growth (Singh et al., 2012). Similar results were also reported in pigeon pea (*Cajanus cajan* (L.) Millspaugh) (Choudhary et al., 2011a) when comparing tolerant and sensitive genotypes (IPA 7-10, T 7 and 67 B). A study in white spruce (*Picea glauca* (Moench) Voss) found that toxic Al does not interfere with seed germination directly, but reduces seedling establishment and the growth of new roots

(Nosko et al., 1988). However, *Pisum sativum* (Var. Arkil and Rachana) showed a reduction in germination rate, seedling height and dry weight when plants from two different varieties were exposed to different Al concentrations (0.8 and 0.6 g Al/kg soil, respectively). Seedling growth and dry weight was reduced to a greater extent in Arkil compared to Rachana (Singh et al., 2011b). Several experiments in soybean also reported a reduction in root length during an Al treatment (Horst et al., 1992; Ferrufino et al., 2000; Villagarcia et al., 2001; Spehar & Copati Souza, 2006; Ojo & Bello, 2010).

The primary effects of Al toxicity are on the roots, making them inefficient in absorbing nutrients and water. High Al concentration produces abnormal root branching and short root lengths in lentil. Purpling of stem also indicates Al induced P deficiency (Singh et al., 2012). Foliar secondary effects in some plants resemble those of P deficiency (overall stunting; small, dark green leaves and late maturity; purpling of stems, leaves and leaf veins; yellowing and necrosis of leaf tips) and Ca deficiency (curling or rolling of young leaves and a collapse of growing points or petioles) (Foy et al., 1978). Excess Al also induces Fe deficiency symptoms in rice, wheat and sorghum (Liu et al., 1993; Ryan et al., 1995b; Kumar et al., 2009). Long term exposure to Al also results in a deficiency of K, Mg, P and Ca (Vitorello et al., 2005). These deficiency symptoms are usually associated with injuries to the root system. The most common responses to Al toxicity in above ground tissues are cellular and ultrastructural modifications in leaves, reduced stomatal opening, decreased photosynthetic activity, chlorosis and foliar necrosis.

1.4 Genetics and inheritance of Al toxicity tolerance

Some cereal crops such as wheat (Somers & Gustafson, 1995), barley, sorghum and oat have simple genetic inheritance, compared with quantitative inheritance in rice (*Oryza sativa* L.) and maize where more loci/genes are involved in Al tolerance. Generally, in wheat Al tolerance is inherited as a single dominant gene as shown in near isogenic lines derived from crosses between an Al tolerant (Carazinho) and an Al sensitive cultivar (Egret). Delhaize et

al. (1993a) conducted experiments with hematoxylin staining and root length measurements in nutrient solution that concluded the single dominant locus (*Alt1*) for Al tolerance, which when activated by Al, results in malic acid excretion from root apices (Delhaize et al., 1993b). A segregating population (F_2) from the cross between two wheat varieties, Druchamp and Brevor were screened following exposure to 1.6 ppm Al solution. The results indicated a single gene tolerance, with Druchamp carrying the dominant gene for Al tolerance. However, despite this, Druchamp was sensitive to very high concentration of Al. This suggests one or more major genes, with several modifying genes may be involved in the process of conferring Al tolerance (Kerridge & Kronstad, 1968). Using different populations of wheat, a major Al tolerance gene was mapped on chromosomes 4DL (cultivar BH1146 and Atlas 66, accounting for 85% and 50% phenotypic variation), 3BL (line originated from Chinese spring wheat accounting for 49% phenotypic variation) and 4BL (population from Carazinho and EGA-Burke, accounting for 50% phenotypic variation) (Riede & Anderson, 1996; Ma et al., 2005; Ryan et al., 2009; Navakode et al., 2010). Another study in wheat also showed a major QTL on 4DL which co-segregated with the Al-activated malate transporter (*ALMT1*) gene in a population derived from FSW and ND35 (Cai et al., 2008). Furthermore, another major *MATE* gene on the 4BL chromosome co-segregated with citrate efflux shown in wheat populations from EGA-Burke/Carazinho (Ryan et al., 2009). A study on ditelosomic and nullisomic-tetrasomic lines of 'Chinese Spring' wheat identified multiple genes on chromosomes 6AL, 7AS, 4BS, 2DL, 3DL, 4DL and 7D which are thought to be important for conferring Al tolerance in this moderately resistant genotype (Aniol & Gustafson, 1984). Complex inheritance of Al tolerance in wheat was found in a population derived from Chisholm, where a second QTL for Al resistance on chromosome 3BL was shown to be effective when a major gene on 4DL was absent, with both loci contributing to 50% of the variation (Zhou et al., 2007).

Among cereals, rye (*Secale cereale* L) is one of the most Al tolerant crops. A study using different mapping populations (F1, F2 and backcrosses (BCs)) derived from a cross between tolerant, Ailes and medium tolerant, Riodeva showed two dominant loci (*Alt1* and *Alt3*) with an additive effect at 150 uM Al in nutrient culture. The linkage analysis in this work showed the *Alt1* locus was linked to several isozymes on chromosome 6RL (Gallego & Benito, 1997). The Al activated organic acid transporter gene *ScALMTA1* at the *Alt4* locus in rye was mapped onto chromosome 7R in different F2 populations. This gene was more expressed in root apices than in non-apical root parts and more abundantly in Al tolerant genotypes than Al sensitive ones. This work also supported the existence of common mechanisms of Al tolerance in four homeology groups of grasses (wheat, barley, rye and rice) and in the dicotyledonous plant common bean by polymerase chain reaction (PCR) amplification of the *ALMT1* gene from wheat (Fontecha et al., 2007).

A high-resolution mapping population in barley identified the candidate gene *HvMATE* on the long arm of chromosome 4H and its relative expression correlated well with Al tolerance and citrate efflux in all the recombinant lines with flanking makers (HvGABP and ABG715) for the *Alp* locus (Wang et al., 2007). A new allele for the Al tolerant *HvACCT1* gene was also identified in moderately tolerant barley genotype (CXHKSL), which failed to amplify a marker (~1kb insert) in the 5' UTR of the *HvACCT1* gene. This study concluded the presence of polymorphisms for CXHKSL in 5'UTR of the *HvACCT1* gene compared to other tolerant lines (Dayton) (Ma et al., 2016).

A study into the genetics and physiology of Al tolerance in *Arabidopsis*, identified two significant QTLs on chromosome 1 and 5, with the closest markers m488/apx1A and marker TSL, respectively in recombinant inbred lines (RIL). These QTLs explained approximately 40% and 95% of the variance for root length and for malate release respectively. This indicates Al tolerance in this population is more genetically complex than physiologically

complex, in that the extent of malate release is controlled by many interacting genetic factors and underlies almost all the differences observed in Al tolerance (Hoekenga et al., 2003).

Large numbers of QTLs have been identified in rice (*Oryza sativa* L.) for different traits (three QTLs for the control of root length, seven QTLs for Al stress responsive root length, ten QTLs for root length ratio) in double haploid populations derived from the breeding lines CT9993 and IR62266. Of the ten QTLs for root length ratio, two QTLs (*qALLR-1-1* and *qALRR-8*) explained 24.1% and 28.7% phenotypic variation and were on chromosome 1 and 9, respectively (Nguyen et al., 2002). Another study also identified a major QTL on chromosome 1 by using different genetic backgrounds (IR1552 X Azucena and OM269 X Chiembau) (Wu et al., 2000; Nguyen et al., 2001), which suggested presence of this genomic region in several Al tolerant rice genotypes.

In maize five QTLs on chromosomes 2, 6 and 8 were detected in a mapping population derived from cross between Al tolerant (Cateto AI237) and an Al sensitive (L53), which explained 60% phenotypic variation (Ninamango-Cárdenas et al., 2003). Similarly in a RIL maize population major loci, *ZmMATE1* and *ZmMATE2* colocalized with an important Al tolerance QTL on chromosome 6 and 5, respectively and explained up to 66.6% of the phenotypic variance (Maron et al., 2010).

When looking at growth related traits for Al tolerance in soybean, seven additive QTLs and 11 epistatic QTLs were identified in RIL populations (Korir et al., 2011). One major QTL on B1 chromosome (GMKF046-Sat) was shared by all the traits tested and explained the largest proportion of phenotypic variation.

In pea (tolerant lines Azad P1 and PC-55-11-1-2; sensitive lines PC-493-5 and PSM-2) and chickpea (tolerant lines ICC14880 and IPC92-39; sensitive lines IPCK96-3 and IPC99-4), different mapping populations were used to study the genetic basis of Al tolerance. Based on haematoxylin staining and root regrowth measurements in nutrient solution containing

30 ppm Al (in pea) and 20 ppm Al (in chickpea), a single dominant gene was reported (Singh & Choudhary, 2010; Singh & Raje, 2011). However using the same measurements in pigeon pea, two dominant genes (*Alp1* and *Alp2*) for Al tolerance were identified in segregating population of F_3 families (Singh et al., 2011a).

Very limited studies have been reported in literature in case of lentil for Al toxicity tolerance. Initial studies have shown the presence of the variation for Al toxicity tolerance and consistent performance of the tolerant lines (L-7903, L-4602 and ILL6002) and sensitive lines (L-4147, BARI Massor-4) from seedling to adult stage (Singh et al., 2012) indicating selection can be made at an early seedling stage, as Al tolerance does not vary with growth stages of the lentil plant. The monogenic dominant inheritance of Al tolerance was reported in lentil by using F_1, F_2 and backcross populations derived from a cross between Al tolerant (L-7903 and L-4602) and sensitive lines (BM-4 and L-4147). Root regrowth after haematoxylin staining and fluorescent signals after aniline blue stain (callose accumulation) in the root tips supported the F_1 3:1 ratio of a single major gene inheritance pattern (Singh et al., 2015). Furthermore, two major QTLs for root regrowth (qAlt_rrg) and fluorescence signals (callose accumulation) (qAlt_fs) on the linkage group (LG-1) were mapped, with a high LOD score of 140.5 for fluorescent signals and 28.8 for relative root growth (RRG) RRG in F2 mapping population of cross BM-4 × L-4602, which explained phenotypic variation of 52% and 11% respectively. The QTL, qAlt_fs was localised between PLC_88 and PBA_LC_373, covering 25.9 cM with an adjacent marker PLC_88 at a distance of 0.4 cM. Another major QTL,qAlt_rrg was in the marker interval of PBA_LC_1247 and PLC_51, covering a distance of 45.7 cM with the nearest marker being PBA_LC_1247 at a distance of 21.2 cM (Singh et al., 2018). This study suggested the possible use of these linked markers in marker assisted selection programmes for Al resistance in lentil.

1.5 Aluminium toxicity tolerance mechanisms

Plant species have different levels of tolerance to Al toxicity, with previous studies ranking tolerance among cereals, rye (*Secale cereal* L.) has a greater tolerance than (>) oats (*Avena sativa* ***L***.) > millet (*Panicum miliaceum* L.) > bread wheat (*Triticum aestivum* L.) > barley (*Hordeum vulgare* L.) > durum wheat (*Triticum durum* Desf.) (Bona et al., 1993). However, Al tolerance also differs between genotypes of the same species giving an opportunity for improvement through breeding. Many hypotheses have been proposed to study Al tolerance mechanisms in plants. In general, there are two categories, external and internal, depending on different forms of Al binding. External (or exclusion) are resistance mechanisms where Al is excluded from the root apex or plant tissues, especially the symplastic portion of the root meristem. An internal (or detoxification) tolerance mechanism involves the ability of plants to tolerate the Al ion in the symplasm system. In this Al ions enters the plasmalemma and chelate with organic acid anions to become non-toxic (Kochian, 1995).

1.5.1 External or exclude mechanism

External structures of the root such as the cell wall, cell membrane or chemical exudates including organic acids (A) and phenolic compounds (B) are involved in preventing Al from entering and accumulating in cells. Plants release organic acid anions (di and tri carboxylic acids) into the rhizosphere which form strong complexes with Al (Kochian et al., 2005). This defence mechanism is documented in the literature and has been shown in families such as Poaceae (wheat, barley, sorghum, maize and rye), Araceae (taro), Polygonaceae (buckwheat), Brassicaceae (*Arabidopsis*) and Fabaceae (soybean) (Ryan & Delhaize, 2010).

1.5.1.1 Organic acid release

In different crops, a number of Al tolerant plants were identified that were able to secrete different organic acid anions (Table 1.4), thus protecting sensitive parts of root tips (Ryan et al., 1995b; Yang et al., 2013; Chen & Liao, 2016). Plants adapt to toxic environmental conditions by secreting different organic acids from their roots, but few are specific and

effective in tolerance to Al. Many types of organic acids are present in root cells and specific ones are released in response to Al toxicity.

Table 1.4. Release of organic acids by different crops in response to low pH and Al in hydroponics experiments.

Plant species	**Organic acid (OA) anion secretion**	**pH and Al concentration (µM $AlCl_3$)**	**Nutrient solution**	**References**
Common bean	Citrate	4.5, 148	Steinberg	(Miyasaka et al., 1991)
Pea	Citrate	4.9, 20	0.2 mM $CaCl_2$	(Ishikawa et al., 2000)
Rye	Citrate	4.5, 50	0.5 mM $CaCl_2$	(Li et al., 2000)
Arabidopsis thaliana	Citrate	4.2, 50	Low-strength hydroponic	(Hoekenga et al., 2003)
Barley	Citrate	5.0, 10	1.0 mM $CaCl_2$	(Zhao et al., 2003)
Rice bean	Citrate	5.0, 50	0.5 mM $CaCl_2$	(Yang et al., 2006)
Maize	Citrate	4.3, 0.6µM Al^{3+} activity	225 µM $CaCl_2$	(Pellet et al., 1995)
Rice	Malate	4.5, 200 and 400	½ strength Kimura B	(Liu et al., 2017)
Wheat	Malate	4.3, 200	200 µM $CaCl_2$	(Ryan et al., 1995a)
Soybean	Citrate	4.3, 1.4µM Al^{3+} activity	0.08 mM $CaCl_2$	(Silva et al., 2001)
Lentil	Malate	4.5, 74 and 148	0.5 mM $CaCl_2$	(Singh et al., 2016)

For example, citrate is released in sickle senna (*Cassia tora)* (Ma et al., 1997b) in the presence of toxic Al. Effective organic acid anions include; citrate, malate, oxalate and acetate which are excellent chelating agents for Al (Ma et al., 1998; Ma & Hiradate, 2000; Matsumoto, 2000; Igamberdiev & Eprintsev, 2016). However they do differ in their binding capacity with Al, with citrate making stronger complexes with Al than oxalate and malate (Ma, 2007). Effective organic acids exclude phytotoxic Al from the roots, most probably due to transformation of mobile Al into insoluble forms via precipitation with phosphates and/or other unknown compounds thus maintaining nutrient uptake (Kichigina et al., 2017). However, it is impossible to detoxify all the Al in soil, using organic acids as they only target the part of Al^{3+} which surrounds the root apex which have the potential to enter the root cells. This neutralisation of the root part could be the first step in Al tolerance (Delhaize et al., 1993a). Organic acid anions such as malate and citrate, are a product of the Krebs or Tricarboxylic Acid cycle (TCA) which takes place in mitochondria. They are involved in several anabolic and catabolic pathways, including cellular metabolism, nutrient acquisition, osmatic balance of the cytoplasm, and the alleviation of nutrient deficiency and heavy metal toxicities (Ryan & Delhaize, 2010). Therefore, they are considered to play an essential role in plants at both cellular and whole organism level.

There are two patterns proposed for organic acid release in relation to time following Al stimulations (Ma et al., 2001) (Figure 1.8). In Pattern I (P-I), no discriminable delay is observed between the addition of Al and the onset of organic acid release, which suggests there is a pre-existing mechanism for organic acids release, i.e. only the response to Al^{3+} is required to activate transporters and does not require stimulation of novel proteins (Ryan et al., 2001). The Al activates an anion channel on the plasma membrane in one of three mechanisms: (1) Al^{3+} interacts directly with the channel protein to trigger its opening; (2) Al^{3+} interacts with a specific receptor (R) on the membrane surface or with the membrane itself to initiate a secondary-messenger cascade that then activates the channel; or (3) Al^{3+}

enters the cytoplasm and activates the channel directly, or indirectly via secondary messengers. It was observed in buck wheat after 15 to 30 min of exposure to high Al (50 and 200 uM), more oxalic acid was observed in the tolerant buck wheat (Ma et al., 1997c). In wheat tolerant line (ET3) 5-10 fold more malic acid was reported than in the sensitive line (ES3) (Delhaize et al., 1993b). This suggests the presence of a specific transporter system for organic acid anions on the plasma membrane in wheat, but the exact mechanism is unknown whereas, in maize Al activated efflux was suggested to occur by mechanism 1 where an anion channels are involved (Ma et al., 2001).

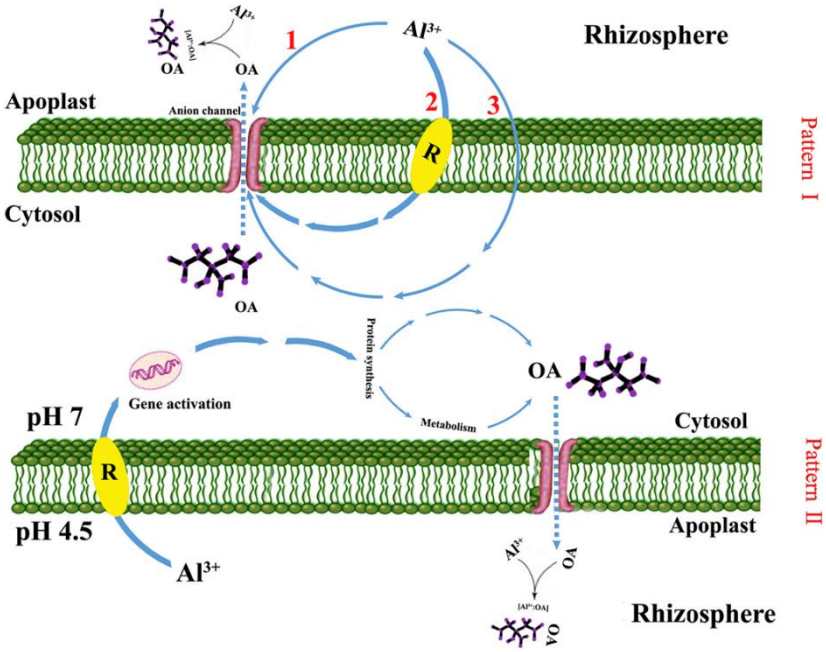

Figure 1.8. Model illustrating the two patterns of Al-stimulated efflux of organic acid anions (OA) from plant roots in response to aluminium (Al^{3+}).

Adapted from the source: (Riaz et al., 2018). Experiments have identified some of the components shown in the model for Pattern I whereas the components depicted for Pattern II are entirely speculative. R – specific receptor.

In Pattern II (P-II), Al interacts with the cell, perhaps via a receptor protein (R) on the plasma membrane, and activates novel proteins and the transcription of genes that are involved in

either, the organic acid metabolism or in the transport of organic acids anions. In this pattern, where organic acid secretion is delayed by several hours, following exposure to Al toxicity and the rate of efflux of organic acids varies with time after exposure to Al (Ma et al., 2001). In *Cassia tora* and *Zea mays*, there was a considerable lag phase before the maximum citrate efflux could be recorded (Pellet et al., 1995; Ma, 2000). In sorghum (Magalhaes et al., 2007), citric acid induction took 4-6 days to induce Al resistance and is closely related to increasing rates of citrate efflux from the root tip. This suggests two Al responses; Al induction of resistance genes and Al activation of organic acid transport proteins.

1.5.1.2 Phenolic compounds

Root exudation of phenolic compounds is another response mechanism used by plants in response to Al toxicity, but it has received less attention compared to organic acid anion release. In maize varieties (*Zea mays* L.Var Clavito, Hs701b and Sikuani), when Al combined with silicon (Al + Si), it induced catechol and flavonoid type (catechin and quercetin) phenolics, which was up to 150 times more than the release of organic acid anions. Even in the absence of Si (Al -Si), phenolic induction was up to 20 times greater than organic acid anions. In these experiments, organic acid and phenolic exudation was observed over a 24hr period following exposure to 20 µM Al, for all varieties, and 50 µM Al for Sikuani, with or without Si pre-treatment. In the resistant variety (Sikuani), Si pre-treatment induced catechin and quercetin after Al exposure increased from 20-50 µM. It is thought that these flavonoid type phenolics play a role in apoplast detoxification of Al in maize root tips. The oxalate organic acid release in this variety did not increase with Al concentration. In contrast, in the sensitive varieties (Hs701b), increasing Al concentration induced the exudation of citrate but not oxalate, following Si pre-treatment. The Si pre-treatment had no effect on phenolic exudation or catechin release, as the exudation of quercetin was observed with Si pre-treatment whether plants had been exposed to Al or not (Kidd et al., 2001).

1.5.2 Internal or Detoxification mechanism

The internal tolerance or detoxification mechanism operate within the root symplasm and include the chelation of Al in the cytosol, compartmentation in the vacuole and use of Al tolerance enzymes. In the case of *Melastoma malabathricum* (a tropical rainforest species and Al accumulator), Al was found as a free ion as well as forming complexes with oxalate in different ratios of 1:1, 1:2 and 1:3. Al-oxalate ratios other than 1:3, are potentially toxic to plants. In these plants, Al is transported in the xylem in the form of citrate, while oxalate is stored in the form of Al-oxalate complex in leaf vacuoles (Watanabe et al., 1998).

Buckwheat leaves accumulate more than 400 mg/kg dry weight of Al when grown in Al solution for 5 days, whilst when grown in acid soils, they accumulate 1,500 mg/kg dry weight of Al. In this species, changes in Al forms occur during the uptake, translocation and accumulation. Buckwheat roots take up the Al in the form of Al^{3+}, yet the exact mechanism for uptake is still unknown. Internally, Al^{3+} will chelate with oxalate by forming an Al-oxalate complex in root cells. In the xylem, Al-oxalate complexes form Al citrate by a ligand exchange reaction. When Al is unloaded from the xylem to leaf cells, Al-oxalate complexes form by another ligand exchange reaction and are stored in vacuoles (Zheng et al., 1998; Ma & Hiradate, 2000).

1.6 Aluminium toxicity tolerance screening methods

Rapid, reliable and effective screening methods are required for selecting and breeding tolerant genotypes which can hopefully enhance lentil production in acid soils. Al toxicity tolerance can be screened based on solution, sand or soil cultures assays, with main measurements being on root regrowth and root staining techniques. Field based screenings are very expensive and time consuming and reliable ranking of tolerance is difficult because of temporal and spatial variation in acidic soils (Choudhary et al., 2011b).

1.6.1 Hydroponics screening - Nutrient solution composition and buffers

Hydroponics screening is an easy and reproducible solution-based assay for Al toxicity where basal macro and micronutrients are added to grow plants. The advantages of using a rapid hydroponic assay is that it allows screening of many plants within a small area, allows easy controlling of pH and nutrient concentrations in solution compared to soil screen. Hydroponic screening has been used in many crops to identify and test for Al toxicity tolerant plants. Species tested include wheat (Baier et al., 1995; Riede & Anderson, 1996; Dai et al., 2009), maize (Poschenrieder et al., 1995; Cançado et al., 1999), barley (Ma et al., 1997a; Lima Echart et al., 2002; Hossain et al., 2005), sorghum (Anas & Yoshida, 2000), rye (Hede et al., 2002) and soybean (Campbell & Carter, 1990; Horst et al., 1992). In some crops like rice, a modified hydroponic solution (Famoso et al., 2010), or a simple calcium chloride ($CaCl_2$) solution, was used to grow plants for a short time in order to reduce the risk of Al forming complexes or precipitates (Ma et al., 2002). There are many factors to consider when testing Al toxicity tolerance in hydroponics. As the speciation of Al depends on the pH of the hydroponic solution, it is very important to maintain an acidic pH with buffers to ensure the intended Al^{3+} concentration in solution. In addition, it is important to frequently monitor the solution pH and adjust or replace solution as plant exudates affect the pH of the solution over time (Samac & Tesfaye, 2003). PIPES buffers have been shown to be useful for maintaining low pH in assays containing Al (Kinraide & Sweeney, 2001). In acidic solutions, Al is predominately in the toxic trivalent cation form (Al^{3+}), however it may form complexes with anions, making Al non-toxic. Thus, chemical speciation programs like Geochem-EZ (Shaff et al., 2009) are useful to predict the activity of toxic Al^{3+} in a given hydroponic solution (Shavrukov et al., 2012). This has been used in rice (Famoso et al., 2010) and maize (Maron et al., 2013) to calculate free ionic activity in the solution, particularly for the rhizotoxic mononuclear Al species. In hydroponics, complete-nutrient solutions with high ionic strength should be avoided as these allow Al to form complexes

with P and S present in the solution at a wide range of pH values. However, when growing plants in hydroponics over a long period, complete-nutrient solution is recommended to meet plant nutritional requirements (Kinraide & Sweeney, 2001). It is always important to conduct Al toxicity test in nutrient solution that approximate the soil solution's compositions, ionic strength and Al activity (Blamey et al., 1991; Kopittke & Blamey, 2016). In the case of soybean, root elongation rate was closely related to Al^{3+} activity when the nutrient solution has pH of ≤ 4.5, P of ≤5 μM and an ionic strength of < ca. 5 mM. Such nutrient solution ensured the solubility of the Al as the toxic dominant species(Al^{3+}) and Al not getting precipitated with P (Kopittke & Blamey, 2016). Macronutrients in the solution such as nitrate (NO_3^-), ammonium (NH^+), K^+ and Mg^{2+} do not cause Al polynucleation (Marschner, 1995). Also, micronutrients do not interact with Al at low pH except for Fe, so it is generally added as $FeCl_3$ or Fe:citrate (Kinraide & Sweeney, 2001).

1.6.2 Al treatment and growing period

The concentration of Al and treatment length are inversely related in their phenotypic effects. A longer treatment of 3-4 weeks needs much lower Al concentrations of about one third of the concentrations used for short period treatments of 24 hours (Wang et al., 2006). In standard hydroponic screenings, seedlings are preconditioned for a few days to a low pH solution and then transferred to the treatment solution with Al at a low pH for quantitative assessment. Root length is measured before and after treatment, and a comparison is made between the treated and control seedlings (Baier et al., 1995; Somers et al., 1996; Samac & Tesfaye, 2003; Hossain et al., 2005). However, in other studies, 2 to 4-day old seedlings were directly transferred to the hydroponic solution along with required Al concentrations and seedlings were assessed for relative root length by comparing with control without Al in hydroponic solution or with high pH (6.0). Here the hydroponic solution will be renewed for every 24 hr to minimise the changes in the pH and Al concentrations (Baier et al., 1995; Gallardo et al., 1999; Aguilera et al., 2016). In wheat and sorghum cultivars, screenings with

low Al concentrations (2 and 1 µM) were found to be effective in a 4 day treatment period (Shuman et al., 1993). This solution with only simple nutrients such as Ca, K, Mg, NO3 and Cl, minimized Al precipitation and represented natural soil conditions compared to the traditional method which uses short term exposures to higher Al concentrations (Shuman et al., 1993; Wang et al., 2006).

1.6.3 Staining and measurements

Hydroponic screening can be combined with qualitative staining methods such as Haematoxylin (Polle et al., 1978) and eriochrome cyanine R (Ma et al., 1997a), or fluorescent stains, such as morin and lumogallion which can give an indication of Al uptake by sensitive plants (Samac & Tesfaye, 2003). The Haematoxylin staining method has been used in wheat, rye, pigeon pea, pea, lentil, chickpea and barrel medic(Polle et al., 1978; Hede et al., 2002; Chandran et al., 2008; Singh & Choudhary, 2010; Choudhary et al., 2011a; Singh & Raje, 2011; Singh et al., 2012). It is a non-destructive method which enables seedlings to be checked for root regrowth after staining. In the staining procedure, seedlings are washed with water following the Al treatment to remove any unbound Al, stained in 0.2% hematoxylin with 0.02% Sodium iodate ($NaIO_{3)}$ or 0.02% Potassium iodate (KIO_3) and rinsed to remove excess stain. Seedlings are then scored for purple coloration; the intensity indicates the amount of Al uptake (Delhaize et al., 1993b; Bona & Carver, 1998; Giaveno & Miranda Filho, 2000). This staining alone or in conjunction with other indexes, is capable of increasing the efficiency, precision and speed of selection thus can be used as robust tools for breeding programs to screen larger number of accessions as shown in case of maize (Cançado et al., 1999). After Haematoxylin staining in chickpea and rice, root tips were excised and soaked in HCl where the stain was released. Then this released stain was quantified for absorbance to indicate Al uptake, where the amount of the dye released was directly proportional to the amount of Al accumulated in root tips (Sharma et al., 2016; Awasthi et al., 2017). Staining techniques are sensitive, however Haematoxylin staining

proved conducive in identifying tolerant and sensitive wheat genotypes after short period of exposure of seedlings to Al, well before differences in the seminal root length become detectable (Delhaize et al., 1993b).

Aluminium toxicity stress also produces callose in seedling roots. This callose biosynthesis has been assessed in lentil and rice plants by fluorescent signals following aniline blue staining (Alvim et al., 2012; Singh et al., 2015). The Al treated seedlings were fixed in 10% formaldehyde, 5% glacial acetic acid and 10% ethanol, and around 1 cm root segments were stained in solution of 0.1% water soluble aniline blue in 50 mM glycine -NaOH buffer at pH 9.5 (Kauss, 1992). Callose accumulation can be readily detected by fluorescence signals, where sensitive genotypes show the strong fluorescence compared to tolerant genotypes (Singh et al., 2015; Singh et al., 2016). The morin staining is another stain which is more sensitive to Al than Haematoxylin hence morin is generally considered as more suitable tracer dye for Al in the histochemical detection of Al in the roots cells (Illéš et al., 2006). With morin stain, the Al treated seedlings emits green colour fluorescence signals in sensitive seedlings in greater intensity compared to tolerant seedlings as observed in lentil root tips (Singh et al., 2015; Singh et al., 2016). However, Al localization using morin staining in maize root tips, detected the presence of Al in the cytosol but not in the cell wall where Al tightly bound to the cell wall pectin. Hence suggested not to be used this stain to determine the relative distribution of Al in different parts of the cell (Eticha et al., 2005).

Another fluorescent stain, lumogallion, has been used in soybean and *Medicago truncatula* (Kataoka et al., 1997; Narasimhamoorthy et al., 2007) as it has a great affinity for low Al concentrations and is able to trace Al in plant tissues over time (Nakanishi et al., 2001). Aluminium was detected in the nuclei of cortical cells 1 and 2 mm from the root tip of sensitive soybean cultivars after 15 min of Al treatment. Symplasm Al was observed in cells 1 mm from the root tip whereas apoplasmic Al was found in the cell wall and cell periphery

at 2mm from the root tip. In addition, Al was detected in the protoxylem, suggesting its transportation to aerial parts after 30 minutes of Al treatment (Kataoka et al., 1997).

Staining and root regrowth after staining in hydroponic and sand assays were consistent in discriminating tolerant and sensitive lines in pigeon pea (Choudhary et al., 2011b). Due to its simplicity and short test time, it is mainly used to study inheritance patterns in large populations. Hydroponic screening in barley populations with root regrowth and staining (eriochrome cyanine R) at the end of the experiment has shown good correlation of relative seminal root regrowth length with plant height, dry weight and grain weight in soil based experiments (Hossain et al., 2005). However, in soybean, Al tolerance ranking was not consistent between the hydroponic and sand assays (Villagarcia et al., 2001). Furthermore, when analysing 32 diverse accessions of *Medicago truncatula* using different methods such as seedling-based hydroponics, soil-based plant method and seedling based lumogallion root staining to detect Al tolerance, the ranking of the genotypes varied across the methods. In these experiments the soil assay very well discriminated the Al response among the genotypes with higher reproducibility (Narasimhamoorthy et al., 2007), as in acid Al toxic soil the plants are grown for a longer period (seven weeks) compared to other methods.

These quantitative and qualitative hydroponic assays are efficient methods for quickly screening species for Al toxicity tolerance. In a few studies, hydroponic assays have had positive correlation with Al tolerance in acid soils (Samac & Tesfaye, 2003). The relative root growth in *Arabidopsis*, exposed to 2.5 µM $AlCl_3$ in solution culture correlated well with Al tolerance when identified ecotypes were grown in acid soil (Toda et al., 1999). However, poor correlation has been observed in barley (Moroni et al., 2010; Ferreira et al., 2017) and wheat (Aguilera et al., 2016), indicating the different levels of stress in hydroponics and soils, which can result in differences in the relative performance of seedling rankings.

1.6.4 Soil screening method

Soil based screenings are generally preceded by hydroponic screenings and are carried out in controlled glasshouses or environments with soil from targeted areas (Carver & Ownby, 1995; Abate et al., 2013). Acid soils, used for Al toxicity tolerance screenings, form the basis of either short term experiments of 1 to 2 weeks, as in the case of maize, barley, wheat and soybean (Sartain & Kamprath, 1978; Urrea-Gómez et al., 1996; Gallardo et al., 1999; Villagarcia et al., 2001; Pereira, 2018), or long term experiments of 4 to 6 weeks as in the case of barley, *Medicago truncatula*, lucerne, alfalfa wheat and soybean (Campbell & Carter, 1990; Toda et al., 1999; Liu, 2005; Narasimhamoorthy et al., 2007; Scott et al., 2008; Khu et al., 2012; Dong et al., 2018). In all these experiments, acid soils were used to grow plants (treatments) and soils treated with lime to increase pH and reduce exchangeable and extractable Al to a non-toxic level, were used as the control. Basic assessment measurements included root growth and architecture, shoot and root fresh and dry biomass and Al and P content in plant tissues.

The advantage of soil-based screening methods compared to nutrient solution culture is that it takes into consideration other soil factors that may influence Al tolerance (Ring et al., 1993). In the case of wheat a short term soil based screening method provided a realistic rooting environment and discriminated between tolerant and sensitive lines at low pH (3.9 and 4.1) (Tang et al., 2003). However, for highly Al sensitive plant species, these soil-based methods could be too stringent as shown in Al toxic Tatum soils, where sorghum varieties were screened at a pH of 3.8 and an increased pH of 4.0, that showed no discrimination between tolerant and sensitive lines based on relative dry weights (Foy et al., 1993). A similar observation was recorded in durum wheat varieties (Foy, 1996), with prominent discrimination observed only when the pH was above 4.3 (Samac & Tesfaye, 2003). The relative root length (RRL) of 20 wheat varieties from a short term soil experiment, was highly correlated with field performance at three developmental stages, tillering, silking and

maturation (Aguilera et al., 2016; Pereira, 2018). This study suggested the use of these short-term soil experiments to select the wheat accessions for improvements in the root system when grown in acid soils. In this study field assessments were used to correlate glass house short term soil assays, in contrast to most studies in wheat, where hydroponic Al tolerance assessments were used to correlate the short term soil assays in glasshouse or growth chambers (Tang et al., 2003; Pereira et al., 2010; Zhou et al., 2013; Pereira et al., 2015). Hydroponics and soil assays in 36 wheat lines (Triticum aestivum L.), showed significant correlation ($r = 0.71$), where root length or root tolerance index from hydroponic assay, and the relative dry weights from an acid soil pot experiment were used (Baier et al., 1995).

1.6.5 Other screening methods for Al toxicity tolerance

An alternative to soil based screening methods (Voigt & Staley, 2004) is the soil-on-agar method as used in white clover. Where a thin layer of acid soil is placed on top of an agar layer. This method was devised to be used for small seeded plants where Al tolerance is reflected in the different lengths of time taken for seedlings to grow on the agar layer. This method has also been used in alfalfa, where absolute root emergence was considered an appropriate index for identifying Al toxicity tolerance (Pan et al., 2008).

Peat and perlite growth media have been used in faba bean (Belachew & Stoddard, 2017) for growing plants for a longer periods of time (58 and 32 days, respectively), where Al and nutrients are supplied through an irrigation system and physiological measurements, along with shoot and root biomass, root length and Al content in shoots, are analysed. Between the two, perlite is a more appropriate medium because of its inert nature and ability to allow monitoring leaf and shoot responses to acidity and Al stress, as well as separation of the root systems for evaluation. Ranking of Al tolerance in peat and perlite was similar to that shown in hydroponic screening, with minor variation due to seed material heterogeneity.

In some cases, sand or soil was treated with $AlCl_3.6H_20$ or $AlCl_3$ in different concentrations to create Al toxicity for screening. Irrigation water was maintained at an acidic pH of 4.5 and an Al treatment solution was provided daily. Pigeon pea plants grown in sand assays were evaluated for shoot and root dry matter weights after approximately three weeks (Choudhary et al., 2011a). The results were shown to be consistent with those from the hydroponic screening, therefore supporting that sand assays could be used for screening pigeon pea genotypes. Similar methods were also used to study the phytotoxic effects of Al on the growth and metabolism of peas, where soil was made toxic by adding Al in varying concentrations (0.2, 0.4, 0.6 and 0.8 g/kg of Al) (Singh et al., 2011b).

1.6.6 Field screening for Al toxicity tolerance

Field based screening for Al toxicity tolerance is not the preferred choice for plants for a few reasons. It is costly, labour intensive and there is often an uneven distribution of nutrients in the soil and other nutritional deficiencies or toxicities (e.g. P, Ca and Mn) may be present in soils. These variations in soil, in addition to Al^{3+} toxicity makes it difficult to interpret the results and correlate between hydroponics and field rankings. As the variation in each environment, in terms of physical and chemical characteristics, could result in the development of different morphological traits in plant roots. This could impact water and nutrient uptake by the plant and thus, influence its performance in the soil (Shavrukov et al., 2012; Aguilera et al., 2016).

In a study, 12 wheat cultivars with previously classes as Al sensitive or Al tolerant were evaluated in a field where the subsoil acidity had a pH of 4.5, and was increased to 5.0 with lime. Further to this using soil collected from near the field area, 116 wheat lines were also screened in a short term glass house assay with soil pH of 3.9 and 4.8. The relative yields from the field were well correlated with the root length per plant ($r = 0.95$) and relative root length at pH 3.9 (as % of pH 4.8) ($r= 0.94$) from the glass house soil assay. Using the soil from the same field site contributed to the high correlation between experiments and this

rapid (6 day) glasshouse screen method was found to be efficient for separating wheat genotypes with varied levels of acid tolerance, thus can be used for preliminary assessment of tolerance (Tang et al., 2003). This study highlighted root length as being suitable measure for tolerance. It is simple and non-destructive and can be used for screening breeding and segregating populations. Effects of the seed size and nutrient reserves on genotype rankings can be minimised by using relative root length and it also makes it possible to compare different experimental results.

Moderate correlation ($r = 0.56$, $p < 0.001$) was observed between relative root growth from hydroponics and field scores at different developmental stages from a two year field experiment for Al tolerance in 338 wheat accessions (Aguilera et al., 2016). The reasons for this moderate correlation could be; (1) Al^{3+} toxicity is the only factor limiting root growth in nutrient solution, whereas a combination of factors may be present in acid soil; (2) uneven distribution of the nutrients and deficiencies and toxicities (e.g: P, Ca and Mn) of other elements in the soil in addition to Al toxicity; (3) different environments (hydroponics and soil) could have resulted in the development of different root morphological traits which could have impacted water and nutrient uptake and therefore, the differences in the plants performance in the soil. Moreover, the Al concentration (74 µM) used in the hydroponic assay may not be sufficient to discriminate between the wheat genotypes used in the study, thus an adjustment of Al concentration and a wider genetic base are required to improve the correlation.

1.7 Selection of the lentil accessions for screening Al toxicity tolerance

There is evidence for high genetic diversity in exotic germplasms, wild species and landraces, however they are underutilised (Prohens et al., 2017) in breeding programs due to lack of efficient strategies to select and introgress the trait of interest into elite gene pools of cultivated crops (Wang et al., 2017). The germplasm collections of various crops continue to grow in global germplasm collection programs, with approximately 7.4 million accessions

being conserved at various genebanks worldwide (Wang et al., 2017). Lentil germplasm comprises approximately 43,214 accessions (U der Schweiz, 2008) and mining for the adaptive traits/rare alleles from such large collection is resource intensive. Therefore, considering economic feasibilities, evaluating a subset of accessions representing maximum genetic diversity of the total collection would be beneficial (Glaszmann et al., 2010).

1.7.1 Focused Identification of Germplasm Strategy (FIGS)

For successful breeding and crop improvement for Al toxicity tolerance, having access to gene banks that have accessions with potential target traits or genes is important. Randomly selecting accessions from genebanks is not an effective way to identify accessions for screening. Thus, the Focused Identification of Germplasm Strategy (FIGS) has been developed for many traits and crops (Street et al., 2016; Dadu, 2018). It is one of the scientific approaches used to mine genebank accessions and is a scientifically proven tool used to help breeding programs identify useful plant traits (Mackay et al., 2004) . It was developed by a group of leading plant scientists led by ICARDA (International Center for Agricultural Research in the Dry Areas) in collaboration with the Vavilov Institute (Russia), the Nordgen genebank (Nordic region) and the Australian Winter Cereals Collection (Tamworth, NSW). It uses applied Bayesian mathematics and geographical information data and works on the premise that the environment strongly influences natural selection and consequently, the geographic distribution of organisms. FIGS create a 'best bet' smaller subset of accessions which will be trait specific. The subset of material passes through accession level information, especially agro-climatic site information, via a series of filters which increase the chance of finding the adaptive trait of interest (Mackay et al., 2004). It enables the rapid identification of varieties that are resistant to biotic and abiotic stress, thereby reducing the time needed to develop new, improved cultivars, as well as costs involved in field experiments and labour by lowering the number of accessions that need to be screened.

Focused Identification of Germplasm Strategy (FIGS) has been used to identify a novel source of resistance in wheat to biotic stresses including Russian wheat aphid (El Bouhssini et al., 2011), stem rust (UG99) (Bari et al., 2012; Endresen et al., 2012), yellow or strip rust (Bari et al., 2014), powdery mildew (Bhullar et al., 2009), and Sunn pest (Bouhssini et al., 2009). It has also been used in barley for net blotch resistance (Endresen et al., 2011), and in lentil for ascochyta resistance (Dadu et al., 2019). Similarly, for abiotic stresses FIGS has been used to identify drought traits in *Vicia faba* (Khazaei et al., 2013) and tolerance to boron toxicity (Mackay et al., 2004) in wheat. These studies demonstrate that FIGS can be used to identify subsets of germplasm that contain novel trait variation for biotic and abiotic stresses from a relatively large genetic base. The FIGS processes were used by ICARDA in lentil to identify an acid soil tolerant FIGS set. This set was made available in AGG for use in this present study and was used in the hydroponic screening for Al toxicity tolerance and detailed in Chapter 3.

1.7.2 Diverse lentil landraces

The landrace sources are the dynamic population(s) of a cultivated plant that has a historical origin and distinct identity but lacks formal crop improvement. Landraces are often genetically diverse, and adapted to local environment and associated with traditional farming systems (Villa et al., 2005). Lentil landraces have the representative variation of the cultivated species and significant variability has been reported for morpho-agronomic traits (Toklu et al., 2009; Cristobal et al., 2014). Landraces also an optimal source of genes/traits, that are linked to biotic or abiotic resistance and to productivity or nutritional quality traits, which are valuable for lentils breeding programmes (Nadia et al., 2019). Hence, there is a need consider lentil landraces from different geographic origins, which may be a source of diversity the diversity for the trait under study.

1.8 Marker-assisted selection (MAS)

Analysis of the genetic background of target species is essential for successful plant breeding programmes. In this, molecular markers play a number of advantages as they are more objective than phenotypic markers, not subject to environmental influence, and theoretically available in vast numbers (Kim et al., 2016). Marker assisted selection (MAS) employs DNA markers associated with traits of interest to select a plant for inclusion in breeding programmes early in their development. MAS has greatly increased the efficiency and precision of plant breeding compared to conventional methods and has been accelerated since the advent of the genomic era (Collard & Mackill, 2008). It is of great benefit mainly for selection of quantitative resistance/tolerance where many genes with each contributing minor effect are considered (Miedaner & Korzun, 2012). Since the development of MAS, several successful selections based on markers have been reported in different crops for different traits including morphological traits, biotic and abiotic resistances/ tolerances, quality and yield attributes (Ragot et al., 2000; Baliyan et al., 2018; Hossain et al., 2018; Sundaram et al., 2018).

1.8.1 Genotyping by sequencing (GBS)

Single nucleotide polymorphism (SNP) markers are abundant in plant genomes among the different marker systems. The advancement of next generation sequencing (NGS) technologies has reduced the sequencing cost and marker discovery time, enabling the development of dense linkage maps, high resolution QTL analysis and fine mapping. Thus plant breeders are utilizing these technologies in their programs to understand several traits in different crops (Varshney et al., 2015). For example, the large-scale identification of SNPs has resulted in mapping of several QTLs and trait linked markers in food legumes (Jaganathan et al., 2015; Kale et al., 2015; Valdisser et al., 2017). The identification of SNPs using high-throughput sequencing technology is known as genotyping-by-sequencing (GBS) (Chung et al., 2017). Advances in NGS technologies have taken the implementation of SNPs

for genetic analysis to a new level and GBS methods are now practicable for highly diverse and large genome species (Malmberg et al., 2018). Genotyping-by-sequencing (GBS) can simultaneously perform SNP discovery and genotyping, which is particularly advantageous for understudied species that lack reference genome sequences (Kim et al., 2016). The main beneficial features of GBS compared to other genotyping methodologies include low cost, the ability to identify and genotype large numbers of SNPs, reduced sample handling, few PCR amplifications and clean up steps, and efficient barcoding enabling multiplexing. Thus, GBS methods have become popular as a cost-effective tool for genomic assisted breeding in plant species (Chung et al., 2017). There are a number of approaches to simplify the analysis in GBS such as target enrichment/capture-based methods and genome complexity reduction-based methods (Malmberg et al., 2018). Of these, the transcriptome-based complexity reduction approach is the most reliable method that allows the detection of sequence polymorphisms, and also splice variants, in gene sequences (Sudheesh et al., 2016; Malmberg et al., 2018). This has been used in many crop species including alfalfa (Yang et al., 2011), maize (Hansey et al., 2012), wheat (Ramirez-Gonzalez et al., 2015), and legume crops such as chickpea (Hiremath et al., 2011) and lentil (Malmberg et al., 2018). The common steps after generating NGS reads includes the read alignment, mapping and identification of the variants. Burrows wheeler alignment tools such as BWA (Li & Durbin, 2009) and Bowtie (Langmead et al., 2009) were developed to align NGS short read data. Spliced Transcripts Alignment to a Reference (STAR) aligner mapping tools were developed for aligning RNA sequencing (RNA-seq) data to the reference genome (Dobin et al., 2013) and bioinformatic pipelines such as SAMtools (Li et al., 2009) were used for calling variants.

1.8.2 Evaluation of germplasm molecular diversity

Genetic diversity is the prerequisite for the crop enhancement and successful plant breeding. Discovery and characterization of useful genes/alleles which can be introgressed into elite germplasm backgrounds gives an opportunity for genetic improvement of crops (Govindaraj

et al., 2015; Khazaei et al., 2016). Crosses involving highly divergent parents can result in the improvement of agronomic characteristics and higher productivity, hence it is essential to characterize the lentil germplasm resources to make sustainable gains in the crop productivity (Fu et al., 2014; Dissanayake et al., 2020). Several studies have used DNA-based markers in both wild and cultivated lentils to evaluate levels of genetic diversity and to assess the relationship among diverse germplasm collections (Ferguson et al., 1998; Fikiru et al., 2007; Lombardi et al., 2014; Idrissi et al., 2015; Wong et al., 2015; Khazaei et al., 2016; Yadav et al., 2016; Pavan et al., 2019; Dissanayake et al., 2020). The identified genetic clusters or population structures or subpopulation specific alleles/genes from such studies are of great interest for conservation genetics, breeding and reliable documentation of genetic resources. The relationship between the molecular diversity and geographical origins or with specific phenotypic traits helps to understand crop evolution and adaptation, which in turn helps to choose appropriate parental lines for hybridization (Pavan et al., 2019; Dissanayake et al., 2020). Further information on genetic diversity and population structures are useful to study the marker-trait association for any trait of interest by genome wide association study (GWAS), where population is usually considered by their large geographic distributions and diverse phylogenetic relationships (Wang et al., 2020).

1.8.3 Genome wide association studies (GWAS)

In plants, quantitative trait loci (QTL) were originally mapped in bi-parental crosses to identify genetic sources of phenotypic variation. This approach is restricted by low allelic diversity limited to those present in the parents, and the genetic resolution determined by the number of progenies recovered from the cross. The GWAS approach has overcome the limitations of traditional gene mapping as it uses natural populations instead of recombinant populations from two parental lines. These natural populations often contain a greater amount of genetic diversity which can be associated with phenotypic variation. They also provide higher resolution, often to the gene level (Brachi et al., 2011). Generally, a GWAS

panel or population of plants is selected by considering high levels of genetic diversity and low levels of population structure(s) (Wang et al., 2020). A high level of genetic diversity means more loci associated with more phenotypic diversity which can be captured by GWAS, while a low or simple level of population structure results in fewer false positive associations. GWAS make use of the high quality dense markers, that are present in sufficient number across genomes to detect the casual variants that control complex traits (Mohammadi et al., 2020). High density coverage is achieved by genotyping every segment of the genome. The LD between the SNP markers can be used as a measure of the effectiveness of the genomic coverage (Zhu et al., 2008), it also determines the mapping resolution, marker density, statistical methods, and mapping power. The LD, or gametic phase disequilibrium, measures the degree of non-random association between alleles at different loci. The r^2 is the most relevant LD measurement to identifying SNPs or haplotypes that are significantly associated with phenotypic trait variation. Typically, r^2 values of 0.1 or 0.2 are often used to describe the LD decay. Genome-wide LD determines the mapping resolution and marker density for a genome scan and generally, LD extends to a much longer distance in self-pollinated crops, such as wheat, than in cross-pollinated species, such as maize (Zhu et al., 2008).

1.8.4 Statistical models in GWAS study

The most frequently used software packages for association analysis are TASSEL (Trait Analysis by Association, Evolution, and Linkage) and GAPIT (Genome Association and Prediction Integrated Tool). The TASSEL package includes general linear model (GLM) and mixed linear model (MLM) models for performing GWAS and can also analyse population structure using kinship and PCA (Bradbury et al., 2007). GAPIT is a useful R package that performs GWAS and genomic selection, it can handle a large amount of SNPs and genotypes and at the same time it reduces the computational time without compromising statistical power (Lipka et al., 2012). Genome Association and Prediction Integrated Tool

(GAPIT) uses different models for GWAS analysis. Common statistical models used in GWAS include a single locus approach where markers are tested individually in one dimensional genome scan. The MLM is popular and considers population structure and family relatedness (Zhang et al., 2005; Yu et al., 2006). Based on MLM framework, other single-locus approaches have been proposed to reduce the computational time and increase statistical power, such as Efficient Mixed Model Association (EMMA) (Kang et al., 2008), EMMA eXpedited (EMMAX) (Kang et al., 2010), Population Previously Determined (P3D) (Zhang et al., 2010), Factored Spectrally Transformed Linear Mixed Models (FaST-LMM) (Lippert et al., 2011), and Genome-Wide Efficient Mixed Model Association (GEMMA) (Zhou & Stephens, 2012). In the Compressed MLM (CMLM) the genetic effects of individuals in the conventional MLM are replaced by the their corresponding kinship groups, and variance components are estimated by using the P3D algorithm (Zhang et al., 2010) resulting in an increase in the statistical power. However, these single-locus models failed to model the genetics of complex traits that are controlled by numerous loci simultaneously, as these only test a single locus at a time. Additionally, multiple test corrections for critical values are usually required to control false positive rates for single-locus GWAS. The widely used Bonferroni correction to modify the threshold value is very conservative and lots of true loci may be eliminated. Therefore, the best solution to overcome these problems is using multi-locus GWAS methods. These methods consider the information of all loci simultaneously and multiple test corrections are not required because of their multi-locus nature (Wang et al., 2016a). The FaST-LMM-Select method uses associated markers as pseudo Quantitative Trait Nucleotides (QTN) and QTNs are considered correlated when they are estimated within a 2 Mb (Listgarten et al., 2012). The Settlement of MLM Under Progressively Exclusive Relationship (SUPER) method sets a threshold between the pseudo QTNs and the testing markers on LD (Wang et al., 2014). The Multi-Locus Mixed-Model (MLMM) conducts genetic marker tests one by one and tests multiple markers

simultaneously by fitting pseudo QTNs and has advantages over MLM (Korte et al., 2012). The Fixed and random model Circulating Probability Unification (FarmCPU), iteratively uses the Fixed Effect Model (FEM) and a Random Effect Model (REM), reportedly improves statistical power, increases computational efficiency, and has the ability to control false positives and false negatives as compared to other models (Liu et al., 2016). Generally these multi-locus methods involve two step algorithms, wherein the first step, the entire genome is scanned with a single locus GWAS method and putative Quantitative Trait Nucleotides (QTNs) are detected with a less stringent critical value such as $p < 0.005$ or $p < 1/m$, where m is the number of markers. In the second step all the selected putative QTNs are examined by multi locus GWAS method to identify true QTNs (Wang et al., 2016a; Wang et al., 2016b; Wen et al., 2018).

1.8.5 GWAS for phenotypic and Al toxicity tolerance trait

GWAS has been used successfully in cereals and pulses to identify significant markers for different traits, including adaptive or agronomic and complex traits such as flowering time, growth rate, yield, plant architecture, disease resistance, stress tolerance and nutritional quality. GWAS has been used in *Arabidopsis thaliana* for number of phenotypes (Atwell et al., 2010) including for Al and proton tolerance (Nakano et al., 2020) that identified significant associations and explained a high proportion of variation. Similarly, in rice, GWAS also has been used for flowering time, yield and agronomic traits (Huang et al., 2010; Huang et al., 2012; Begum et al., 2015), for plant architecture (Famoso et al., 2011; Yano et al., 2019), for salt, salinity and Mn tolerance (Cui et al., 2018; Shrestha et al., 2018; Lekklar et al., 2019) and aluminium tolerances (Zhang et al., 2016; Tao et al., 2018; Zhao et al., 2018; Zhang et al., 2019). Most of the GWAS for rice Al tolerance was carried out using Ting's core collection (Zhang et al., 2016). The phenotype was based on measuring relative root elongation (RRE) in the seedling stage. and the markers explained low to medium levels of phenotypic variation (Zhao et al., 2018). The rice Al tolerance GWAS study also

identified important candidate genes (*NRAT1*, *ART1* and *STAR1*) along with new candidate genes. These candidate genes showed significant upregulation of the lipid metabolism, abiotic stress responses and membrane proteins in tolerant varieties while no significant differences was observed for sensitive varieties between the Al treatments by transcriptome sequencing (Zhang et al., 2019). The study with different subpopulations of rice showed the relative degree of Al tolerance in five rice subpopulations (*temperate japonica* > *tropical japonica* > *aromatic* > *indica* = *aus*) which was correlated with genetic relatedness among them and indicated subpopulation specific Al tolerance in *aus* and *indica*. This suggests Al tolerance in a given subpopulation is largely controlled by alleles that are unique to that subpopulation (Famoso et al., 2011). In a wheat GWAS study for Al toxicity tolerance significant associations were identified on chromosomes 1A, 1D, 3B and 6A (Navakode et al., 2014). Although this study did not identify the major 4DL locus as reported by many classical QTL mapping studies, it identified the 3B locus (*wpt0021)* which lies approximately 50 cM away from the markers *Xwpt-1625/Xwpt-4597*. This corresponds to a previously identified QTL in a biparental study on chromosome 3B (Zhou et al., 2007; Cai et al., 2008; Navakode et al., 2010).

The GWAS study in lentil for Al toxicity tolerance was designed in this present work, with more details are given in the Chapter 4. The genomic evaluation with dense SNPs and genome wide association study will identify the tolerant trait associated genomic regions which will help to develop markers for breeding programmes. These identified accessions with favourable alleles could be incorporated into ongoing lentil breeding programmes to develop new varieties with acid soil aluminium tolerance.

1.9 Lentil low pH and Al toxicity tolerance breeding programs

Lentil is cultivated worldwide and is adapted to a diverse range of agro climatic conditions. Generally, increases in crop production over recent decades has largely relied on increases in yield (harvestable product per unit area) rather than increases in the area of cultivation.

Individual crop cultivation can be expanded if breeding or management can overcome specific biotic or abiotic constraints (Ryan, 2018). In lentil production, among other biotic and abiotic constraints, the low pH and Al toxicity are also major concerns on acid soils. In pulses including lentils, some level of genetic variation in Al resistance has been found based on short-term studies measuring Al uptake into the root tissues or root regrowth after Haematoxylin staining or induction of callose (indicator of stress) (Singh et al., 2012; Singh et al., 2015; Singh et al., 2016; Singh et al., 2018). Further in lentil, bulk segregant analysis (BSA) was employed to identify SSRs linked with resistance to Al stress based on root regrowth and fluorescent signals in F2 mapping populations (Singh et al., 2018). However, in lentil not much progress has been made, although observed variation in Al resistance is promising for the breeding purposes. It is unclear whether the variation in Al resistance is large enough to be useful in agriculture and whether in-vitro measurements in seedlings can be translated to differences in whole plant biomass and yield on acid soils (Ryan, 2018). Nevertheless, there is a need for lentil crop improvement for Al resistance which can provide important opportunities for increasing production and cultivation area.

In this thesis work, hydroponics method was established to screen lentils for Al toxicity tolerance by using root length parameters, which are detailed in Chapter 2. Putative acid tolerant lentil accessions along with local varieties were screened based on the established methodology for Al toxicity tolerance and results were supported by different staining tests and soil screens, that are detailed in Chapter 3. In the Chapter 4 more diver accessions evaluated for Al toxicity tolerance variation and linked molecular markers were identified that are further can be used for MAS and breeding programmes.

1.10 Aim of the thesis

The aim of the work described in this thesis is to establish an efficient screening method for Al toxicity during the early stage of lentil development. This method will then be used to identify Al tolerant accessions, and informative markers linked to Al toxicity tolerance. These can then be deployed using MAS in lentil breeding programmes.

Objectives

1) Establish a high throughput phenotypic screening method to evaluate lentil accessions for acidity and Al toxicity tolerance in large numbers to enable GWAS.

2) To phenotype lentil accessions identified as putative acid tolerant FIGS accessions and a diverse lentil landrace collection for Al toxicity tolerance to assess the variation for tolerance and identify tolerant accessions to be included in the lentil breeding program for the Al toxicity tolerance breeding.

3) Validate the hydroponics screening results in selected subset of the accessions by histochemical and biochemical analyses, and acid soil screening. Test the tolerance mechanisms in selected contrasting accessions to gain some insights about tolerance mechanisms in lentil.

4) Genotype and evaluate the diverse lentil collection that are screened for Al toxicity tolerance, for population structure, genetic diversity, and selection signatures, which enabled the identification of marker-trait associations for Al toxicity tolerance trait by using GWAS.

1.11 References

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CHAPTER 2: Establishment of a hydroponic screening method for the evaluation of acidity and Aluminium toxicity tolerance in lentil

Chapter preface

This chapter established a high throughput hydroponic screening method. Al treatment was optimised, and acidity was evaluated. This method was used to assess Al toxicity tolerance in a diverse set of lentil accessions. The developed method is simple and screens a large number of lentil seedlings at early stage of development. This method was used further in Chapter 3 and 4 for phenotyping large number of lentil accessions which enabled the genome wide association (GWAS) study for Al toxicity tolerance trait. In this chapter, acid and Al toxicity tolerant accessions were identified. These identified tolerant and sensitive accessions were tested by histochemical and biochemical analyses, and for Al content to provide insights into Al tolerance mechanisms of lentil.

Publication details

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Statement from co-author confirming the contribution of the PhD candidate:

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I confirm that Vani Kulkarni has made the contributions listed above."

Dr Tim Sawbridge 16/11/2020

Abbreviations: AGG, Australians Grain Genebank; ΔRL, Change in Root Length; DI, deionised water; ICP-OES, inductively coupled plasms-optical emission spectrometry; LSD, least significant difference; NaOCl, sodium hypochlorite; OD, optical density; OA, oxalic acid; RRG%, Relative Root Growth%; RRG1%, Relative Root Growth for acidity; RRG2%, Relative root Growth for Al tolerance; SDS, sodium dodecyl sulphate;

Abstract

Lentil production is greatly affected by abiotic stresses such as acid soils with toxic levels of aluminium (Al). Tolerance/resistance breeding for acid soil and Al toxicity is critical, as liming, to ameliorate these soil constraints is not an effective long-term strategy. To accelerate breeding for these traits, an effective screening method to assess for acidity and Al toxicity tolerance is an essential first step. An effective screening method relies on the appropriate methodology, optimal Al concentration and reliable measurements of acidity and Al toxicity tolerance. This study reports a hydroponic screening method where the effective Al treatment was optimised for lentil by using four-day old seedlings and the method subsequently used to evaluate acidity and Al toxicity tolerance in diverse lentil accessions. Tolerance mechanisms were also evaluated for the identified tolerant lentil accessions. In all Experiments, the change in root length (ΔRL) were calculated from the pre and post treatment root length, with the relative root growth (RRG%) used as a tolerance index. Acidity tolerance was measured as RRG1% where ΔRL at pH 4.5 was compared to ΔRL at pH 6.0, while Al toxicity tolerance was measured as RRG2% across different Al treatments compared to control pH4.5 without Al. In Experiment 1, nine landraces were tested at different Al concentrations to optimise Al treatment for further screening. This showed that, the low Al treatment of 2 µM increased Al tolerance in accessions AGG71377 (104%) and AGG71438 (112%), however high Al treatments (10, 20 and 30 µM) drastically reduced the Al tolerance of all lentil accessions to an average of 8.6%. In Experiment 2, the optimised 5 µM along with 10 µM Al treatments were used to screen a wider set of 35 diverse

lentil accessions for acidity and Al toxicity tolerance. From this Experiment, accessions were grouped by K-means clustering based on RRG1% and mean RRG2% (5 and 10 µM Al), where high acidity tolerant accessions (Digger, AGG70305 and AGG70085) were grouped in cluster 1, while high Al tolerant accessions (AGG70137, AGG70164, Northfield, Cassab and PBA Jumbo2) were grouped in cluster 3. In Experiment 3, commercial Australian PBA varieties were tested for Al toxicity tolerance at 5 µM Al, which showed no significant difference between PBA Flash, PBA Blitz, PBA Herald and PBA Hurricane, with an average of 30% Al tolerance. Two tolerant and two sensitive accessions identified in Experiment 2 and 3 were used to study the tolerance mechanism determined through stains, and root and shoot Al content with different treatment durations at 5 and 10 µM Al in Experiment 4. The result showed an average 2-fold increase in Haematoxylin stain (Al content) in sensitive accessions (Precoz and AGG70530) at 5 µM Al after 1 day's treatment, which increased to 2.5-fold after 2 days treatment compared to tolerant accessions. A similar trend was also reported for Evans blue stain in these lines. Both the tolerant Northfield and sensitive Precoz lines showed an average of 1055 ug/g DW root Al content at 5 µM Al , however Northfield showed more root growth than Precoz and it also accumulated more shoot Al (74 ug/g DW) at higher 10 µM Al treatment. Overall, this work showed 5 µM Al as optimal Al treatment in lentil. Among the varieties tested, Digger and PBA Jumbo2 are the high acidity and high Al toxicity tolerant accessions respectively. The sensitive accessions Precoz and AGG70530 showed more intense stain indicating greater Al accumulation and plasma membrane damage compared to tolerant lines Northfield and AGG70137.

2.1 Introduction

Lentil (*L. culinaris ssp. Culinaris*) is an annual and highly economical food crop. It is an important global crop for the human diet as it is an affordable source of carbohydrates (53-63%), proteins (20-30%), minerals (1.78-3.1%), oil (0.70-2.0%), trace elements and fibre (Qaim et al., 2007; Kumar et al., 2015; Ates et al., 2016). This nutrient combination in lentils

provides the recommended daily nutritional balance thus playing a significant role in reducing malnutrition and micro-nutrient deficiencies (Karaköy et al., 2012) in humans worldwide. Lentil global cultivation is 6.58 Mha with production of 7.59 Mt (FAOSTAT, 2017). The major lentil growing countries include Canada, India, Turkey, Syria, Australia, Nepal and the United States where some areas of production are significantly affected by acid soils and associated aluminium (Al) toxicity (Singh et al., 2012). Approximately 30% of the total land area and up to half of the potentially arable land in tropical and sub-tropical regions (Von Uexküll & Mutert, 1995; Kochian et al., 2004) are affected by soil acidity. In Australia, ~50% of agricultural land has surface pH values ≤ 5.5 which is below optimum for extremely acid sensitive agricultural plants such as lentil. Of this 12–24 Mha is extremely to highly acidic with pH values ≤ 4.8 and in addition 23 Mha is with pH ≤ 5.5 (NLWR-Audit, 2001).

Australia's annual total agriculture losses due to soil acidity are estimated to be AU $900-1585 million, with the major grain crops including bread wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), canola (*Brassica napus* L.) and pulses significantly affected by soil acidity (Hajkowicz & Young, 2005; Dang, 2013). Lentil is highly adapted in Australia to well drained, neutral to alkaline loam and clay loam soil types with the main production areas in Victoria and South Australia (Siddique et al., 1998; GRDC, 2017). A drop in pH below the threshold value (pH 5.5) or < 6.0 can cause more than 86% reduction in lentil seed yield which is largely related to an inherent susceptibility of the plant and/or the nitrogen fixing rhizobia to soil acidity (Siddique et al., 1999).

Acid soils contain high concentrations of hydrogen ions (H^+) which can inhibit plant growth, as well as several ions (such as Al and Mn) that become toxic to the plant and further limit the availability of some nutrients (especially phosphorus (P), magnesium (Mg) and molybdenum (Mo)). However, the primary reason most plants grow poorly on acid soils is the increased concentrations of the soluble Al cation (Al^{3+}) (Ryan, 2018). Aluminium is

mainly present in mineral soil as harmless aluminosilicate, however it becomes phytotoxic at a pH of <5.0 forming Al^{3+} (Kinraide, 1997; Silva, 2012; Schmitt et al., 2016). Al toxicity is the major factor causing production losses on 67% of the total acid soils worldwide (Hede et al., 2001), particularly in the developing countries of South America, Central Asia, and South East Asia (Kochian et al., 2015).

Lentil is highly sensitive to acid soils and Al toxicity with critical concentrations of calcium chloride extractable Al (Al_{Ca}) of 0.1 to 0.4 ppm, capable of reducing plant growth by 10% (Upjohn et al., 2005). The solubilized toxic Al^{3+} in acid soil typically affects the viability of the root apex of sensitive plants and inhibits root elongation and alters the architecture and morphology of the roots, resulting in reduced water and nutrients uptake, ultimately hindering plant growth and development (Kochian et al., 2015). Depending on the Al concentration and duration of exposure, it may increase cell wall rigidity, causing the rupture of the rhizodermis and outer cortex of the meristem, which inhibits elongation of the root tips (Blarney et al., 2004; Jones et al., 2006; Kopittke et al., 2008).

The primary management tool to neutralize acid soils is the application of lime, however this can be costly, and can take years to correct acidity at depth (Wayima et al., 2019). A complementary strategy for improving crop production on acid soils is by growing Al tolerant varieties/cultivars. This involves identification of new germplasm sources with Al toxicity tolerance, understanding its genetic variability, genetic inheritance and tolerance mechanisms for incorporation of favourable alleles through molecular assisted breeding. In several crop species, variation in Al toxicity tolerance has been reported, and in addition to interspecific variability, a large degree of intraspecific variability has been observed (Parentoni et al., 2001; Samac & Tesfaye, 2003). Simple inheritance with a single gene was reported in wheat (Delhaize et al., 1993a; Delhaize et al., 1993b), barley (Wang et al., 2007), and sorghum (Magalhaes et al., 2004) whereas multiple genes/ QTLs were reported in rice (Nguyen et al., 2001; Nguyen et al., 2002) and maize (Ninamango-Cárdenas et al., 2003).

Some plant species growing on acid soils have developed tolerance mechanisms to overcome and resist Al toxicity (Ma et al., 2001; Kochian et al., 2015). The mechanisms include avoiding the Al ions by exclusion from root tips, or intrinsic detoxification of Al ions absorbed by plant roots (Sade et al., 2016). The most studied mechanism is exclusion by release of organic acids (citrate, malate, oxalate and acetate) that are excellent Al chelating agents which are involved in the adaptation of plants to Al stress environments (Ma et al., 1998; Ma & Hiradate, 2000; Matsumoto, 2000; Igamberdiev & Eprintsev, 2016). This type of tolerance mechanism has been reported in wheat (Ryan et al., 1995) and rice (Liu et al., 2017) where malate is released in response to Al, whereas citrate was reported in barley (Wang et al., 2007), sorghum (Magalhaes et al., 2007) and maize (Pellet et al., 1995).

Variation for Al toxicity tolerance has also been reported for lentil, as measured by root regrowth (short and long term) after Haematoxylin staining (Singh et al., 2012). The presence of a single major gene has been reported for root regrowth and callose accumulation (Singh et al., 2015) with an exclusion type of tolerance mechanism by release of malate organic acid also being described (Singh et al., 2016; Singh et al., 2018). In all above-mentioned studies, nutrient solution based hydroponic systems were used to screen Al tolerance as it provides easy access to the root system and allows control over the pH and nutrient availability. Furthermore, it can be used in conjunction with non-destructive measurements of root growth (Hede et al., 2002; Bidhan & Bhadra, 2014; Awasthi et al., 2017), or root re-growth after staining with Haematoxylin (Singh et al., 2012; Singh et al., 2016; Singh et al., 2018) in Al treatments. Hematoxylin staining is a simple and easy method to detect Al, where the intensity of the stained roots correlates directly to Al accumulation in the root tissues. This has been widely used to discriminate plant genotypes for tolerance to toxic Al (Miftahudin et al., 2007). Many other studies (Xu et al., 2017; Motoda et al., 2010) have assessed Al toxicity tolerance by investigating relative root growth (RRG), where the root growth under Al treatment was compared with respect to a control with no Al

treatment supplied over the time period. The use of RRG as a selection index for Al toxicity tolerance or resistance is preferred as it allows for better differentiation of the genotypes and has been used extensively in many crops (Pineros et al., 2005; Famoso et al., 2010; Raman & Gustafson, 2011; Matonyei et al., 2014).

To date, there has been no study in lentil for Al toxicity tolerance based on RRG measurements, and very limited information on acidity and Al toxicity tolerance in lentil. Hence this study was conducted with following objectives; 1) Establish an effective and robust high throughput hydroponic screening method with optimised Al concentration for evaluating lentil landraces, adapted cultivars and varieties; 2) Evaluate diverse accessions for acidity and Al toxicity tolerance and 3) Study the tolerance mechanisms in identified tolerant accessions.

2.2 Material and methods

2.2.1 Plant material and growth conditions

Diverse lentil accessions (Table 2.1) comprised of landraces, adapted cultivars and varieties sourced from the Australians Grain Genebank (AGG), Horsham, Australia. In all screenings known lentil tolerant (ILL6002), and wheat tolerant (yitpi) and sentitive (chara) lines were used as check lines. Data from wheat lines were not used for analysis however their performance was used as guide for the working condition of the nutrient solutions. Seeds (40-50 per accession) were sterilized with 1% NaOCl (sodium hypochlorite) (w/v) for 5 min before being rinsed with deionised water (DI) 3-4 times. Approximately 10-20 seeds per accession (depending on seed size) were germinated in rolled paper towels at 20-22 °C in darkness for 4 days. Seeds were placed onto moist paper towel, which was then rolled, and two to three rolls were kept vertically in plastic containers containing a small amount of water. Germinated seedlings with uniform root length were selected for transfer into single holes in a 96-capacity polyethylene float. The float was placed into a 13 L capacity tub (L

432 mm x W 320 mm x H 127 mm), containing 10 L low ionic strength nutrient solution. The solution was continuously aerated with 35.5 cm air stones connected to a 50 W air compressor. Plants were grown in a controlled environment under natural light with 24/15℃ day/night temperature for a period of three days. All Experiments were conducted in split-plot randomized design with three to four replications as blocks, treatments were applied to tubs that were considered as main plots and accessions within each tub were considered as subplots. Each time depending on the number of technical seedlings (four or three), 24 or 32 accessions were screened in each Experiment and details are given in further sections. Supplementary Figure 2.1 shows the detailed hydroponic setup used in this study.

2.2.2 Nutrient solution and Al treatment

Nutrient solutions were considered based on previous research in cereals and other crops (Delhaize et al., 2004; Rossello, 2011). These nutrient solutions were selected and nutrient concentrations were analysed in the GeochemEZ (Shaff et al., 2010) chemical speciation programme for Al activity and precipitation. The inductively coupled plasma mass spectrometry (ICPMS) analysis of pre and post treatment solution (20 ML) confirmed the maintenance of the toxic Al levels and less variations in other nutrients compositions. The final selected nutrient component solutions used in the hydroponic screening is given in Table 2.2. Aluminium was supplied as $AlCl_3.6H_2O$ (Molecular weight 241.43 g/mol). The pH of the solution was maintained by 0.1M HCl and 1M KOH solutions by adjusting daily, as pH varied between ±0.02 to ±0.1 over 24 hours period. Different concentration of Al treatments and pH controls (pH 6.0 = control 1 and acidic pH 4.5 = control 2) were used for the three-day treatment across different Experiments.

All nutrient components were the same for both controls with the exception of the source of iron. For the neural pH solution (control 1), Fe:EDTA was used which is stable at pH 6.0

(Kasozi et al., 2019), whereas $FeCl_3.6H_2O$ was used for the acidic solution (control 2) which ensures maximum availability of Fe at acidic pH.

Table 2.1. List of diverse lentil accessions used in the present study

Sl.no	Genotypes	Country of origin	Level of improvement
1	AGG70305	Afghanistan	Landrace
2	AGG70455	Algeria	Landrace
3	AGG71377	Chile	Landrace
4	AGG71438	Chile	Landrace
5	AGG71501	Iran	Landrace
6	AGG71512	Turkey	Landrace
7	AGG71717	Iran	Landrace
8	AGG72372	Egypt	Landrace
9	ILL6002	Syria	Landrace
10	07H062L-08HS2004	Australia	Breeding line
11	AGG70023	Ethiopia	Landrace
12	AGG70024	Afghanistan	Landrace
13	AGG70085	Morocco	Landrace
14	AGG70138	Turkey	Landrace
15	AGG70145	Morocco	Landrace
16	AGG70163	Tunisia	Landrace
17	AGG70164	Tunisia	Landrace
18	AGG70247	Afghanistan	Landrace
19	AGG70249	Ethiopia	Landrace
20	Boomer	Australia	Advanced Cultivar
21	Cassab	Australia	Advanced Cultivar
22	CIPAL1301	Australia	Advanced Cultivar
23	Digger	Australia	Advanced Cultivar
24	ILL0213A		Landrace
25	ILL2024	Ethiopia	Landrace
26	ILL6788	Syria	Unknown
27	ILL7537	Syria	Landrace
28	Indianhead	Canada	Advanced Cultivar
29	Nugget	Australia	Advanced Cultivar
30	PBA Ace	Australia	Advanced Cultivar
31	PBA Blitz	Australia	Advanced Cultivar
32	PBA Bolt	Australia	Advanced Cultivar
33	PBA Greenfield	Australia	Advanced Cultivar
34	PBA Jumbo2	Australia	Advanced Cultivar
35	AGG70137	Lebanon	Landrace
36	Northfield	Ethiopia	Landrace
37	Ansak	From ICARDA	Advanced Cultivar

Sl.no	Genotypes	Country of origin	Level of improvement
38	CDC Matador	Canada	Advanced Cultivar
39	CIPAL0501	Australia	Breeder's Line
40	Cobber	Australia	Advanced Cultivar
41	Cumra	Turkey	Advanced Cultivar
42	Emerald	United States	Advanced Cultivar
43	ILL0061	-	Landrace
44	ILL0214	-	Landrace
45	Nipper	Australia	Advanced Cultivar
46	AGG70530	Jordan	Advanced Cultivar
47	PBA Flash	Australia	Advanced Cultivar
48	PBA Herald	Australia	Advanced Cultivar
49	PBA Hurricane	Australia	Advanced Cultivar
50	PBA Jumbo	Australia	Advanced Cultivar
51	Precoz	Argentina	Advanced Cultivar

1-9; Used in Experiment 1, 1-36; Used in Experiment 2 except AGG72372, 35-51; Used in Experiment 3 including PBA Blitz

Table 2.2. Nutrient components of the hydroponic solution

Component	Molecular weight g/mol	Final concentration µM	Working mg/L
KNO_3	101.1	500	50.56
$CaCl_2$	110.9	500	55.45
NH_4NO_3	80.04	500	40.02
$MgSO_4.7H_2O$	246.47	150	36.97
KH_2PO_4	136.09	2.0	0.27
$FeCl_3.6H_2O$	270.3	2.0	0.54
FeEDTA	367.1	2.0	0.73
H_3BO_3	61.83	11.0	0.68
$MnCl_2.4H_2O$	197.9	2.0	0.40
$ZnSO_4.7H_2O$	287.56	0.35	0.10
$CuSO_4.5H_2O$	249.69	0.20	0.05
$Na_2MoO_4.2H_2O$	241.9	0.33	0.08

2.2.3 Al tolerance screening in lentil

Different Experiments were designed with wide rang accessions using different Al treatments. Experiment 1 was deigned to determine the screening method and Al concentration. Experimental 2 and 3 were designed mainly to screen for variation for Al tolerance.

Experiment 1: Determination of optimal Al concentration for hydroponic screening (Accession numbers 1-9; Table 2.1): This Experiment consisted of eight treatments in total; six comprising of the differing Al concentrations (2, 3, 5, 10, 20 and 30 µM) at a pH of 4.5, plus the two controls (1 and 2). For each accession x treatment combination, a maximum of 10 seedlings were used and replicated four times.

Experiment 2: Screening for acidity and Al toxicity tolerance in a wider set of 35 accessions at two Al concentration (Accession numbers 1-36, except AGG72372; Table 2.1): This Experiment consists of two independent hydroponics Experiments, one Experiment had 22 accessions and other had the 21 with seven common lines between them. As the known tolerant lentil line (ILL6002) performed consistently similar and all other experimental conditions were the same between the two independent Experiments, for easy presentation they are put together in Experiment 2, and further data were merged and average was considered for analysis. Each accession was screened at an Al concentration of 5 and 10 µM, and compared to the controls (1 and 2). A total of four seedlings per accession were used in each of the treatments, with each accession x treatment combination replicated three times.

Experiment 3: Al toxicity tolerance screening of 18 varieties and landraces screened at the optimized 5 µM Al along with control 2 (Table 2.1: 35-51 including PBA Blitz accessions): This was conducted with three replications with four seedlings per accession in each treatment x replication combination.

Germinated seedlings were transferred directly to respective treatment solutions after recording initial (pre-treatment) root length and all seedlings were harvested after three day of treatment where main root length measures (post treatment) were recorded. These root lengths were measured on individual seedling from all accessions by using a ruler (in mm). The mean change in root length (ΔRL) was calculated by Equation 1 (Dai et al., 2009). The acidity tolerance (Equation 2) and Al tolerance (Equation 3) were expressed as relative root growth percent as shown below.

Equation 1: Change (Δ) in RL = Post treatment RL – Pre-treatment RL

Equation 2: Acidity tolerance (RRG1%) = (Δ RL at control 2 / Δ RL at control 1) *100

Equation 3: Aluminium tolerance (RRG2%) = (Δ RL at Al treatment / Δ RL at control 2) *100

In different hydroponic Experiments the known tolerant line (ILL6002) was used which showed the consistent performance. Similarly, the accessions (Northfield, Precoz, AGG70137 and AGG70530) also showed the consistent performance in term of root growth and tolerances (RRG%). This indicates reliability of screening method and nutrient solution.

2.3 Al tolerance mechanisms in lentil

Based on morphological evaluation of root length in previous hydroponics screens (Exp 2 and 3) Al tolerant (Northfield and AGG70137) and Al sensitive (Precoz and AGG70530) accessions were selected to study the tolerance mechanism, where histochemical and ICP-OES analysis were conducted (Experiment 4). Each analysis was completed as separate Experiment that were designed in split plot with two or three replications and details are given in the Experiment 4a - 4d. Al treatments of 5 and 10 µM were applied at pH 4.5 for one, two and three days and harvested accordingly. In each experiment (Experiment 4a and

4b) at each harvest time after recording the post treatment root lengths, seedlings were further used for histochemical or ICP-OES analysis.

2.3.1 Al uptake by Hematoxylin stain

Experiment 4a: Haematoxylin staining – In this Experiment 21 seedlings per accessions were used for each of the three replications in each treatment of 5 and 10 µAl at pH of 4.5 along with control. Set of 21 seedlings of per accessions were transferred as set without randomisation with other accession seedlings. During each harvest time seven seedlings were harvested from each treatment and post treatment root length were recorded with ruler in mm of which three where used for stereomicroscopic (ISCapture V4.1, Tucsen Photonics Co., Ltd) observations and other four were used for stain quantification for Al uptake after staining with the Haematoxylin.

Al treated and untreated (control) roots of intact seedlings were washed in distilled water for 15 min and stained with Haematoxylin solution for 15 min (0.2 % aqueous stain containing 0.02 % potassium iodide) at room temperature. After washing with distilled water for 15 min, four root tips (5 mm) from each accession x treatment combination were excised and soaked in 200 µl of 1 M HCl for 1 hour. The optical density (OD) of the released stain was measured at 490 nm using a spectrophotometer (UV-1800 Shimadzu spectrophotometer). Aluminium uptake was determined by comparing treated and control OD values. The amount of dye released (as observed in terms of absorbance at 490 nm) was directly proportional to the amount of Al accumulated in the root tips.

2.3.2 Plasma membrane integrity by Evans blue stain

Experiment 4b: Evans blue staining – This Experiment was also designed in the same way as Experiment 4a with three replication and 21 seedlings per accessions in each treatment of 5 and 10 µAl at pH of 4.5 along with control. At each harvest time seven seedlings were harvested to record the post treatment root length and stained with 10 ml Evans blue. Among

which three were used for stereomicroscopic observations for stain accumulation and four were used for stain quantification for loss of plasma membrane integrity.

Intact seedling roots were stained with 0.025% (w/v) Evans blue stain in 100 µM $CaCl_2$ (pH 5.6) for 15 min . The stained roots were then washed three times with 200 ml of 100 µM $CaCl_2$ (pH 5.6), after which the dye no longer eluted from the roots (Yamamoto et al., 2001). After washing, four root tips (5 mm) were excised and homogenized with 1 ml of 1% (w/v) aqueous sodium dodecyl sulphate (SDS) at room temperature. The homogenate was centrifuged at 13,500 rpm for 10 min. The optical density of the supernatant was measured at 600 nm by a spectrophotometer (Awasthi et al., 2017).

2.3.3 Determination of Al uptake in whole root and shoot by ICP-OES

Experiment 4c: Root and shoot Al content – This Experiment was designed with two replication and 24 seedling per accessions in each Al treatment of 5 and 10 µM along with control at pH 4.5. To determine the Al content in whole roots and shoots, all 24 seedlings per accessions were harvested after the three-day Al treatment (5 and 10 µM) and dried at 70˚C for 72 hours. After grinding dried samples, a representative sub-sample from the bulk of ground dried plant tissue was weighed (0.5 g) into a pyrex tube and digested with 5 ml of 3:1 mixture of nitric and perchloric acids (V/V) in a heated aluminium block (Zasoski & Burau, 1977). In this process the digestion mixture (2 ml) was held at different increasing temperatures (Supplementary Table 2.1) until white fumes of perchloric acid were seen in the test tubes. After cooling it was diluted to 20 ml with 1% V/V perchloric acid and the concentrations of analytes in the digest were determined by ICP-OES (Agilent 5100 DV, Agilent Technologies Australia Pty Ltd). The amount of Al was expressed in µg per g of the dry weight of the tissue.

2.3.4 Organic acid release

Experiment 4c: Organic acid release from roots – This Experiment was done with two replication that were spread over time. The 14 seedlings per accessions per Al treatment (0, 100 and 200 µM Al) X elution time (1, 3, 6, 24 and 48h) combinations were used. Root exudates were collected following the method described in lentil (Singh et al., 2016) with modifications. The 14 four day old seedling with similar root length were placed into separate plastic containers (500 ml volume) and exposed to 200 ml $CaCl_2$ (0.2 mM) solution with 0, 100 and 200 µM Al (pH 4.5) to collect the root exudates under differing Al concentrations (50 ml from each container). These containers were maintained in a growth chamber (Bio Chambers, Canada) under constant conditions of 18°C and 14 h day/10 h night cycle; with light intensity maintained at 510 µmol m-2 s-1. Root exudates were collected at 1, 3, 6, 24 and 48h after the start of Al treatments. Eluted solution was sent to the Soil Science Department, DJPR, Macleod, Victoria for HPLC analysis for malate, citrate and oxalate acid analysis.

2.4 Statistical analysis

For all the screening Experiments (1, 2 and 3) the average of Δ RL of control and Al treatment along with RRG1% and RRG2% across all the replications was used for analysis of variance in GenStat edition 18.2 (VSN-International, 2015) and least significant difference (LSD) was used to compare the means. Before the ANOVA, all the data was checked for the assumptions and distributions by checking the graphs of residuals, fitted values, and normal plots. The relationship between acidity (RRG1%) and mean Al tolerance (RRG2% at 5 and 10 µM Al) was analysed by K means clustering method. The clustering was performed with "cluster" and visualised by "factoextra" libraries in R programme. For the histochemical stains (Haematoxylin and Evans blue), the absorbance data from all the replications were converted to fold change by comparing the Al treatment of 5 and 10 µM

with respect control 2 at pH 4.5. Further this fold change was also analysed in GenStat edition 18.2 for analysis of variance. The Al content of root and shoot data from three replication were also analysed in GenStat edition 18.2 for analysis of variance and further Fisher protected test was used to compare the means.

2.5 Results

2.5.1 Experiment 1: Determination of optimal Al concentration for hydroponic screening

The main effects of the Al treatments, the accessions and their interactions were significantly different ($p < 0.001$) for measured ΔRL. The Al treatment significantly affected root length, with a linear decrease in ΔRL observed with increase in Al concentrations (Figure 2.1). The acidity alone reduced the ΔRL to 57 mm compared to the 90 mm in the control 1 (pH = 6.0), while the Al treatment reduced the ΔRL on an average to 16 mm compared to the control 2 (pH = 4.5) of 57 mm.

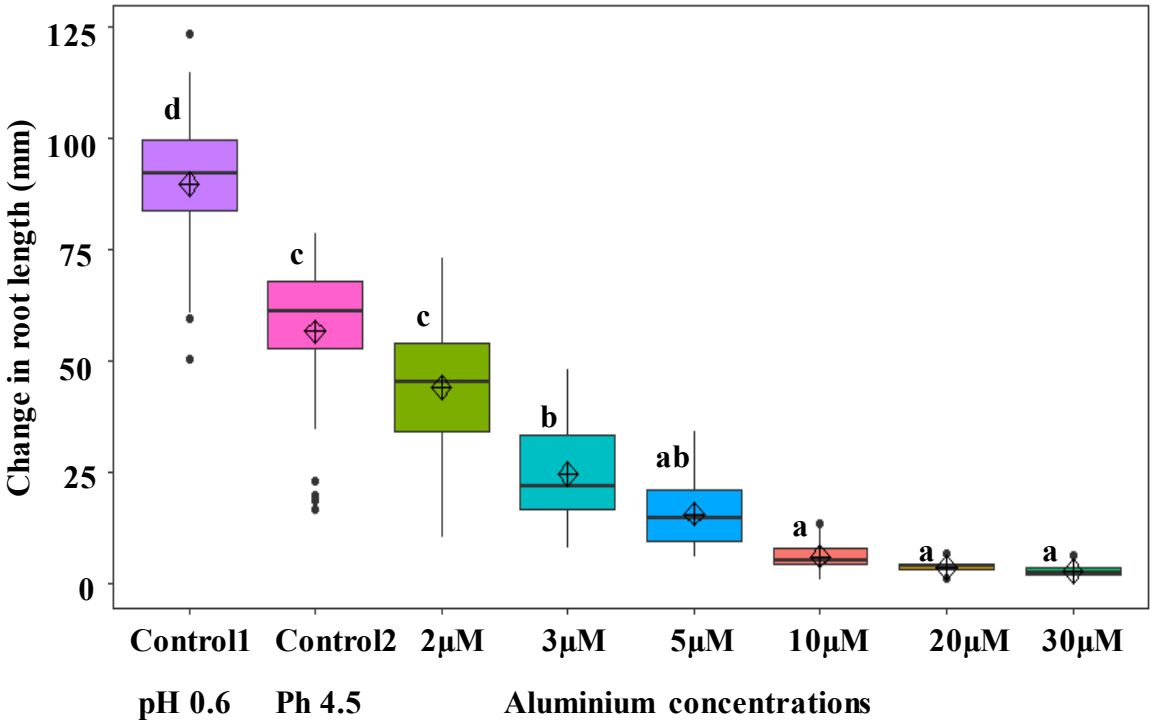

Figure 2.1. Inhibition of change (Δ) in RL at different pH and Al concentrations during three-day Al treatment in hydroponic solution.

The pH and Al treatments with same letters are not significantly different at $p = 0.01$ by Fishers protected test

Acidity tolerance (RRG1%) was not significant among the accessions whereas accessions showed variation for Al tolerance (RRG2%) (Table 2.3). Significant ($p < 0.001$) interaction has been observed between Al treatment and genotypes. Al tolerance at 2 µM was significantly ($p < 0.001$) different than the other concentrations, and increased Al tolerance by 1 and 1.4 times in accessions AGG71377 and AGG71438 respectively, while it significantly ($p < 0.001$) reduced the tolerance by half in accession AGG72372 (58%) compared to the acidic control (control 2). At 3 µM Al exposure, accession AGG71501, was significantly ($p < 0.001$) higher than all other accessions. Aluminium tolerance at 5 µM ranged from 15.7% (AGG72372) to 43.9% (AGG71717) with an average of 28.8%. All the accessions showed reduced RRG2% at higher concentrations of 10, 20 and 30 µM, with little variation between them (Table 2.3).

Table 2.3. Aluminium (Al) tolerance (RRG%) of the lentil accessions after three day of treatment at different Al concentrations (Experiment 1)

	Relative root growth (RRG%)						
Accessions	**2 µM Al**	**3 µM Al**	**5 µM Al**	**10 µM Al**	**20 µM Al**	**30 µM Al**	**Mean**
AGG70305	97.5	35.3	23.3	7.0	7.0	4.7	29.1
AGG70455	82.3	49.5	28.4	8.3	7.5	8.7	30.8
AGG71377	104.1	41.3	40.4	9.0	10.5	4.3	34.9
AGG71438	112.1	48.9	20.8	14.6	8.4	8.6	35.6
AGG71501	99.4	73.2	38.5	19.6	7.9	5.6	40.7
AGG71512	82.4	41.5	20.4	11.8	5.5	4.8	27.7
AGG71717	91.9	54.9	43.9	13.5	5.7	3.8	35.6
AGG72372	58.1	44.2	15.7	14.8	8.6	8.4	25.0
ILL6002	79.8	54.9	27.4	10.9	11.5	9.0	32.3

Mean	89.7	49.3	28.8	12.1	8.1	6.4	
p value (LSD at 5%)							
Al treatment	<0.001, (34.2)						
Accessions	<0.001 (6.2)						
Al treatment X Accessions	<0.001 (36.5, 15.2)						

The values inside the bracket are the LSD (LSD of 36.5 is for between Al treatments and 15.2 is for between genotypes within Al treatment).

2.5.2 Experiment 2: Acidity and Al toxicity tolerance in wider set of accessions at two Al concentrations

The landraces and varieties screened at 5 and 10 µM Al treatments with showed significant ($p < 0.001$) reduction in ΔRL compared to controls 1 and 2 (Table 2.4). The average ΔRL reduced to 71 mm compared to pH 6.0 (control 1, 99 mm), and further under Al treatment ΔRL reduced to an average of 17 mm compared to pH 4.5 (control 2, 71 mm). Seedlings exposed to control 1 showed an average ΔRL of 99 mm, with AGG70247 showing the highest (126.3 mm) and AGG70305 the lowest (62.3 mm). Contrastingly, seedlings exposed to the control 2 showed an average ΔRL of 71 mm, with AGG70085 showing the highest (95 mm) and PBA Bolt the lowest (52 mm). On average, the 10 µM Al treatment reduced the ΔRL to 8 mm compared to 27 mm for the 5 µM Al treatment. Highest ΔRL was observed in Northfield for both Al treatments whereas the lowest ΔRL was observed in AGG71438 (7 mm) and AGG71377 (2 mm) for the 5 µM and 10 µM Al treatments, respectively.

The accessions significantly ($p = 0.009$) differ based on acidity tolerance (Figure 2.2). The AGG70085 (106 RRG1%) showed high acidity tolerance whereas Nugget had the lowest (55 RRG1%). Among the local varieties, Digger showed an acidity tolerance of 87% which was significantly higher ($p = 0.009$) compared to PBA Ace (65%), AGG71717 (62%),

Nugget (55%), ILL6788 (61%) and ILL0213A (62%). Furthermore, Al tolerance at 5 µM ranged from 13 to 87%, with Northfield, AGG70137 and Cassab showing the highest tolerance, averaging 76% and were significantly ($p < 0.001$) higher to other accessions. However, in 10 µM Al, Northfield (24%) was significantly ($p < 0.001$) higher to the very sensitive accessions AGG71377 (3%) and AGG70305 (5%) whereas other accessions did not differ significantly as all had reduced root growth. There was significantly high correlation ($p < 0.001$, r = 0.79, n = 35) observed between 5 and 10 µM Al, however no correlation was observed between the mean Al tolerance (5 and 10 µM Al) and acidity tolerance (Supplementary Figure 2.2).

The clustering by K-means grouped accessions in three clusters, cluster 1 has the three accessions (Digger, AGG70305, and AGG70085) with high means of 95.9% for acidity (Figure 2.3). Whereas five accessions (AGG70137, AGG70164, Northfield, Cassab and PBA Jumbo2) were the grouped in cluster 3 with acidity mean of 72.7%. Other 27 accessions were grouped in cluster 2 which had the acidity mean of 70.7%. Mean Al tolerance was high in cluster 3 (44.4%), where other clusters had nearly similar level of acidity tolerance (21% in cluster 1, 21.3% in cluster 2).

2.5.3 Experiment 3: Al toxicity tolerance in lentil varieties at 5 µM Al

In this, varieties were tested for Al toxicity tolerance at 5 µM. Accessions significantly ($p < 0.001$) differed in Al tolerance, with high tolerance observed in Northfield (75.4 RRG2%) and AGG70137 (40.96 RRG2%) while a low tolerance of 10.2% RRG2% was reported in ILL0213. The varieties PBA Flash, PBA Blitz, PBA Herald and PBA Hurricane did not differ significantly and showed an average of 30% RRG2%, however these were significantly different compared to Emerald, Cumra and CDC Matador which showed average RRG2% of 12.7% (Figure 2.4 and Supplementary Table 2.2).

Table 2.4. Mean change in root length (Δ RL) of lentil accessions in pH 6.0, 4.5 and in aluminium treatment of 5 and 10 µM after three-day treatment (Experiment 2)

Accessions	pH 6.0 Δ RL	pH 4.5 Δ RL	Δ RL in 5 µM Al	Δ RL in 10 µM Al
07H062L-08HS2004	89.78	66.11	33.17	9.25
AGG70023	101.08	77.83	21.67	5.83
AGG70024	110.33	85	24.31	5.33
AGG70085	109.69	95.5	31.33	8.58
AGG70137	109.75	72	61.08	13.08
AGG70138	111.67	76.67	23.5	6.33
AGG70145	125.08	84.31	33.67	8.5
AGG70163	114.33	91.92	38.75	11.17
AGG70164	102.33	72	37.08	14.58
AGG70247	126.33	90.42	28.67	6.83
AGG70249	94.17	70.58	19	5.83
AGG70305	62.28	56.19	16.72	2.94
AGG70455	93.03	68.58	13.25	4.58
AGG71377	76.72	57	18.67	2.33
AGG71438	72.58	58.33	7.92	6.08
AGG71501	102.58	73.83	22.69	12.25
AGG71512	104.92	70.33	12.78	8.17
AGG71717	92.24	63.75	22.33	6.33
Boomer	94.21	73	31.04	9.96
Cassab	111.08	79.67	52.33	14.67
CIPAL1301	90.17	59.22	22.08	7.67
Digger	72.67	73.19	37.75	10.7
ILL0213A	102.25	63.56	12.14	5.75
ILL2024	87.22	61.65	21.61	6.07
ILL6002	94.4	70.81	19.94	7.16
ILL6788	115.07	70.65	18.42	6.06
ILL7537	99.43	66.65	10.69	4.12
Indianhead	115.25	77.67	24.17	7.58
Northfield	120.17	85.81	65.33	21.58
Nugget	101.42	55.33	19.58	5.42
PBA Ace	112.07	73.46	31.00	10.75

Accessions	pH 6.0 Δ RL	pH 4.5 Δ RL	Δ RL in 5 µM Al	Δ RL in 10 µM Al
PBA Blitz	95.92	73.58	25.92	8.83
PBA Bolt	77.56	52.58	18.92	5.78
PBA Greenfield	105.78	69.42	36.85	12.5
PBA Jumbo2	74.14	62.33	38.03	11.5
Mean	99.07	71.39	27.21	8.40
Treatment	< 0.001(4.48)			
Treatment X Accessions	< 0.001 (13.28, 12.97)			

Al = Aluminum , Control 1- pH6.0 without Al, Control 2- pH4.5 without Al
For Treatment (pH and Al concentrations), values inside the bracket are the LSD to compare the main means For Treatment x Accessions the values inside the bracket are the LSD. For comparing between treatments LSD is 13.28 and to compare within treatment LSD is 12.97

Al toxicity reduced the ΔRL in all accessions compared to control. Other visible symptoms observed were the swollen, discolored, hard root tips, ruptures on root surface.

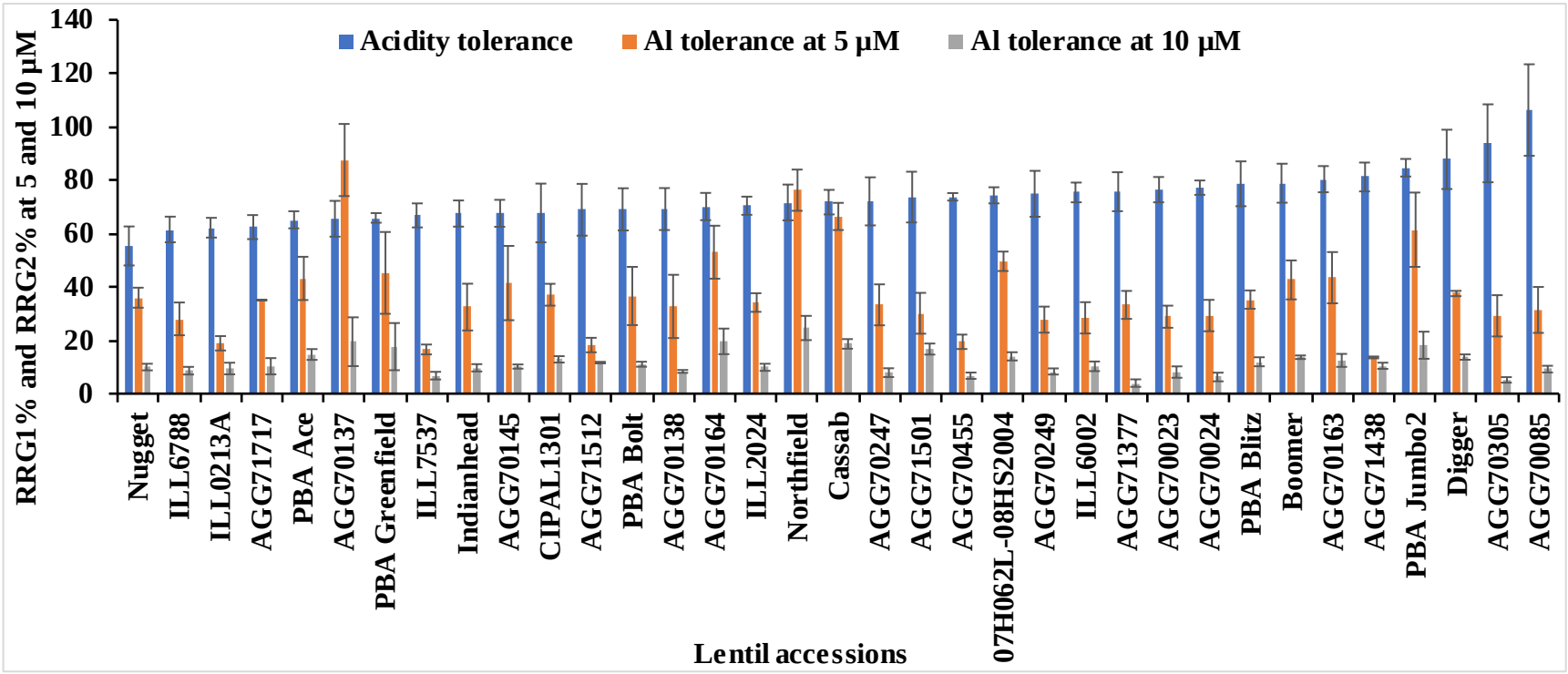

Figure 2.2. Mean acidity (RRG1%) and Al toxicity tolerance (RRG2%, at 5 and 10 µM Al) of lentil accessions in three-day hydroponic screening (Experiment 2).

Data points are mean ± SEM of three replications (n = 4)

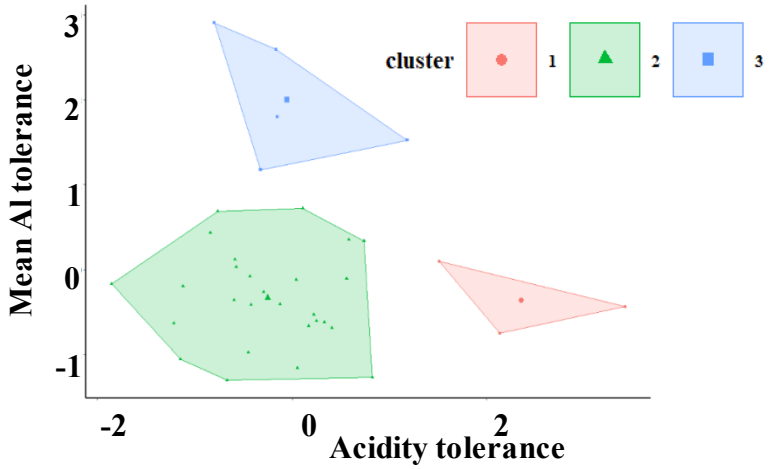

Figure 2.3. K means cluster analysis for acidity and mean Al tolerance for 35 lentil accessions from Experiment 2.

Mean Al tolerance is the Al tolerance at 5 and 10 µM Al after three-day Al treatment in hydroponic solution

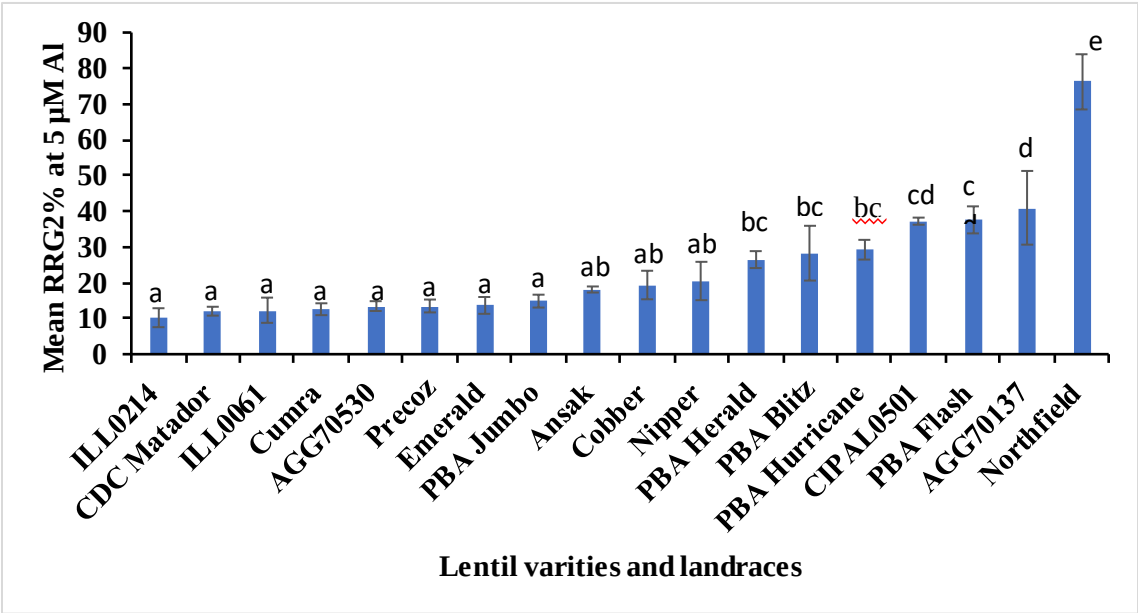

Figure 2.4. Mean Al toxicity tolerance (RRG2%, at 5 µM Al) of lentil varieties and landraces in three-day hydroponic screening (Experiment 3).

Data points are mean ± SEM of three replications (n = 3) and same letters above the bars are not significantly different at 5% level of significance

2.6 Al tolerance mechanisms in lentil - Root length

From the hydroponic experiments two tolerant (Northfield and AGG70137) and two sensitive (Precoz and AGG70530) accessions were tested at 5 and 10 µM Al for one, two and three days of treatment. Al treatment adversely affected the ΔRL of both the tolerant and sensitive accessions compared to the control 2. The ΔRL decreased linearly with an increase in both Al concentration and duration (Figure 2.5). There was no significant difference in ΔRL between accessions following 1 day of Al treatment, and there were no significant interactions between accessions and treatments (p = 0.61) indicating all four accessions have similar levels of reduction in ΔRL. However, following two days of 5 µM Al exposure, the average ΔRL of tolerant accessions was significantly higher (p = 0.006) (3.1 times) compared to sensitive accessions, this was further increased by 5.2 times after three days of treatment.

Following the exposure to 10 µM Al the difference in ΔRL between the contrasting accessions (tolerant and sensitive) was less compared to 5 µM. Following the exposure to 10 µM Al the tolerant accessions showed 2.1 and 2.9 times higher average ΔRL compared to sensitive accessions following 2 days and 3 days of treatment respectively. Accession AGG70530 showed to be the most sensitive to Al treatment having the lowest average ΔRL of 6.4 and 6.9 mm in 5 and 10 µM Al treatments respectively across all durations.

2.6.1 Al tolerance mechanisms in lentil – Haematoxylin staining

The Haematoxylin stain produced intense brown/purple colour in sensitive accessions compared to tolerant accessions in both Al treatments (Figure 2.6). Ruptures on root surface with hard root tips were observed more prominently in sensitive accessions. This was further supported by the quantification of stain released in terms of absorbance and fold increase which was linear with an increase in Al concentration and duration (Table 2.5, Supplementary Figure 2.3). There was significant (p = 0.024) interaction between accessions and Al treatment as shown in Precoz with

a 10.2 and 19.7 fold increase in Al accumulation (stain intensity) compared to the control in 5 and 10 µM Al treatment respectively after one day of Al exposure, which was higher than other accessions. After two days of treatment, a significant ($p < 0.001$) fold increase was observed in 10 µM compared to 5 µM across all accessions. After three days of treatment the fold increase was not significant ($p = 0.221$) for any of the accessions in any of the Al treatments. An average of 2-fold increase in stain (Al content) was observed in sensitive accessions at 5 µM Al following the one-day treatment, which increased to 2.5 after two days of treatment, however it was reduced to 2.2 following three days of treatment. A similar trend was also observed at 10 µM Al where there was a fold increase of 1.5, 1.4 and 1 for days one, two and three respectively (Table 2.5).

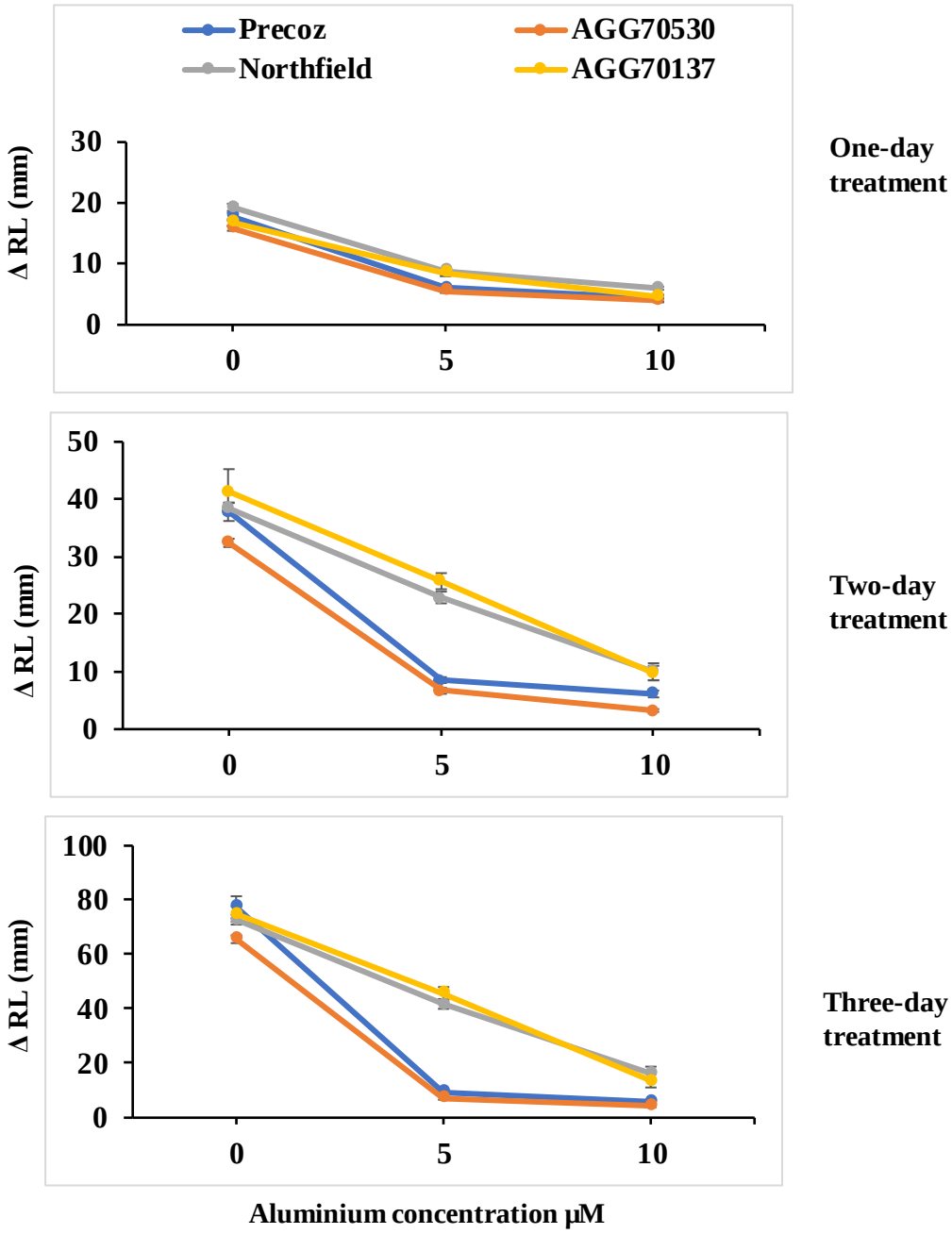

Figure 2.5. Change (Δ) in RL of Al tolerant and sensitive lentil accessions exposed to different Al concentrations for different durations.

Data points are mean ± SEM of two replications (n=6). Accessions - Al tolerant (Northfield and AGG70137) and Al sensitive (Precoz and AGG70530). The Al concentrations (0, 5 and 10 µM) and Al durations (one, two and three day).

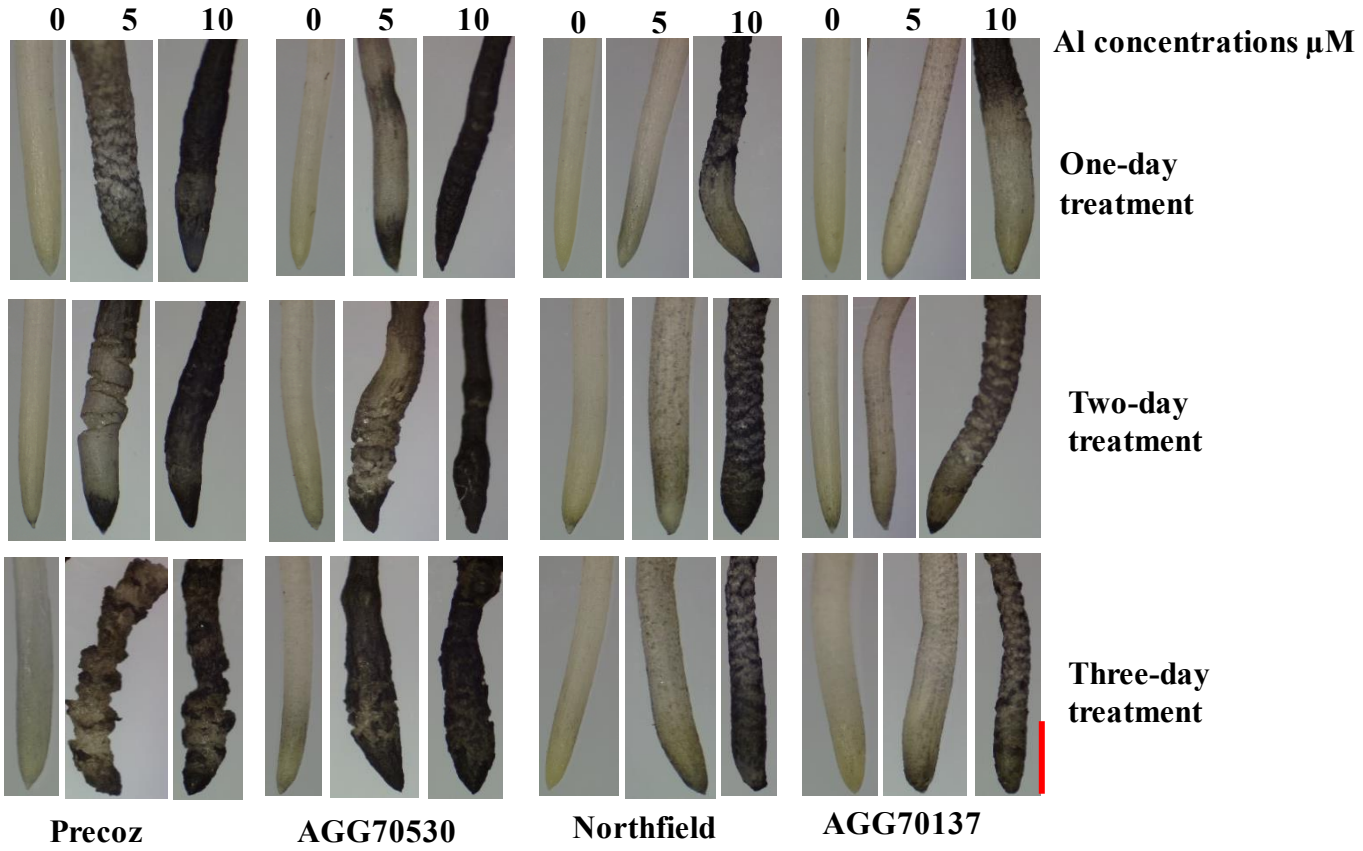

Figure 2.6. Haematoxylin staining of root tips for Al accumulation in tolerant and sensitive lentil accessions in control, 5 and 10 µM Al treatments during one, two and three day treatment.

The accessions – Al tolerant (Northfield and AGG70137) and sensitive (Precoz and AGG70530), Al concentrations - Control (pH4.5 + 0 Al) and Al treatments (pH4.5 + 5 or 10 µM Al), Duration - one, two- and three-day treatment. Red scale bar is 1mm

Table 2.5. Haematoxylin and Evans blue stain quantification as fold change in absorbance compared to control in tolerant and sensitive accessions exposed to different Al concentrations, after growth durations of 1, 2 and 3 days

Haematoxylin	**One day**		**Two day**	
Accessions	**5µM Al**	**10µM Al**	**5µM Al**	**10µM Al**
Northfield	3.417	10.583	2.2	13.4
AGG70137	4.5	11.5	4.324	12.552
AGG70530	5.667	12.933	7.867	15.467
Precoz	10.211	19.717	8.033	21.7
p value (LSD at 5%)				
Al treatment x Accessions	0.024 (3.3, 3.5)		< 0.001 (1.8, 1.9)	
Evans blue	**Two day**		**Three day**	
Accessions	**5µM Al**	**10µM Al**	**5µM Al**	**10µM Al**
Northfield	2.917	5.972	2.771	5.067
AGG70137	2.128	4.867	2.722	3.759
AGG70530	6.444	8.194	4.938	14.817
Precoz	5.233	6.217	9.65	7.607
p value (LSD at5%)				
Al treatment x Accessions	0.001 (1.4, 1.2)		< 0.001 (1.7, 1.8)	

Haematoxylin: Three-day treatment is not significant, Evans blue; One day treatment is not significant hence data nor presented.

Accessions – Al tolerant (Northfield and AGG70137) and sensitive (Precoz and AGG70530), Al concentrations – 0 (control), 5 and 10 µM, Durations – one, two and three day. For Al treatment x Accessions the values inside the bracket are the LSD. Fist value inside bracket is the between treatment LSD and second value inside bracket is within treatment LSD

2.6.2 Al tolerance mechanisms in lentil – Evans blue staining

Visual assessment of plasma membrane damage and its quantification by Evans blue stain increased with Al concentration and durations in all accessions, but damage was the highest in sensitive accessions (Figure 2.7 and Supplementary Figure 2.4). The fold increase after 1 day at either of the Al treatments was not significant ($P = 0.139$) for any accessions. The average fold increase of the stain (plasma membrane damage) in sensitive accessions was increased by 1.3, 2.3, and 2.6 during day 1, 2, and 3 respectively in 5 µM Al treatment. Similarly, in 10 µM Al the average fold increase was 0.72, 1.3 and 2.5 in 1, 2 and 3 day respectively in sensitive accessions compared to tolerant accessions. However, the fold increases were relatively less compared to 5 µM Al, due to lower differences between the contrasting lines (Table 2.5).

2.6.3 Al tolerance mechanisms in lentil – Root and shoot Al

Root and shoot Al content detected by ICP-OES, after 3 days of Al exposure. The main effects of Al treatment were significant for both root ($p = 0.047$) and shoot Al content($p < 0001$). and significantly higher root Al was observed compared to the shoots. There was significant interaction (root Al $p = 0.042$, shoot Al $p = 0.03$) between accessions and the Al treatment. A lower root Al content was observed in the 5 µM Al treatment in accessions AGG70530 (639 µg/g DW) and AGG70137 (865 µg/g DW) compared to Northfield and Precoz which showed an average Al content of 1055 µg/g DW. However, in the 10 µM treatment, Precoz was the only accession that showed a significantly higher root Al content (1410 µg/g DW) compared to the other sensitive accession (AGG70530;1249 µg/g DW). The average shoot Al content was less in sensitive lines (25.4 and 32.1 µg/g DW) compared to tolerant lines (41.7 and 62.0 µg/g DW) in 5 and 10 µM Al treatment respectively (Figure 2.8). The tolerant line Northfield had

significantly higher shoot Al content (74 µg/g DW) at 10 µM Al treatment compared to 5 µM Al treatment (45 µg/g DW). Accessions did not differ significantly in shoot and root dry weight (Data not shown).

2.6.4 Al tolerance mechanisms in lentil – organic acid release

The release of organic acids (malate, citrate and oxalate) were analysed for different Al treatments (0, 100 and 200 µM Al at pH 4.5) and time points (1, 3, 6, 24 and 48h). The release of oxalic acid (OA) was observed only in the tolerant accession AGG70137 after 1 h (35.6 mg/L with retention time 4.4) and 3 h (42.3 mg/L with retention time 4.2) of 100 µM Al treatment. As other tested accessions did not show any release of the organic acids; hence results were only used to support discussion of Al tolerance mechanism.

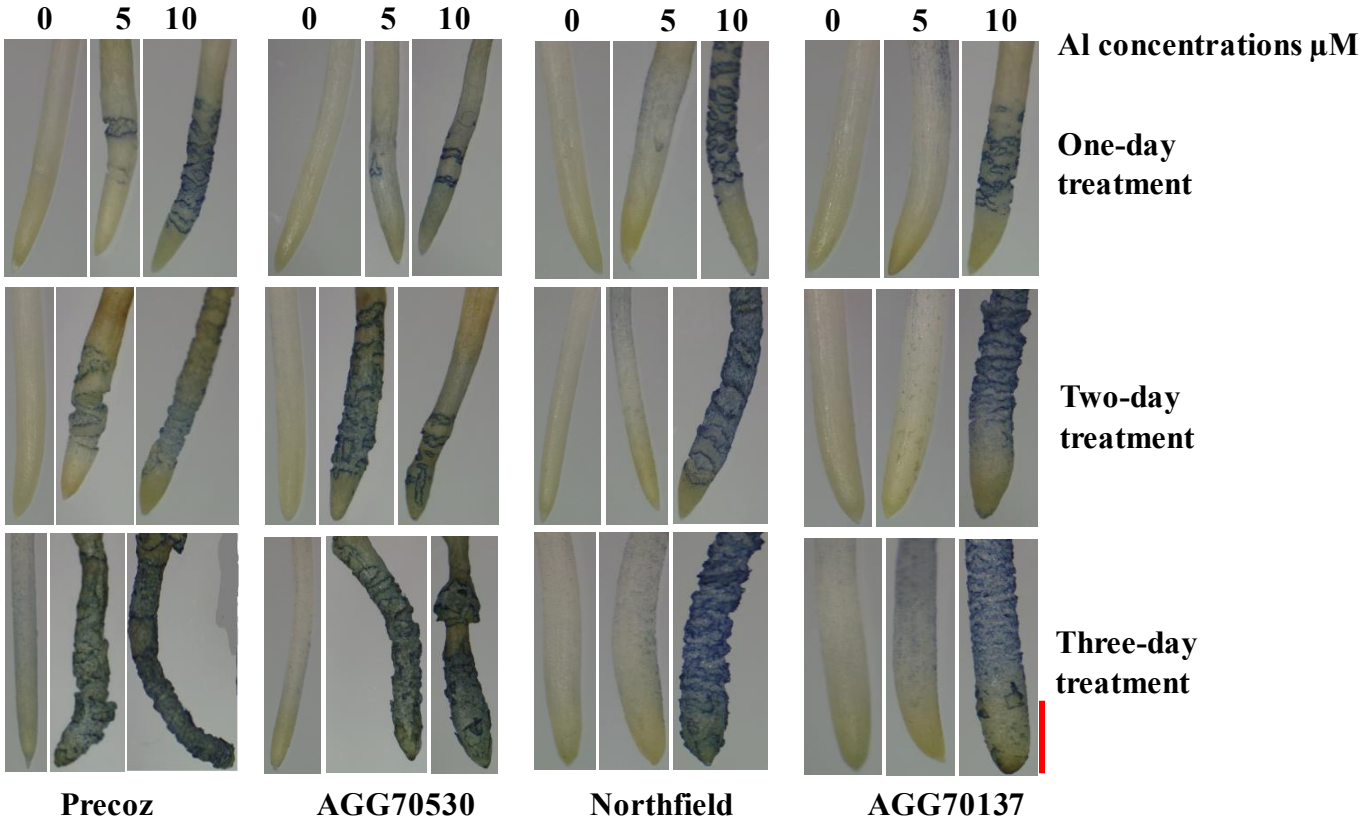

Figure 2.7. Evans blue staining to show plasma membrane damage in tolerant and sensitive lentil accessions in control, 5 and 10 µM Al treatments during one, two and three day treatment.

The accessions – Al tolerant (Northfield and AGG70137) and sensitive (Precoz and AGG70530), Al concentrations - Control (pH4.5 + 0 Al) and Al treatments (pH4.5 + 5 or 10 µM Al), Duration - one, two- and three-day treatment. Red scale bar is 1mm.

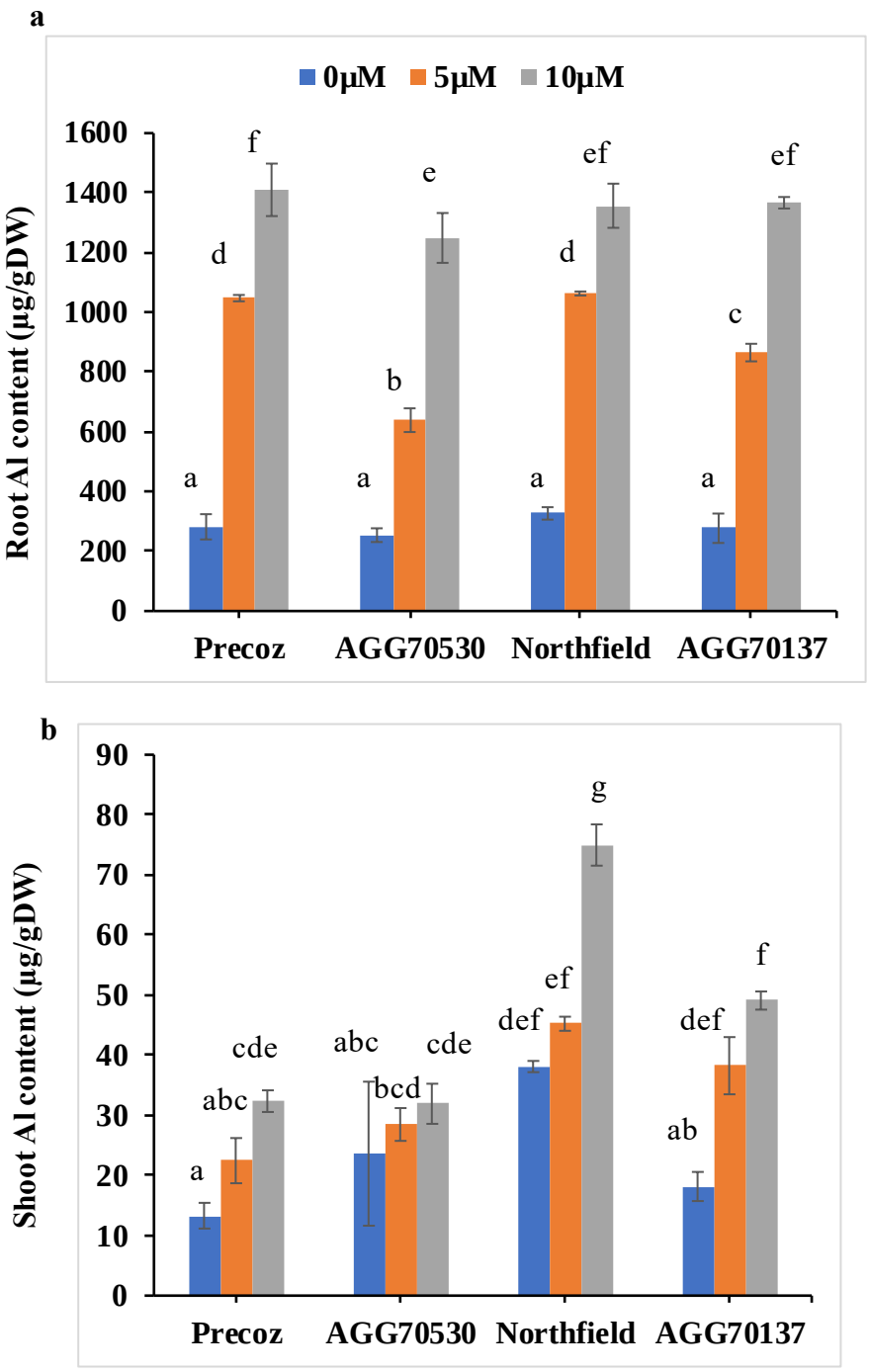

Figure 2.8. Al content in tolerant and sensitive accessions exposed to different Al concentrations after three-day treatment.

Data points are mean ± SEM of two replications (n=24) and same letters above the bars are not significantly different at 5% level of significance. The accessions – Al tolerant (Northfield and AGG70137) and sensitive (Precoz and AGG70530), Al concentrations (0, 5 and 10 µM), a- Root Al content, b- Shoot Al content.

2.7 Discussion

The high throughput screening method developed in this study consistently and reliably assessed Al toxicity tolerance at an early seedling stage of lentil. The use of low ionic strength nutrient solution has the advantage of increasing the activity coefficient of trivalent ions (Al^{3+}) compared to the full strength nutrient solution (Famoso et al., 2010). As the low ionic solution has lower concentrations of other cations, there is greater chance of Al accumulation on negatively charged sites within the root cell wall and root plasma membrane thus maintaining Al^{3+} activity to cause toxicity (Famoso et al., 2010). Hence all experiments were conducted in low ionic strength hydroponic solution with three days of Al treatment to ensure toxic Al^{3+} activity.

In the present study, RRG% and ΔRL were used as a measure of Al toxicity tolerance, where the RRG% of the genotype is relative root growth of ΔRL in Al solution compared to control without Al at pH 4.5. This method differs from an earlier lentil study (Singh et al., 2012), where the measure of root regrowth (length) under Al stress was used which is the combination of root vigor (long roots) and Al tolerance. This type of measurement failed to detect Al tolerance in genotypes with poor root vigor in rye (Hede et al., 2002). The RRG% used in our present study is a more reliable and reproducible phenotypic index as it can eliminate genotype-specific differences in root growth and normalize comparisons between genotypes (Baier et al., 1995) resulting in better separation of the genotypes for Al tolerance, as has been seen in maize (Xu et al., 2017). The most obvious Al toxicity symptoms include stunted root growth with reduced or absence of lateral roots, root tips becoming thick and brittle, and browning of the roots as reported in earlier studies (Mossor-Pietraszewska et al., 1997). They are the result from complex interactions of Al with apoplasmic (cell wall), plasma membrane, and symplasmic (cytosol) targets (Kochian et al., 2005).

The key observation from Experiment 1 was the linear decrease in the ΔRL with an increase in Al concentration during three days of Al treatment. A similar reduction in root elongation at different Al concentrations was reported in maize (Al tolerant), sorghum (Al sensitive) and soybean (intermediate Al tolerant) (Akhter et al., 2009). At high Al concentrations (10, 20 and 30 µM) root growth was totally inhibited causing no variation among the lentil accessions for Al tolerance. Similar observations were reported in sorghum (Akhter et al., 2009), that is also sensitive to Al, where highly toxic Al concentrations (20 µM) added to the solutions could not differentiated Al tolerance between cultivars. The increased Al toxicity tolerance (RRG2%) in AGG71377 and AGG71438 at low (2 µM) Al concentration was similar to observations in soybean where increased root elongation and activity of the root was reported at 10 ppm Al (Hui et al., 2011). Low concentrations of Al can stimulate root growth for either short term, as in wheat (Kinraide, 1993) or for longer term, as in silver birch (Kidd & Proctor, 2000) by prevention of H^+ toxicity and induction of root elongation. Hence for optimal Al concentrations there is a need to consider the level of tolerance of each crop species and potential genotypic variation in responses, and whether or not complete inhibition of root growth occurs (Akhter et al., 2009; Xu et al., 2017). In our study, the Al treatment at 5 µM (1.2 ppm) presented more variation (15.7 to 43.9%) among the accessions for Al toxicity tolerance (RRG%) and reliably discriminated tolerant and sensitive accessions. Generally, a soil Al concentration of 2 – 5 ppm is toxic to sensitive species (SoilQuality, 2013) and lentils are generally considered as sensitive to soil acidity (Helen Burnes, 2017) and Al toxicity (Singh et al., 2012). Based on the variation observed at 5 µM (1.2 ppm) treatment in the present study, it was selected as the optimal concentration for further screening experiments in lentil. This is in agreement with the consensus that low Al concentration treatments are recommended for Al tolerance studies in sensitive species (Akhter et al., 2009).

Further screening of a wider set of lentil landraces and varieties at 5 and 10 µM Al treatment showed the Al toxic effect in all the accessions by reducing the root growth, which further supported the result of Experiment 1, with 5 µM Al displaying the better variation of Al tolerance than the higher Al concentration (10 µM). Acidity and mean Al tolerance (5 and 10 µM Al) were not correlated in the accessions tested. This indicates that the mechanisms for acidity and Al toxicity tolerance are likely to be different in lentil and implies an independent nature of the two tolerance factors (acidity and Al). This was also shown in Arabidopsis, with proton resistance (acidity) and Al resistance being regulated by different genetic mechanisms (Ikka et al., 2007) and in faba bean where independent responses of acidity and Al have also been reported (Belachew & Stoddard, 2017). Studies in Yorkshire-fog grass and Silver Birch trees showed that accessions adapt to (acidity) H^+ and Al^{3+} toxicity as a result of differences in the nature of soil materials, whereby accessions from acidic organic soils were H^+ tolerant while those from acid mineral soils were Al^{3+} tolerant but not necessarily H^+ tolerant (Kidd & Proctor, 2001). Similarly, in this present study, adaptability to different toxicities might depend on the soil characteristics at their collection sites. The cluster 1 acidity (Figure 2.3) tolerant landraces are from Afghanistan (AGG70305) and Morocco (AGG70085), and Al tolerant cluster 3 landraces are from Lebanon (AGG70137), Jordan (Northfield) and Tunisia (AGG70164), where differences in genotypic origins and ecological zones might have contributed to their respective tolerance adaptability. However, varieties (acidity tolerant – Digger, Al tolerant – Cassab, PBA Jumbo2) from both clusters were well adapted to Western Australia (Garlinge, 2005) and some of the differences in the tolerance in these varieties could be attributed to pedigree of varieties, for example, PBA Jumbo2 has Northfield (IPAustralia, 2016) in its pedigree from which Al tolerance background might has contributed. This was also observed among the tested varieties in Experiment 3, where Australian PBA based varieties were more sensitive to Al toxicity

compared to Northfield and AGG70137. This might be partly due to the CDC Matador and Cumra pedigree in these Australian PBA based varieties (PBA jumbo and PBA Blitz) (PulseAustralia, 2016; Dadu, 2018) as, CDC Matador and Cumra are very sensitive to Al toxicity (~12% RRG%), as shown in our study.

2.7.1 Al tolerance mechanisms in lentil

Some plant species growing on acidic soils have developed tolerance mechanisms to overcome and mitigate toxic Al (Ma et al., 2001; Kochian et al., 2015). There is some controversy over whether Al-induced growth inhibition is attributable to cytosolic or extracytosolic injuries and whether the mechanisms of Al toxicity tolerance involve exclusion (Delhaize et al., 1993b) or internal detoxification (Ma et al., 1998). The mechanism for Al toxicity tolerance observed in the tolerant (AGG70137 and Northfield) and sensitive (AGG70530 and Precoz) accessions were tested by root stains (Hematoxylin and Evans blue), root and shoot Al content, and release of organic acids. Although all the accessions had root surface ruptures and hard root tips in Al treated roots compared to the control, these were more prominently observed in sensitive accessions in both Al treatments, indicating Al toxicity effects. Most of the accumulated Al in the roots bound to pectin constituents of the cell walls (Yang et al., 2008) and has been shown to modify cell wall composition and properties such as its extensibility (Jones et al., 2006; Ma et al., 2014). These factors may have contributed to the observed morphologic effects in the accessions tested in the present study. The ruptures observed in the root tip (10 mm) of sensitive accessions under the stereomicroscope were similar to those observed in pea (Yamamoto et al., 2001; Motoda et al., 2011; Motoda et al., 2010), cowpea (Kopittke et al., 2008) and maize roots (Jones et al., 2006). These transverse ruptures may be caused by the increase in root diameter and the tearing of the external cortex and rhizodermic cells of the elongation zone (Blarney et al., 2004; Kopittke et al., 2008; Motoda et al., 2010). According to these studies, root elongation

inhibition and rupturing is the result of Al linkage to cell wall components and increased lignin biosynthesis resulting in cell wall rigidity.

In the Haematoxylin staining process, oxidized Haematoxylin (hematein) binds to the constituents of the cell wherever there is accumulation of Al, which act as mordant, and results in the formation of coloured complexes. This method has been used in previous studies to evaluate Al tolerance (Al accumulation) in wheat (Delhaize et al., 1993a), sorghum (Anas & Yoshida, 2000), maize (Cançado et al., 1999; Xu et al., 2017) and lentil (Singh et al., 2016). Similar observations were made in the sensitive lentil accessions (Precoz and AGG70530) where more intense brown and blue stain was observed indicating greater Al accumulation and plasma membrane damage (Figure 2.6 and 2.7). The staining was concentrated in the meristematic and elongation regions (<10 mm from tip), as the stain intensified with Al concentrations and growth duration under treatment indicated their inability to protect the root surface at both Al treatments. However, the tolerant accessions at high (10 µM Al) concentration also showed some stain accumulation which was totally absent in 5 µM Al, which increased with duration, indicating prominent tolerance at 5µM, where more significant root growth difference was observed between contrasting accessions. These observations were supported by quantification of the stains and ΔRL measurement indicating that tolerant accessions might have the ability to exclude Al from the root tips and elongation region. These tolerant accessions (Northfield and AGG70137)did not absorb stain even after three days of Al (5 µM) treatment, as generally, Haematoxylin stain in root tips is inversely proportional to both the ability of the accessions to exclude Al from the root apex and its Al resistance/ tolerance (Polle et al., 1978). In contrast to staining qualitative and quantitative results, tolerant accessions accumulated the same amount of root Al as sensitive accessions when analysed in ICP-OES analysis mainly at 5 µM Al. This could be due to the differences in the regions considered for analysis, as for staining

we considered mainly the root tip up to 10 mm from tip, whereas for ICP-OES the whole root was considered, this suggests the root tip is the main tolerance region. Similar observation has been made in wheat sensitive genotypes which accumulated 8-fold more Al in root apex (2mm root tip) than Al tolerant wheat genotype, whereas no differences reported in more mature tissue (Rincón & Gonzales, 1992). The root apex is the critical site for Al toxicity and in this region Al tolerant gene are likely to express (Delhaize & Ryan, 1995). In this regard selective hematoxylin staining of Al-sensitive accessions is the result of direct damage by Al to root cells, leading to leakage of phosphorus (PO_4) into the cell wall region where accumulated Al will immobilize ($AlPO_4$) in the apoplast thus reacts with Haematoxylin stain (Ownby, 1993).

Despite the high root Al content, tolerant lentil accessions maintained significantly higher root growth compared to sensitive accessions at 5 µM Al, which indicates exclusion or detoxification of Al that was taken up into the root cells. Furthermore, lentil cultivar Northfield accumulated more shoot Al than other accessions at high (10 µM) Al concentration, indicating its tolerance by an internal detoxification mechanism which translocates Al to shoots after a certain amount of Al accumulation in the roots. This likely mechanism is supported by the fact that it did not release any organic acid in the organic acid exclusion test (data not shown). Rice has been reported to have a similar type of Al uptake with internal detoxification in the variety Modan, where root Al storage capacity was saturated after 24 hour Al exposure, with Al accumulation observed in the shoots after 48 h exposure (Roselló et al., 2015).

The tolerant AGG70137 lentil accession may exhibit a different tolerance mechanism compared to cultivar Northfield through avoidance of Al uptake into roots as it showed efficient restriction of Al transport to shoots at 10 µM Al (Figure 2.6). Furthermore, in the organic acid exclusion test (data not shown), OA was released after 1 hour (35.6 mg/L with retention time 4.4) and 3

hour (42.3 mg/L with retention time 4.2) of Al treatment with this delay in exudation of organic acids suggesting a pattern II type of exclusion. This type of tolerance was reported in earlier *Lens* species (Singh et al., 2016), in which citrate and malate peaks were reported at 3 h of Al treatment with a lag between Al treatment and OA release. In the present study we observed OA, which forms a high stability complex with Al, that is usually reported in tolerant species, like buckwheat (Zheng et al., 1998a, 1998b). Lentil is a sensitive species with a tolerance level generally similar to the sensitive wheat variety (ES8), hence the observed oxalic acid is a surprising result in the AGG70137 tolerant accession. There are also other instances that do not support the hypothesis that organic acids efflux enhances Al resistance of plants (Parker & Pedler, 1998; Ishikawa et al., 2000; Wenzl et al., 2001). Citrate efflux did not explain the difference in Al resistance in some maize cultivars (Piñeros et al., 2005). Recent findings show that oxalate efflux plays only a minor role in the high Al tolerance of buckwheat as no correlation was observed between Al tolerance and oxalate efflux in seven cultivars that were tested (Zheng et al., 2004). Therefore, the role of organic acid secretion in Al toxicity resistance should not be overemphasized, as alternative mechanisms may play an equal or even more important role in some plants. Hence AGG70137 might have other tolerance (internal detoxification) mechanisms along with an exclusion type of mechanism, however this needs further experimental support.

There is great genetic variation, both among and between plant species for Al resistance, suggesting that Al resistant species, cultivars or lines possess several mechanisms for detoxifying Al. It was also suggested in maize that, although organic acid release is the main tolerance mechanism, the internal detoxification which allows the root tip to cope with the ongoing Al accumulation, is also likely to be present (Piñeros et al., 2002; Piñeros et al., 2005; Giannakoula et al., 2008). In this study tolerant cultivar Northfield might have internal

detoxification, while exclusion could be the suggested type of tolerance mechanism in lentil accessions AGG70137. The AGG70530 and Precoz accumulated Al mainly in roots and showed reduced root growth indicating sensitivity. Further investigation in these contrasting accessions is required to define clear tolerance mechanisms that exist in lentil.

2.8 Conclusion

A robust, high throughput hydroponic method for screening lentil seedlings using an optimal Al concentration (5 µM) and a three-day treatment for Al toxicity tolerance has been developed. The RRG% has been determined as the best measure for an Al toxicity tolerance index. Acidic pH reduced the ΔRL in all lentil accessions and it was further reduced by Al treatment. The elite varieties Cassab and PBA Jumbo2 showed high Al toxicity tolerance, with little variation reported between other PBA based varieties. The identified tolerant Northfield and AGG70137 accessions exhibit potential internal detoxication and exclusion type of tolerance respectively, however further work is needed to understand the tolerance mechanism in these accessions.

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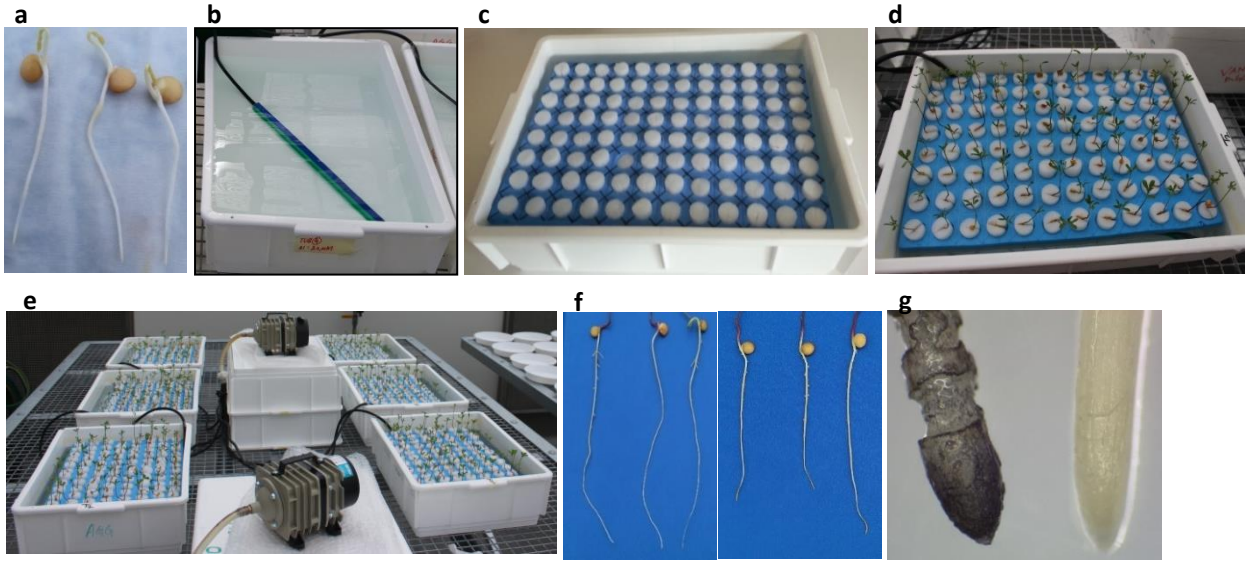
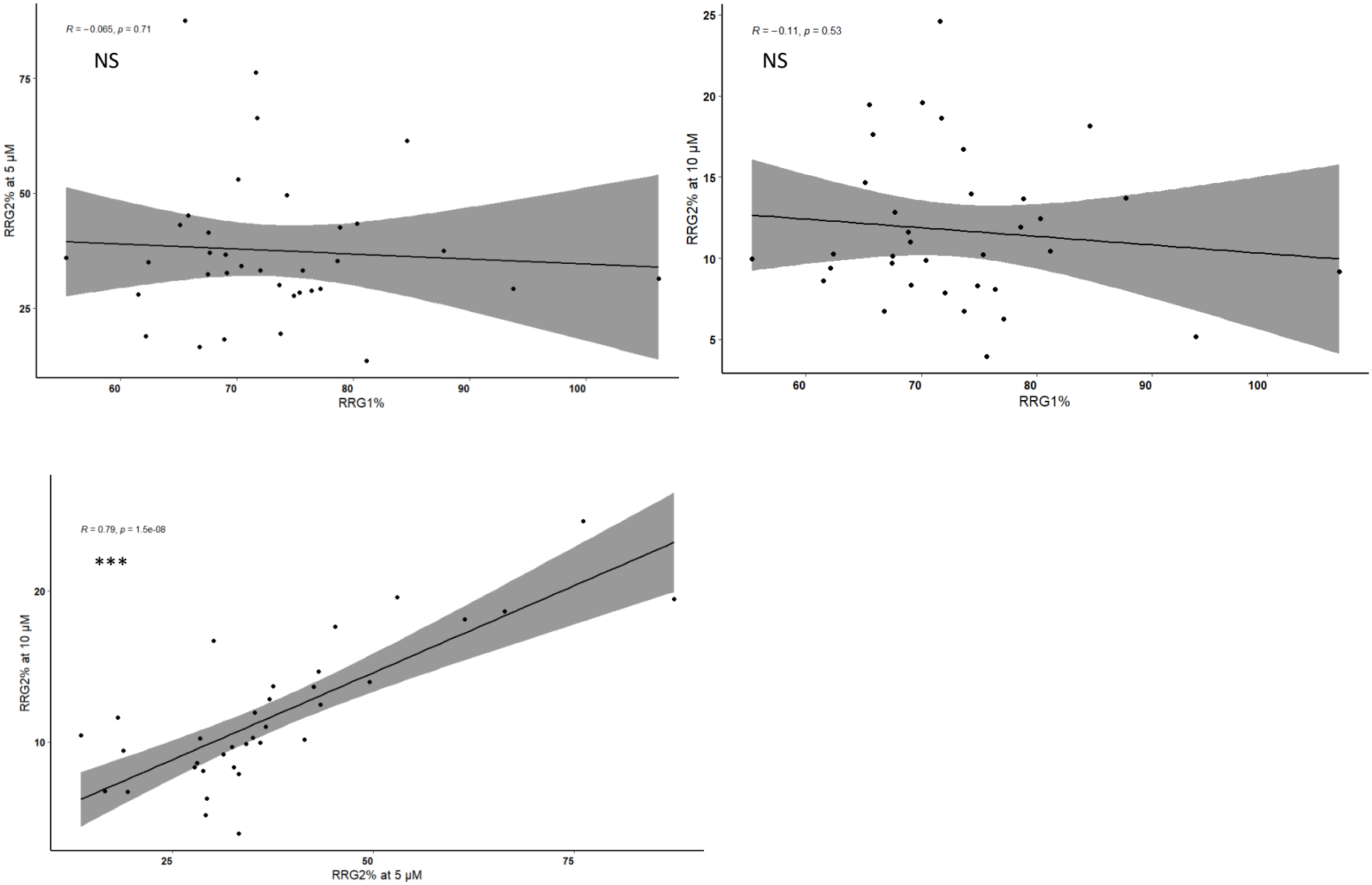
2.10 Supplementary data

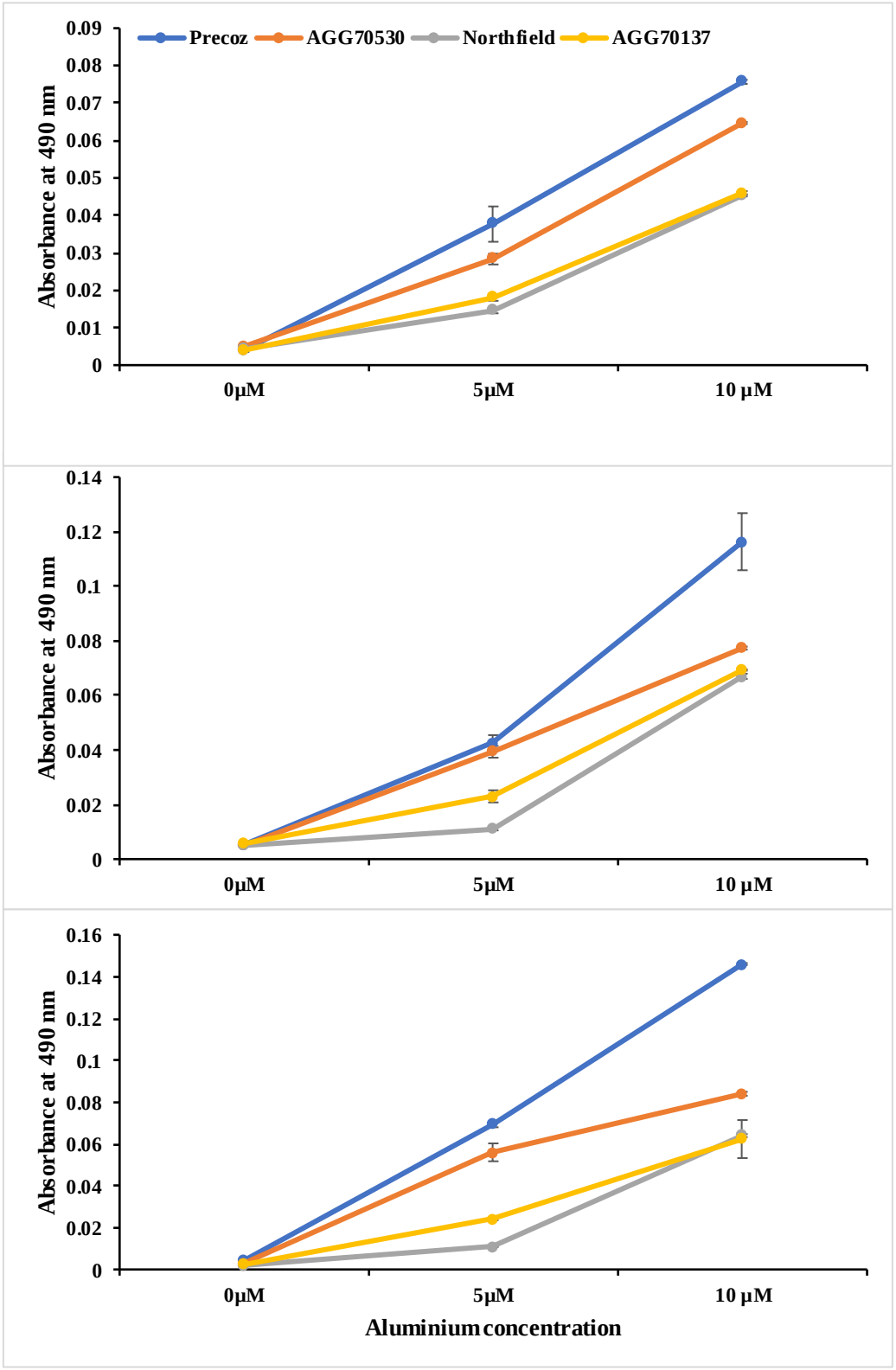


Supplementary Figure 2.1. Established high throughput hydroponic screening setup for Al toxicity tolerance screening in early stage of lentil accessions.

a- Four day old seedlings; b- 13L tote box with 14 inch air stone; c- Polyethylene floating foam with backer rod material; d- Floating foam holding 96 seedlings; e- Arrangement of tote boxes in three replications and two treatments in a split plot experimental design; f- Measuring post treatment root length in control and Al treatment; g- Stereo microscopic observation after Haematoxylin staining

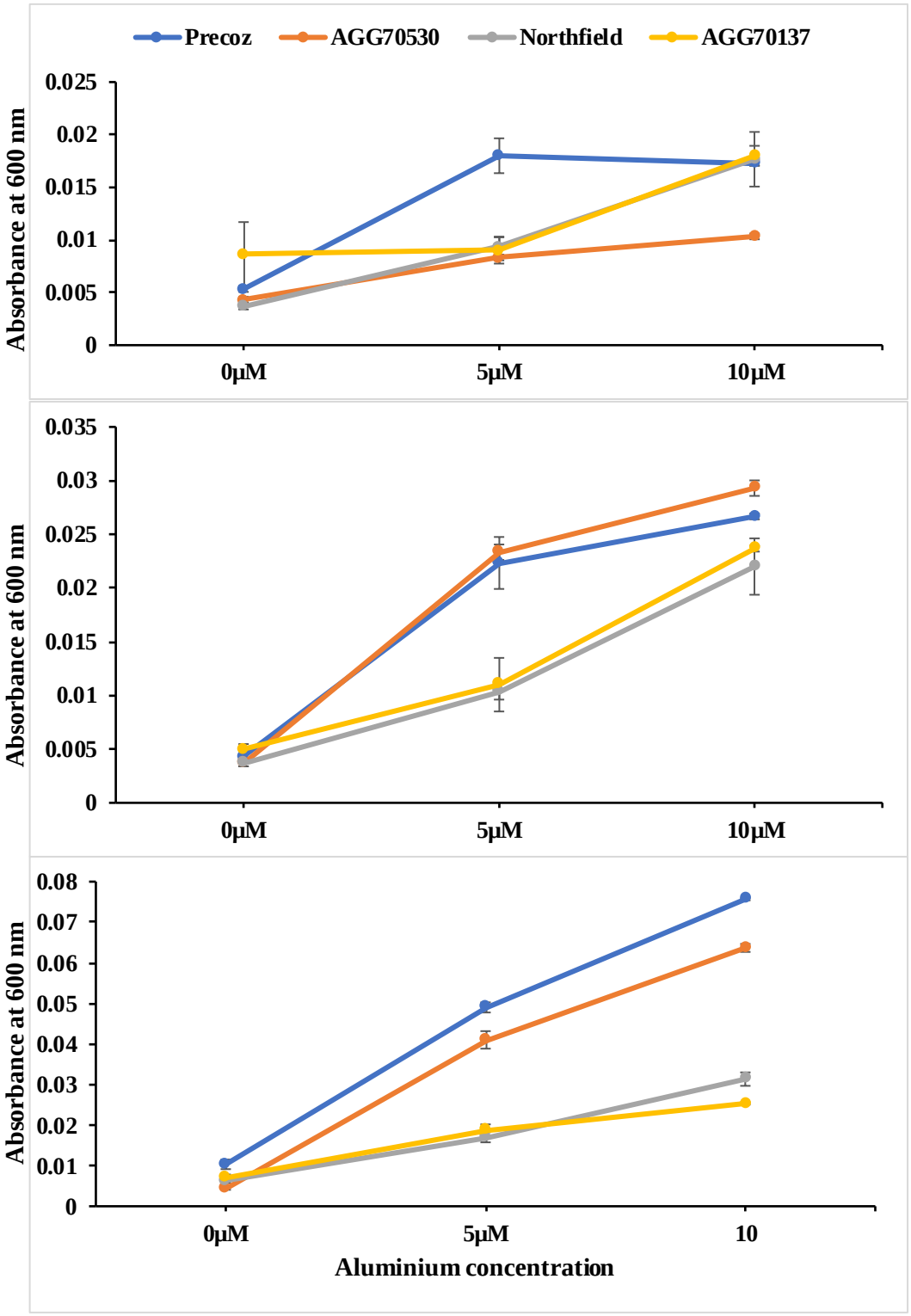


Supplementary Figure 2.2. Correlation between acidity (RRG1%) and Al toxicity tolerance (RRG2% at 5 and 10 µM Al) after three-day treatment (Experiment 2).



Supplementary Figure 2.3. Al uptake assessed by Haematoxylin stain in 5mm root tip of Al tolerant and sensitive accessions exposed to different Al concentrations for different durations.

Data points are mean ± SEM of three replications (n=9), Accessions – Al tolerant (Northfield and AGG70137), Al sensitive (Precoz and AGG70530), Al concentration – control (0), 5 and 10 µM, Treatment duration - one, two and three day.



Supplementary Figure 2.4. Al damage assessed by Evans blue stain in 5mm root tip of Al tolerant and sensitive accessions exposed to different Al concentrations for different durations.

Data points are mean ± SEM of three replications (n=9), Accessions – Al tolerant (Northfield and AGG70137), Al sensitive (Precoz and AGG70530), Al concentration – control (0), 5 and 10 µM, Treatment duration - one, two and three day.

Supplementary Table 2.1. ICP-OES digestion process at different temperature

Temperatures ˚C	Time min	Process
80	30	To subside the frothing in the digestion mixture
150	60	To reflux the sample mixture
185	120	To reduce the digest volume to 2 ml and samples were held at this temperature until white fumes of perchloric acid are seen in the test tubes

Supplementary Table 2.2. Mean change in root length (Δ RL) and relative root growth (RRG2%) in lentil genotypes in acidic control and 5 µM Al during three day of treatment (Experiment3)

Accessions	Mean ΔRL in control	Mean ΔRL in Al treatment	Mean RRG2%
AGG70137	62.11	25.67	40.96
AGG70530	50.89	6.78	13.46
Ansak	71.22	13	18.1
CDC Matador	65.67	7.89	12.03
CIPAL0501	53.22	19.72	37.22
Cobber	70.89	13.44	19.35
Cumra	53.33	6.78	12.58
Emerald	76.11	10.44	13.65
ILL0061	66.56	8	12.3
ILL0214	62.89	6.33	10.21
Nipper	61.22	13.11	20.48
Northfield	85.47	65.33	76.2
PBA Blitz	69.44	19.67	28.26
PBA Flash	66.22	25.44	37.58
PBA Herald	66.22	17.56	26.47
PBA Hurricane	61.22	18	29.23
PBA Jumbo	62.33	9.33	14.85
Precoz	65.56	8.78	13.49
p value (LSD at 5%)			
Aluminium treatment X Accessions	< 0.001 (11.5, 11.6)		<0.001 (11.5)

For Al treatment x Accessions the values inside the bracket are the LSD. To compare between Al treatments the LSD is 11.5 and within Al treatment LSD is 11.6. For mean RRG2% column, there is only within treatment LSD (11.5).

CHAPTER 3: New sources of lentil germplasm for Aluminium toxicity tolerance identified by high throughput hydroponic screening

3.1 Chapter preface

This Chapter uses the optimised Aluminium (Al) treatment and established high throughput hydroponics screening method for Al toxicity tolerance screening from the Chapter 2 to screen a set of putative acid tolerant lentil accessions for Al toxicity tolerance. Further hydroponics results were validated in subset of accessions by acid soil screening, histochemical and biochemical analyses. This work identified Al tolerant accessions that are more tolerant than the known lentil line. Accessions from this Chapter were used for the genotyping and marker trait association study in Chapter 4.

3.2 Publication details

Title: **New sources of lentil germplasm for aluminium toxicity tolerance identified by high throughput hydroponic screening.**

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Author contribution

All the authors of this manuscript contributed to the study concept and design. Material preparation, data collection and analysis were performed by Vani Kulkarni. The first draft of the manuscript was written by Vani Kulkarni and all the co-authors, Tim Sawbridge, Sukhjiwan Kaur, Matthew Hayden, Anthony T. Slater and Sally Norton provided their comments on the previous versions of this manuscript. All the authors read and approved the final version of the manuscript.

Statement of authorship

Statement from co-author confirming the contribution of the PhD candidate:

"As co-author of the manuscript 'New sources of lentil germplasm for aluminium toxicity tolerance identified by high throughput hydroponic screening**.** Vani Kulkarni[1, 2], Tim Sawbridge[2, 3], Sukhjiwan Kaur[3], Matthew Hayden[2, 3], Anthony T. Slater[3], Sally L Norton[1]', I confirm that Vani Kulkarni has made the contributions listed above."

Dr Tim Sawbridge 16/11/2020

Abbreviations: AGG, Australian grains genebank; Al, aluminium; ANOVA, analysis of variance; Ca, calcium; CEC, cation exchange capacity; ΔRL, change in root length; DI, deionised; EC, electrical conductivity; Fe, iron; FIGS, Focused Identification of Germplasm Strategy; FRW, fresh root weights; HCl, hydrochloric acid; LR, number of lateral roots; Mg, magnesium; Mn, manganese; MDA, malondialdehyde; NaOCl, sodium hypochlorite; OD, optical density; P, phosphorous; RL, root length; RRG, relative root growth; SDS, sodium dodecyl sulphate; TBA, thiobarbituric acid; TCA, trichloroacetic acid;

3.3 Manuscript

Abstract

Aluminium (Al) toxicity in acid soils inhibits root elongation and development causing reduced water and nutrient uptake by the root system, which ultimately decreases crop yield. This study established a high throughput hydroponics screening method and identified Al toxicity tolerant accessions from a set of putative acid tolerant lentil accessions. Four-day old lentil seedlings were screened at 5 µM Al (pH 4.5) for three days in hydroponics. Measured pre and post treatment root length was used to calculate the change in root length (ΔRL) and relative root growth (RRG%). A subset of 15 accessions were selected from previous screen of 111 accessions for acid soil Al screening, and histochemical and biochemical analyses based on their differing responses to Al challenge. Al treatment significantly reduced the ΔRL with an average of 32.3% reduction observed compared to the control. Approximately 1/4 of the FIGS accessions showed higher RRG% than the known tolerant line ILL6002 (37.9%). Very tolerant (VT) accessions with RRG% of > 52% were observed in 5.4% of the total accessions. A selection index calculated based on all root traits in acid soil screening was highest in AGG70137 (636.7) whereas it was lowest in Precoz (76.3). All histochemical and biochemical analyses supported the hydroponic results as Northfield, AGG70137, AGG70561 and AGG70281 showed consistent good performance. The identified new sources of Al tolerant lentil germplasm can be used to breed new Al

toxicity tolerant lentil varieties. The established high throughput hydroponic method can be routinely used for screening lentil breeding populations for Al toxicity tolerance.

Keywords Focused Identification of Germplasm Strategy . relative root growth . histochemical analysis . Haematoxylin . low pH

1. Introduction

Cultivated lentil (*L. culinaris ssp. culinaris*) is a diploid ($2n = 2x = 14$) annual cool season crop. It is cultivated globally over 6.58 Mha with a production of 7.59 Mt (FAOSTAT, 2017). Lentil is considered as a high value pulse crop in Australia with approximately 95% of the total production exported to the Middle East and South Asia (PulseAustralia, 2015). The estimated annual production of lentil in Australia is 485 Kt, which production primarily from Victoria (200 Kt) and South Australia (250 Kt) (ABARES, 2018). Lentil is highly sensitive to low pH soils hence cultivation is mostly restricted to cropping regions with higher pH (> 5.0) and low aluminium (Al) content soils (Ryan, 2018). Half of the agricultural soils in Australia (~50 Mha) are affected by surface soil acidity with pH < 5.5 and around 12 - 24 Mha of soils are more problematic with pH < 4.8 (AACM-International, 1995; NLWRA, 2001).

Acid soils are characterized by a deficiency of major nutrients and toxicity of metals, such as manganese (Mn), iron (Fe) and Al; with toxicity of Al being the main limiting factor for plant growth in acid soils (Kochian et al., 2004; Gupta et al., 2013; Bojórquez-Quintal et al., 2017). Aluminium solubilises at low pH (≤ 5.0) to release phytotoxic, monomeric Al^{3+} from non-phytotoxic oxide and aluminosilicate forms. Phytotoxic Al^{3+} is absorbed easily by plant roots and primarily inhibits root elongation even at micromolar concentrations within a few minutes of exposure (Kochian, 1995; Kochian et al., 2005; Matsumoto & Sivaguru, 2008), with subsequent effects on plant development (Kochian, 1995; Ryan et al., 2001; Panda et al., 2009; Silva et al., 2012; Schmitt et al., 2016). A secondary effect is the induction of

nutrient deficiency of phosphorous (P), magnesium (Mg), and calcium (Ca) in shoots (Foy et al., 1978; Singh et al., 2012).

Lime application is primarily used to manage soil acidity, whilst the Al^{3+} toxicity can be reduced by the addition of P-containing fertilizers that increase bioavailable P in the soil (Liao et al., 2006; Atemkeng et al., 2011). However, as liming is not effective for subsoil acidity (Hede et al., 2001; Brown et al., 2008; Zheng, 2010), and is less effective in sensitive crops (Sun et al., 2008), the development of acid soil Al toxicity tolerant crops is the most efficient long term solution.

The Focused Identification of Germplasm Strategy (FIGS) is an approach for selecting accessions from genebanks with targeted traits and genes (Mackay et al., 2004; Bari et al., 2012). It works on the premise that the expression of specific adaptive traits is determined by the selection pressures of the environment in which the population is grown. In practice, it uses environmental variables, such as climatic and soil data, to filter germplasm collections sites for probable locations having the selection pressure for a given trait (Bouhssini et al., 2009). FIGS has identified resistant wheat germplasm for biotic stress traits such as powdery mildew, sun pest, Russian wheat aphid and stem rust (Kaur et al., 2008; Bouhssini et al., 2009; Endresen et al., 2011; Bari et al., 2012). The lentil acid tolerant FIGS set was developed by considering georeferenced accessions which were further filtered by using the collection site information and environmental data related to acid soil, such as detailed physical and chemical properties of top and subsoil (Street et al., 2016). This set was used in the present study for Al toxicity tolerance screening in hydroponics.

Hydroponic screens are rapid, simple and convenient at the seedling stage, and generally provide similar rankings of germplasm performance compared to short term soil screening (Camargo, 1981), although some exceptions occur where a medium correlation ($r = 0.5$) between screening methods have been noted (Moroni et al., 2010; Aguilera et al., 2016).

Hydroponic screening can detect the effects of toxic Al^{3+} in isolation of other stresses which is not possible in soil based pot or field evaluation because of the complexity of multiple other environmental stresses (Spehar & Copati Souza, 2006), as well as temporal and spatial variations in soil acidity and Al toxicity. Germplasm accessions have been successfully evaluated for Al toxicity tolerance in several cereal and legume crops using hydroponic screening (Sledge et al., 2005; Voss et al., 2006; Singh et al., 2012; Belachew & Stoddard, 2017) with the tolerance measured by the inhibitory effects of toxic Al on the root growth. Histochemical analysis of Haematoxylin and Evans blue stained roots are generally used to support hydroponic screenings and visualise variation in Al uptake and damage to the root plasma membrane (Singh et al., 2016; Awasthi et al., 2017). The Haematoxylin technique is a potentially reliable index for Al toxicity tolerance and has been successfully used in other crops (Ruiz-Torres & Carver, 1992; Cançado et al., 1999). Lipid peroxidation is oxidative stress caused by Al toxicity, which affects membrane fluidity, limits ionic transport capacity by protein degradation and triggers cellular death. It is measured by malondialdehyde (MDA) content, where low levels of MDA indicates increased activity of the antioxidant system thus is correlated to Al toxicity tolerance (Giannakoula et al., 2008; Awasthi et al., 2017) in tested lines.

Our present study established the high throughput hydroponics screening method for lentil. This method was used to evaluate Al toxicity tolerance in a set of putative acid tolerant lentil FIGS accessions to identify novel tolerance for use in the development of new varieties. The hydroponic results were further supported by acid soil screening, histochemical and biochemical analysis in the selected accessions.

2. Material and Methods

2.1 Plant material

In this study a geographically diverse set of 111 accessions including landraces, advanced cultivars and breeding lines were used (Figure 3.1). Among these accessions 98 were

putative acid tolerant FIGS accessions and 13 were non-FIGS accessions selected based on the seed availability, which were sourced from the Australian Grains Genebank (AGG) and the Agriculture Victoria lentil breeding programme at Horsham. A subset of 15 accessions from the hydroponics screen across different relative root growth (RRG%) tolerance classes were selected for acid soil screening, histochemical and biochemical experiments (Table 3.1). Details of the accessions along with the country of origin and level of improvement (IPGRI, 2015) is given in the Table 1 in Online Resource 1.

2.2 High throughput hydroponic screening method

A hydroponic system was established to screen large numbers of accessions at an early stage of seedling development. For each accession, 40-50 seeds of uniform size and colour were disinfected with 1% sodium hypochlorite (NaOCl) (w/v) for 5 min and rinsed with deionised (DI) water three to four times. Depending on seed size, an average of 10 -12 seeds were rolled in wet paper towel and incubated at 20-22 °C in darkness for four days. Seedlings with uniform root length were then transferred to 13 L capacity plastic tubs (L 432mm x W 320mm x H 127mm), containing 10 L low ionic strength nutrient solution with polyethylene floating foam (96 holes per box), with each seedling stabilised in the hole using polyethylene backer rod foam material. The solution was continuously aerated through a 35.5 cm long air stone connected to a 50W air compressor. All the experiments were designed in split plot with three replications and two treatments. The treatments were assigned to the plastic tubs as the main plots and accessions as subplots, both were randomized in each experiment (Figure 1 in Online Resource 2). The experiment was conducted in a controlled environment under natural light with 24/15°C day/night temperature. In total, 111 accessions were screened in four different sets (set 1-4 with 21 or 32 accessions) with a known tolerant check line, ILL6002 (Singh et al., 2012) in each experiment. The accessions Northfield, AGG70137 and AGG70530 were used repeatedly in different sets. Three or four uniform, four-day old seedlings per accession were grown for each of the treatments in each

121 replication and were used to measure pre and post treatment root length (RL). Main root
122 length was measured with ruler (in mm).

123 Nutrient solution compositions were adapted from published Al toxicity tolerance studies of
124 wheat, barley (Delhaize et al., 2004), and white clover (Rossello, 2011) and modified for
125 lentil by reducing the concentration of phosphate as high phosphate concentration can reduce
126 Al activity. The hydroponic solution used in our study contained; 500 µM KNO_3, 500 µM
127 $CaCl_2$, 500 µM NH_4NO_3, 150 µM $MgSO_4.7H_2O$, 2 µM KH_2PO_4, 2 µM $FeCl_3.6H_2O$, 11 µM
128 H_3BO_3, 2 µM $MnCl_2.4H_2O$, 0.35 µM $ZnSO_4.7H_2O$, 0.20 µM $CuSO_4.5H_2O$ and 0.33 µM
129 $Na_2MoO_4.2H_2O$. The pH and Al concentration of the three-day treatment were adjusted to
130 4.5 pH without Al (control) and 4.5 pH with 5 µM Al (Al treatment). An earlier pilot study
131 using a range of Al concentrations (2, 5, 10, 20, and 30 µM) identified 5 µM Al as an optimal
132 concentration for screening lentils, as it presented variation for Al tolerance among the
133 accessions tested and also discriminated tolerant and sensitive accessions (Figure 2 in Online
134 Resource 2). Nutrient solution components were tested using the Geochem-EZ speciation
135 programme (Shaff et al., 2009) to check the availability of free Al^{3+} activity which was 1.86
136 µM. The Al was supplied as $AlCl_3.6H_2O$ and acidity of the solution was adjusted daily with
137 0.1M HCl.

138 2.3 Soil screening experiment
139 An acidic sandy loam soil was collected from the Grahms Dip field (36.970544S,
140 142.604386E, pasture) which is located 50 km south of Horsham, Victoria. The soil was
141 tested for chemical and physical properties by sending the representative soil samples to
142 commercial lab (CSBP limited, Bibra, WA, Australia). The soil had pH_{ca} of 4.5, electrical
143 conductivity (EC) of 0.09 ds/m, organic carbon of 1.6%, P by Colwell method of 15 mg/kg,
144 $CaCl_2$-extractable Al of 2.8 mg/kg and exchangeable Al as % of CEC (% Al_{ex}) of 8.9%.
145 Based on the EC and % Al_{ex}, the acid soil Al concentration was considered as critical level

for highly sensitive species such as lentils (Upjohn et al., 2005). Based on the initial tests, soil pH was increased to 6.0 by applying lime ($CaCO_3$) at the rate of 4.70 g/1kg of soil and incubated at 22 °C for 25 days and considered as control. For the incubation soil was wetted by spraying a small quantity of water (~10 ML). Limed soil had the pH 6.0 before potting and there were no major changes in the tested chemical properties except reduced Al concentration (0.9 % Al_{ex}). The lime treatment as main plot and accessions as subplot were arranged in split-plot design with five replications. Five seeds of each of the subset of 15 accessions were sown in each treatment for each replication in a pot (Width 55 mm, Height 12.5 mm and Volume 0.26 L) containing 360 g of soil and thinned to four seedlings after three to four days of germination. The plants were grown at constant 18°C, under 14 h daylength with measured light intensity of 643 µm/m^2/s in growth chamber (Bio Chambers, Canada). Pots were watered to 70% field capacity by weighing the pots every day. Plants were harvested 10 days after germination. The intact roots were washed to remove soil and observations on root length (RL), number of lateral roots (LR) and fresh root weights (FRW) were recorded. Root length was measured with ruler in mm and was the main root length.

2.4 Histochemical analysis for Al uptake and plasma membrane integrity

The roots of intact seedlings of subset of 15 accessions from the hydroponic experiment (control and Al treatment) were washed in DI water for 15 min and stained with 0.2 % aqueous Haematoxylin solution containing 0.02 % potassium iodide for 15 min at room temperature. After washing stained roots with DI water, 5 mm root tips from 10 plants per accession were excised and soaked in 200 µl of 1 M HCl for 1 h. The optical density (OD) of the released stain was measured at 490 nm using a spectrophotometer (Shimadzu UV-1800). The amount of Haematoxylin stain accumulated is directly proportional to the amount of Al uptake in the root tips. Al concentration was determined by comparing treated and control OD values (Sharma et al., 2015; Sharma et al., 2016; Awasthi et al., 2017).

171 The localization of the loss of plasma membrane integrity was detected by Evans blue
172 (0.025%, w/v) staining. Four intact seedlings per accession from hydroponics were
173 suspended in 10 ml of Evans blue solution for 15 min. The stained roots were then washed
174 three times with 200 ml of 100 µM $CaCl_2$ (pH 5.6), after which dye was no longer eluted
175 from the roots (Yamamoto et al., 2001). The washed 5 mm root tips were excised and
176 homogenized with 1ml of aqueous 1% (w/v) sodium dodecyl sulphate (SDS) at room
177 temperature. The homogenate was then centrifuged at 13,500 rpm for 10 min, and the OD
178 of the supernatant was measured at 600 nm using a spectrophotometer (Shimadzu UV-1800)
179 (Awasthi et al., 2017).

180 2.5 Biochemical analysis of lipid peroxidation

181 Lipid peroxidation was expressed as malondialdehyde (MDA) content and its concentration
182 in roots was estimated after reaction with thiobarbituric acid (TBA) (Heath & Packer, 1968).
183 Fresh root tissue (0.1 g) was homogenized in 2 ml of 1% (w/v) trichloroacetic acid (TCA)
184 and centrifuged (Eppendorf) at 12,000 g for 10 min. The collected 0.5 ml supernatant was
185 added to 1.5 ml solution of 0.5% TBA (w/v) in 20% TCA (w/v) and incubated in a water
186 bath at 95°C for 30 min. The reaction was stopped by cooling on ice and centrifuging again
187 at 10000 g for 10 min. The absorbance of the supernatant was determined in a
188 spectrophotometer (Shimadzu UV-1800) at 530 and 600 nm. Non-specific absorbance
189 measured at 600 nm was subtracted from 530 nm and extinction coefficient of 155/mm/cm
190 was used to determine MDA content and expressed µMol/g of fresh weight (Ribeiro et al.,
191 2012).

192 2.6 Statistical analysis

193 For hydroponic screening, the mean change in root length (Δ RL) was calculated by
194 subtracting the pre-treatment RL from the post treatment RL for each seedling (Dai et al.,
195 2009; Xu et al., 2017). The Al toxicity tolerance was expressed as relative root growth

196 (RRG%) for each accession where mean Δ RL of Al treatment was compared with mean Δ
197 RL of the control. The calculated RRG% was similar to RRG of the longest root in maize
198 (Xu et al., 2017) and was also similar to relative tolerance index as described in other crops
199 such as rye (Hede et al., 2002), rice (Nguyen et al., 2002) and wheat (Akhter et al., 2009).
200 Mean Δ RL and mean RRG% across all the three replications were used for the analysis of
201 variance (ANOVA) in GenStat 18.2 (VSN International, UK) in each set of experiments.
202 The ANOVA was completed separately in each set of experiment and data was checked for
203 all the assumptions of ANOVA, including normal distribution and residual spread of the
204 data, hence data was not transformed. The five arbitrary tolerance classes were defined
205 (Table 3.1) for RRG% from all sets (Table 1a-d in Online Resource 1) to classify the
206 accessions for Al tolerance. Such arbitrary classes were also defined in rye (Hede et al.,
207 2002) and maize (Xu et al., 2017). For the soil screening experiment, the average and relative
208 root traits data (comparing acid soil treatment to the control lime treatment) of the subset of
209 15 accessions were also analysed for ANOVA. As the relative performances of the
210 accessions were not consistent among the traits tested, a selection index was calculated
211 (Leonforte et al., 2013). For the selection index, the relative measurements of the trait were
212 converted to the 1 – 9 scale. Then weights were assigned to the traits (RRL = 10, RLRs = 8
213 and RFRW = 7) based on the importance of the trait for Al tolerance, which were summed
214 for each accession to use as selection index. Finally, ranks assigned for selection index from
215 the acid soil experiment and the average RRG% from hydroponics were compared for the
216 overall performance of these accessions. For qualitative assessment of the stains, stained
217 roots were observed under a stereomicroscope and for quantification of these stains the fold
218 change of stain absorbance (between the Al and control treatment) (Awasthi et al., 2017)
219 was used for ANOVA. In all the cases after ANOVA the least significant difference (LSD)
220 was used to compare the means.

221 **3. Results**

222 3.1 Hydroponics screening

223 The hydroponics screening method used in the present study was a simple and quick method.
224 It had the optimal Al concentration (5 µM) in low ionic strength solution which facilitated
225 the evaluation of young lentil seedlings for Al tolerance. It accommodated the 96 seedlings
226 in each screen where individual seedlings were stabilised in backer-rod material for three
227 day treatment without any damage to the root system. Lentil seedlings started to show visible
228 Al toxicity damage such as ruptures on the root surface, hardness and brittleness of the root
229 tips along with discolouration (browning) after two days of Al treatment, this becoming more
230 prominent by end of the third day. Along with inhibition of root growth, reduction in lateral
231 root initiation as well as elongation, and swelling in the root tips were also observed (Figure
232 3.2a, b). Root growth performance as assessed by ΔRL was significantly affected by Al
233 treatment (p = 0.013, 0.005, 0.002 and < 0.001 from sets 1 to 4 respectively), with an average
234 reduction of 32.3% compared to the control treatment. The control ΔRL ranged from 41.3
235 mm (AGG74300) to 96.3 mm (AGG75392) that showed a normal frequency distribution
236 (Figure 3.3a), whereas Al treatment ΔRL ranged from 8.7 mm (Precoz) to 59.5 mm
237 (Northfield) with nearly normal frequency distribution (Figure 3.3b). The ILL7537 (11.5
238 mm), AGG74299 (10.1 mm), AGG74295 (11.4 mm) and AGG74341(9.5 mm) showed
239 lowest ΔRL in Al treatment in different set of experiments compared to ILL6002, Northfield
240 and AGG70137 suggesting the presence of significant interaction between accessions and
241 Al treatment (Table 1a-d in Online Resource 1).

242 Al tolerance expressed as RRG% ranged from 14.6 to 77.5% (Figure 3 Online Resource 2)
243 with significant differences between the accessions ($p \leq 0.001$ in all sets except set 3, p =
244 0.021). Based on RRG% tolerance classes, around 5.4% of the accessions belong to the very
245 tolerant (VT, RRG% >52) class (Northfield, Cassab, AGG70137, AGG70340, 07H062L-
246 08HS2004 and AGG70164) with 76.03 mm and 47.6 mm ΔRL in control and Al treatment
247 respectively (Table 3.1). Tolerant (T, RRG% 42-51%) and moderately tolerant (MT, RRG%

248 32-41) classes contained 9.0% and 27.9% of the accessions, while the largest fraction of the
249 accessions (34.2%) were found in sensitive (S, RRG% 22-31) class with lowest ΔRL of
250 control (68.2 mm) and Al treatment (18.2 mm). The 23.4% of the accessions were classified
251 as very sensitive (VS, RRG% <22%) with lower ΔRL of control (74.2 mm) and Al treatment
252 (12.5 mm) as observed in AGG74295, AGG74369, AGG70530 and Precoz. The VT and T
253 class had the higher mean RRG% of 63.4 and 45.7 respectively compared to other classes
254 (Figure 3.3c). These class accessions had 1.6 and 1.2 times higher Al tolerance (RRG%)
255 than the known tolerant line ILL6002 (37.9%) and 23.4% of the total accessions had the
256 higher RRG% than ILL6002. Low correlation ($r^2 = 0.36$, $p < 0.001$) was observed for ΔRL
257 between control and Al treatment, whereas high correlation ($r^2 = 0.93$, $p < 0.001$) was
258 observed between the Al treatment ΔRL and RRG% which shows the reliability of these
259 parameters in assessing aluminium toxicity tolerance. The RRG% of the subset of 15
260 accessions selected for acid soil and staining experiments ranged from 24.4 to 86.07 with
261 Northfield (86%) and AGG70137 (73.4%) significantly ($P = < 0.001$) higher than other
262 accessions (Table 1e in Online Resource 1).

263 3.2 Soil screening experiment

264 A subset of 15 accessions were evaluated for root growth in a short-term soil screening
265 experiment. The main effects of accession ($p < 0.001$ for all traits) and lime control treatment
266 were significant for the RL ($p = 0.017$), LRs ($p = 0.009$) and FRW ($p = 0.026$). There was
267 significant ($p = 0.002$ LRs and 0.012 FRW) interaction for all the traits except for the RL
268 (Table 3.2) as the acid soil with Al content did not significantly affect RL in any of the
269 accessions. However, relative root length (RRL) was significant with high RRL of 112.3%
270 observed in AGG70281. Acid soil significantly ($p = 0.002$) reduced the average number of
271 LRs to an average of 4.5 in Precoz, AGG74341 and AGG74259 compared to the lime control
272 treatment, which also reduced the relative number of lateral roots (RLR) in these accessions
273 by 54.0%, 66.2% and 71.2% respectively, compared to Northfield (102.2%) and

AGG70137(111.4%). The average FRW was reduced significantly (p = 0.012) in Precoz (0.0379 g), AGG70530 (0.0151 g) and AGG70334 (0.0141 g) compared to other accessions in the acid soil, which was also depicted by their lower relative fresh weight (RFRW) (Table 3.2).

Among the root traits RRL in the soil screening experiments did not show correlation with hydroponics RRG%, whereas RLRs showed medium correlation (r^2 = 0.58, p < 0.001, n = 15) with average hydroponic RRG%, hence a selection index was calculated by considering all the relative measurements. The selection index was low (76.3) in Precoz whereas it was high (636.7) in AGG70137. Similar rankings for average RRG% and selection index was observed for Northfield, AGG70137, AGG70281, Precoz, AGG74367, AGG70334 and AGG74341 accessions (Table 3.3), however it differed for other accessions between the hydroponic and acid soil screening. The AGG70256 and AGG74249 accessions showed high hydroponics RRG% of 45.7 and 43.8 respectively but in soil they had low selection index (352.9 and 281.7), whereas the opposite was observed for AGG70530 (20.6 RRG%) accession as it showed high selection index of 376.2 in acid soil. Medium correlation (r^2 = 0.54, p < 0.001, n = 15) was observed between the rank assigned based on average RRG% and selection index from hydroponics and acid soil screening respectively.

3.3 Histochemical and biochemical analysis

Stereomicroscopic observations of Haematoxylin stained roots showed dark purple to brown colouration compared to control roots after three days of Al treatment. Among the subset of 15 accessions, the colour was more intense in the VS and S class accessions (Precoz, AGG70530 and AGG74367, AGG70334, AGG74341) with ruptures on the root surface and in the tip (Figure 3.4). The MT class accessions, AGG74268 and AGG74287 were also stained however to a lesser extent, compared to the T class, AGG70256, AGG70281 and AGG74249, and VT class, Northfield and AGG70137. A similar trend was also observed

for Evans blue staining by forming intense blue colour on the root surface as observed in Precoz and AGG70530, AGG70334 and AGG74268 compared to the VT and T class accessions where there was no or minimal blue colour on the root surface (Figure 4 in Online Resource 2). Quantification of these stains and MDA content of lipid peroxidation showed a significant (Al content; $p = 0.011$, plasma membrane damage; $p < 0.001$, and MDA content; $p = 0.002$) fold increase in all the MT, S and VS class accessions due to Al treatment compared to control treatment (Table 2 in Online Resource 1). An average fold increase of 1.5, 2.4 and 1.8 was observed for Al content, plasma membrane damage and MDA content respectively in VS and S class compared to the VT and T class. The MDA content was significantly higher in VS and S class except AGG74259 which showed no difference between control and Al treatment.

4. Discussion

This study reports the establishment of a new higher throughput hydroponics method compared to those previously reported that has been used successfully to screen 111 accessions, including 98 putative FIGS acid tolerant accessions for Al toxicity tolerance. The results from the hydroponics screen were supported by acid soil screening, histochemical and biochemical analysis in subset of 15 accessions. The hydroponics method established is a simple and quick screening at the early seedling stage. A three-day treatment efficiently differentiated the tolerance of the lentil accessions at optimal, 5µM Al treatment in low ionic strength solution. This type of solution is necessary to increase or maintain the Al^{3+} activity and also to increases likelihood of Al accumulation on negatively charged sites within the root cell wall and root plasma membrane to cause toxicity (Famoso et al., 2010). The established hydroponics method is high throughput in terms of the number of accessions that can be screened in each run, as it accommodated large number of seedlings (96) in stable condition during Al treatment. This type of screening for Al tolerance is beneficial in case

of large breeding populations as it facilitates the screening of the large number of accessions in short time.

Previous studies in rice, spring rye, lentil, *Medicago truncatula* and *Medicago sativa* demonstrated the primary effects of Al toxicity on root growth with roots becoming shorter and the absence of normal branching patterns compared to control (Hede et al., 2002; Sledge et al., 2005; Singh et al., 2012; Wang et al., 2016; Awasthi et al., 2017). Swollen root apices and inhibited lateral root growth with necrotic or brown tips were reported in French bean and wheat (Foy, 1984). Similar observations were reported in the present study (Figure 2 and Figure 5 in Online Resource 2). The reduced ΔRL of Al treatment compared to control indicates toxic Al effect in all accessions with more severe reduction in VS and S classes. The RRG% is the most widely used phenotypic index for Al toxicity tolerance, as it eliminates any genotype-specific differences, in terms of seed size and root growth among the accessions which are important for assays on young seedlings (Hede et al., 2002). Additionally, comparing Al treatment root growth with the control (low pH without Al) aids reliable separation of genotypes for tolerance to Al toxicity. In maize, the RRG of the longest root was used to classify 141 germplasm lines into three groups after exposure to 60 µmol Al for three days (Xu et al., 2017). In the present study, 42.3% of an acid tolerant lentil FIGS set were successfully identified as VT, T and MT. The 23.4% accessions had a higher (> 38 RRG%) tolerance than the known tolerant line, ILL6002 (37.9 RRG%, classed as MT in the present study). This validates the presence of superior aluminium toxicity tolerant accessions in the FIGS acid tolerant set compared to the check line. Most of the VT and T class accessions are from Afghanistan, Lebanon, Morocco, Tunisia, Turkey, Jordan and Syria indicating their adaptability to these Mediterranean and semi-arid conditions. In these areas, lentil often faces terminal drought hence well-developed deep root system or high root vigour (Ghanem et al., 2017) could be an adaptive trait for drought tolerance. The VT and T class accessions from such areas have shown the high ΔRL or root growth in both the control

(>78mm) and Al treatment (>36 mm). Hence adaptive trait with high root vigour or root growth in these classes might have helped to overcome Al toxicity tolerance in the present study. This indicates the likely presence of Al tolerant gene/alleles in addition to high root vigour trait in VT and T classes. This type of demonstration with superior alleles has been also reported in rye for Al toxicity tolerance (Hede et al., 2002). The majority of the Nepalese and Ethiopian accessions are in VS and S class and showed better root growth in control with high average (71.6 mm) but failed to grow in Al treatment (15.6 mm) indicating their adaptability to only acid conditions but not for toxic Al. These collection sites had acidic soil (Kharal et al., 2018; Mosissa, 2018) but may not have had toxic Al as a selection pressure. This shows that low pH is necessary for Al^{3+} toxicity, but not all soils with low pH contain toxic levels of Al^{3+}, especially as its concentration in acid soil also depends on other soil factors (Ryan, 2018). Even though we identified the majority of the FIGS accessions as acid tolerant based on root growth in low pH control (pH 4.5), tolerance to Al toxicity may vary as we observed in this study. Accessions AGG74257, AGG74299 and AGG74300 from VS and S class suggest their adaptability could be for non-acidic conditions (pH of > 4.5) as they showed very low root growth in control with average of 43.8 mm (Table 1b, c in Online Resource 1). Thus, hydroponic screening and RL measurements (ΔRL and RRG%) for Al toxicity tolerance were successful in identifying Al tolerant accessions with high root vigour trait and potential Al tolerance gene/alleles.

Among the subset of 15 accessions, Northfield, AGG70137, and AGG70281 had high tolerance based on RRG% in hydroponics compared to Precoz and AGG70530. In acid soil, only AGG70137 and AGG70281 showed significant high tolerance based on RRL, but not Northfield or other accessions. The acid soil screening provided a realistic rooting environment in contrast to hydroponics but was not as efficient in differentiating the level of tolerance, as RRL was not successful in differentiating all the accessions (except extreme classes) compared to RLRs. This could be due to the short duration of the soil screening

experiment, as adaptation to Al-toxic soils might need a longer time to express the RL tolerance responses. This was observed in maize and soybean, where a four day initial contact with Al toxic subsoil was not effective in expressing the RL tolerance and hence suggested that such adaptive tolerance will be missed in short term tolerance screening experiments (Bushamuka & Zobel, 1998). The most obvious symptom of Al toxicity along with reduction in RL is the inhibition of lateral root formation (Fleming & Foy, 1968) which was observed in our hydroponic study for all accessions. However, in the acid soil experiment, accessions produced lateral roots with differential response, where the VT and T class accessions produced more laterals in acid soil whilst the VS and S accessions failed to produce them, suggesting the importance of lateral root formation in Al toxicity tolerance screening. Generally, the formation of lateral roots is a developmental process, but it may also be adaptive in response to environmental influences within the rhizosphere (Jung & McCouch, 2013). The high RLR in Northfield and AGG70137 indicates their tolerance as they produced more laterals in acid soil compared to lime treatment. This phenotypic effect was also reported in vertically split root system study in a tolerant and sensitive soybean cultivar, where the lateral root production and length were more significantly and differentially affected by Al concentrations than the tap root elongation (Bushamuka & Zobel, 1998). In Al sensitive wheat (ES8 and Janz) and barley (Pallas and Salka) lines, lateral root length were more reduced than the primary roots at high Al concentration (> 3 - 6 mg/kg) (Haling et al., 2010) compared to tolerant lines in acid soil screening. In wheat, the apparent differences in lateral and primary roots may be due to differences in the levels of *TaALMT1* expression, however in barley it could be because of other factors as barley does not normally possess *TaALMT1* (Haling et al., 2010). These results along with those seen in our soil screening illustrate the importance of considering lateral roots along with main root system in Al toxicity tolerance screens. The observed medium correlation of ranks assigned

for 15 accessions based on hydroponics (average RRG%) and soil (selection index) in our experiments can be explained as;

1) Al^{3+} toxicity was the only root growth limiting factor in hydroponics whereas multiple factors would have been present in the acid soil test, as soil is complex in terms of Al distribution and concentration. Al content in the acid soil could be more toxic to the T accessions AGG70256 and AGG74249 which showed relatively lower performance in terms of the ranks, in acid soil (Table 3.3) than in hydroponics. In contrast the VS accession AGG70530 performed better in acid soil than in hydroponics, indicating acid soil Al content was not as critical to this accession. This illustrates that different screening methods may show different levels of Al^{3+} toxicity among the accessions. 2) The hydroponic solution with or without Al had the same pH of 4.5, whereas in the acid soil experiments, pH differed between acidic soil (pH 4.5) and limed control (pH 6.0) treatment. As sensitive accessions Precoz, AGG74259 and AGG74341 responded well under lime control (pH 6.0) (Table 3.2) by producing more laterals compared to the acid soil, the same accessions showed inhibition of the laterals in the hydroponics control with lower pH (based on the visual observations made during the root length measurements). A similar, poor correlation was also observed in barley (Moroni et al., 2010; Ferreira et al., 2017) and in *Medicago truncatula* (Narasimhamoorthy et al., 2007) with inconsistency between screening methods, however good correlation has been observed in wheat (Rengel & Jurkic, 1992) and soybean (Horst et al., 1992).

The tolerant nature of the accessions (Northfield, AGG70137, AGG70281 and AGG70561) from both screening methods were further supported by the quantification of histochemical stains and biochemical analyses as they had the low average Al accumulation (2.4 fold), plasma membrane damage (1.5 fold) and oxidative stress (1 fold) compared to average fold changes in MT, VS and S classes. In addition, they did not show any staining (Haemato xylin

426 and Evans blue) and root surface damage (Figure 3.4 and Figure 4 in Online Resource 2)
427 which can suggest an Al exclusion type of tolerance mechanism, which also reported in early
428 lentil study by release of citrate and malate in resistance lines (Singh et al., 2016). However
429 higher intensity of the stains (Haematoxylin and Evans blue) in the VS and S accessions is
430 associated with Al susceptibility and indicates more Al accumulation (Table 2 in Online
431 Resource 2) and cell death. Similar reports were also reported in maize (Pineros et al., 2005;
432 Giannakoula et al., 2008), wheat (Ye et al., 2011), other crops where Haematoxylin has been
433 widely used to discriminate between plant genotypes with respect to Al toxicity tolerance
434 (Polle et al., 1978; Miftahudin et al., 2007; Castilhos et al., 2011; Awasthi et al., 2017). Most
435 of the accumulated Al in the roots bound to the pectin constituents of the cell walls which
436 alters the composition, cell extensibility (Ma et al., 2004; Jones et al., 2006; Yang et al.,
437 2008), and increases root diameter causing transverse ruptures as observed in external cortex
438 and rhizodermic cells of the elongation zones in other crops (Blarney et al., 2004; Kopittke
439 et al., 2008; Motoda et al., 2010). It is likely that these factors may have contributed to the
440 observed morphological changes such as hard, brittle root tips and ruptures on root surface
441 in the VS, S and a few of the MT accessions (Figure 3.4 and Figure 4 in Online Resource 2).
442 These results are similar to earlier work in pea (Yamamoto et al., 2001; Motoda et al., 2011;
443 Motoda et al., 2010), cowpea (Kopittke et al., 2008) and maize roots (Jones et al., 2006).
444 Similar Evans blue and lipid peroxidation results were reported in rice (Wu et al., 2014), pea
445 (Yamamoto et al., 2001; Motoda et al., 2011), maize (Wang et al., 2015), wheat (Aggarwal
446 et al., 2015) and soybean (Cakmak & Horst, 1991; Horst et al., 1992).

447 **5. Conclusions**

448 The screening methods, histochemical and biochemical analyses have validated the presence
449 of aluminium toxicity tolerant accessions within the FIGs set screened in our study. The
450 accessions Northfield, AGG70137, AGG70281 and AGG70561 consistently performed
451 better than the known tolerant line ILL6002 and could be considered for Al toxicity tolerance

breeding. The high throughput hydroponics Al toxicity tolerance screening method can be adapted to screen large breeding populations and new germplasm. Future research could include validating Al toxicity tolerance by growing in acid soil field conditions or pot-based glasshouse experiments over a longer duration to further validate hydroponic results and to see the plant growth responses for Al toxicity over the life cycle of the plant.

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714

3.4 Figures and tables of the manuscript

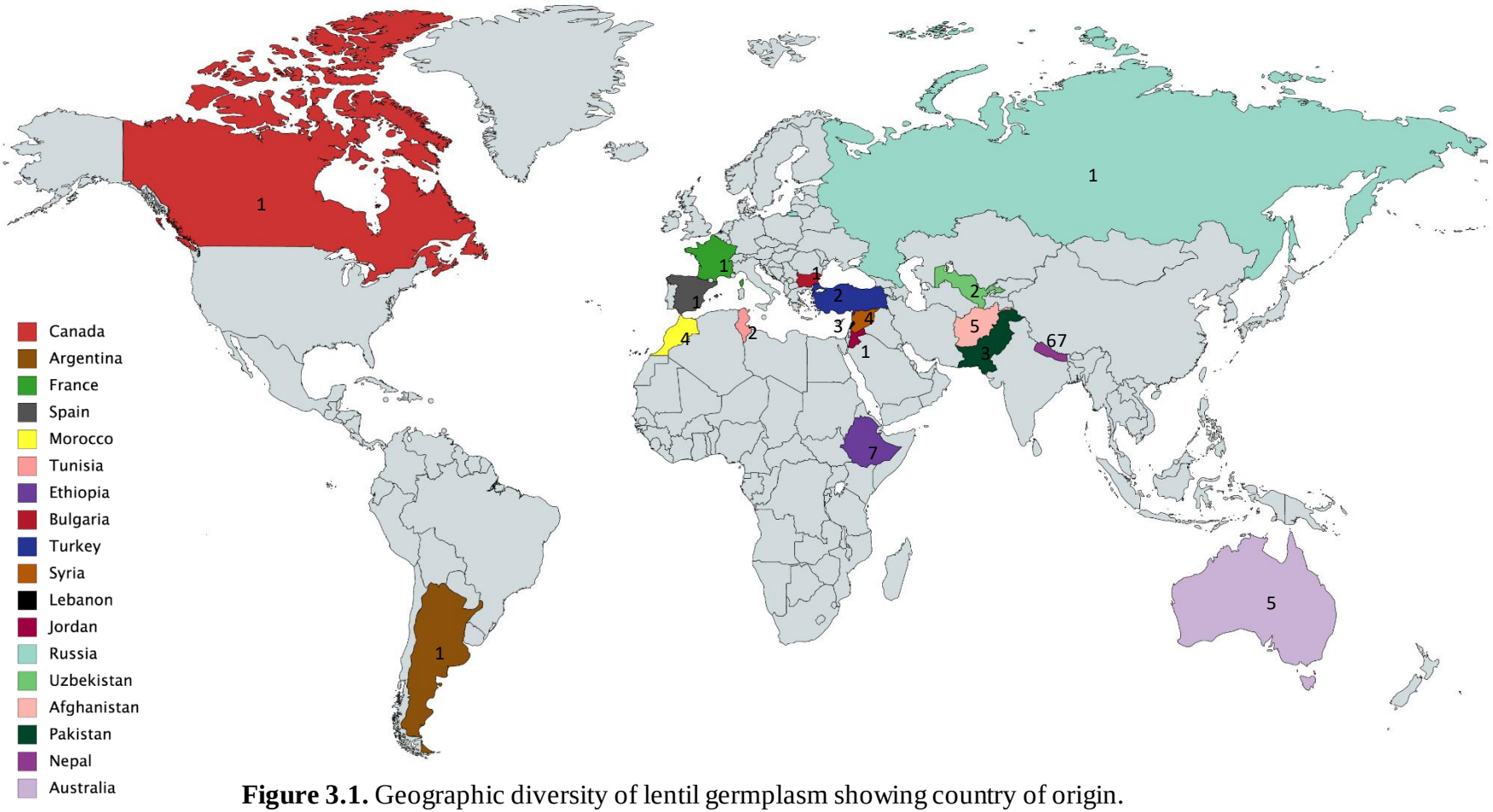


Figure 3.1. Geographic diversity of lentil germplasm showing country of origin.

Numbers of accessions shown on each country.

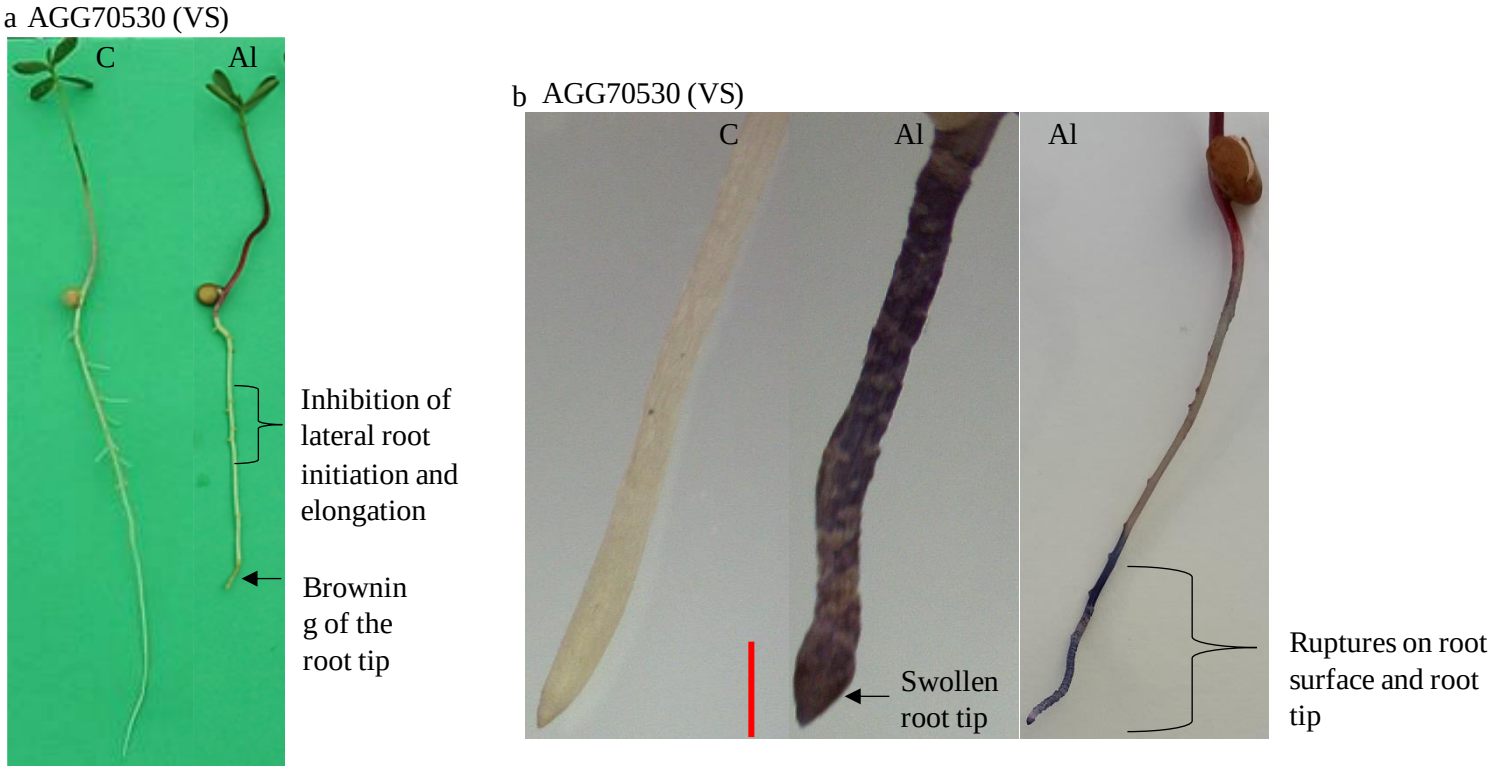


Figure 3.2. Haematoxylin stained roots of accession AGG70530 showing Al toxicity symptoms after 3 days growth in the presence of 5 µM Al at pH 4.5.

a- symptoms of inhibition of lateral root initiation and elongation; b- swelling of the root tips and raptures on root surface and root tip,

C - control, Al – aluminium treatment, VS - very sensitive tolerance class, Red colour scale bar is 1 mm.

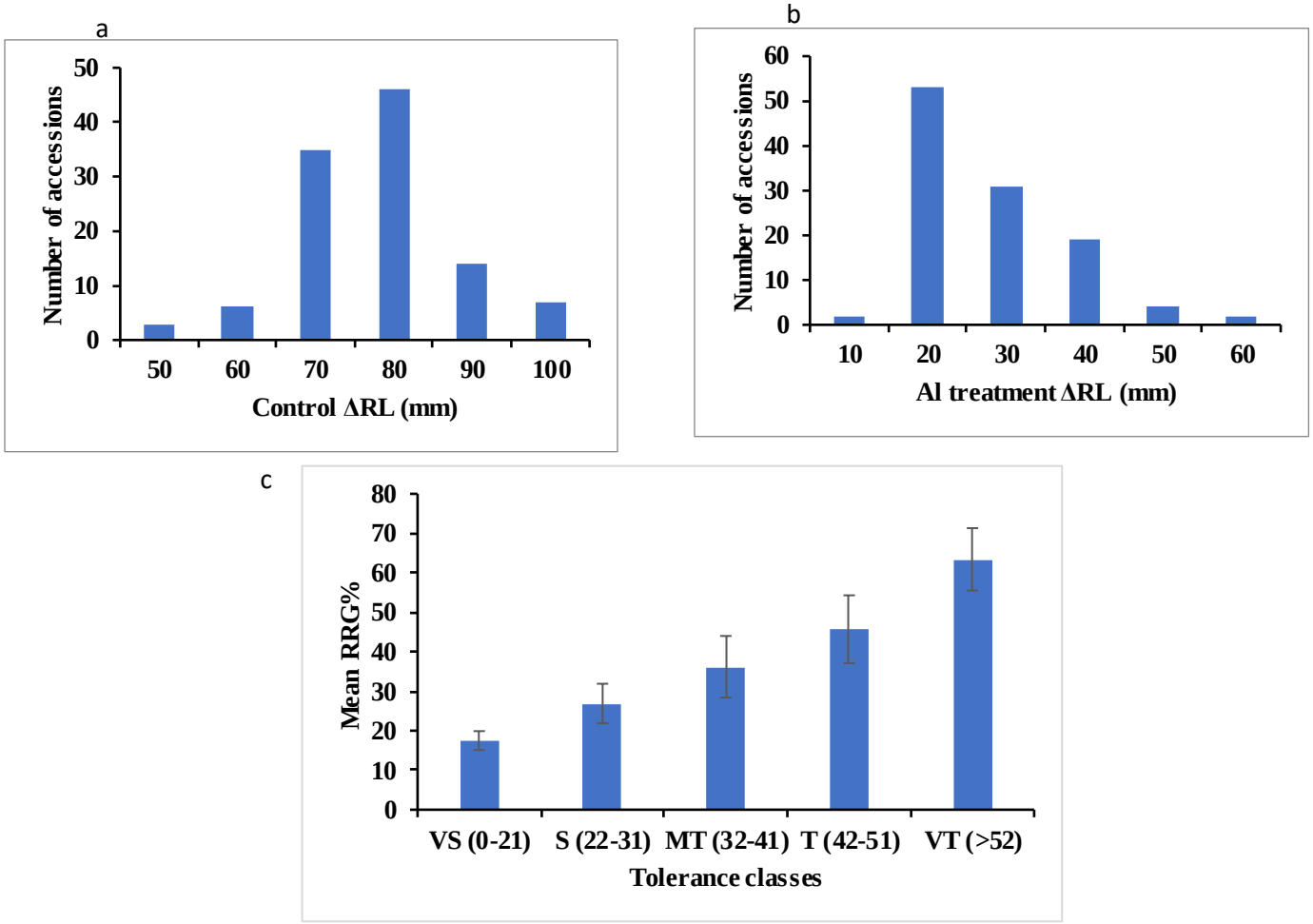


Figure 3.3. Frequency distribution of change in root length (Δ RL) of (a) control treatment, (b) aluminium (Al) treatment and (c) mean of tolerance classes of 111 accessions.

Tolerance classes: VS = Very sensitive, S = Sensitive, MT = Moderately tolerant, T = Tolerant and VT = Very tolerant, numbers inside the bracket are the range of mean relative root growth (RRG%), values are means of each tolerance class ± standard error of mean (SEM)

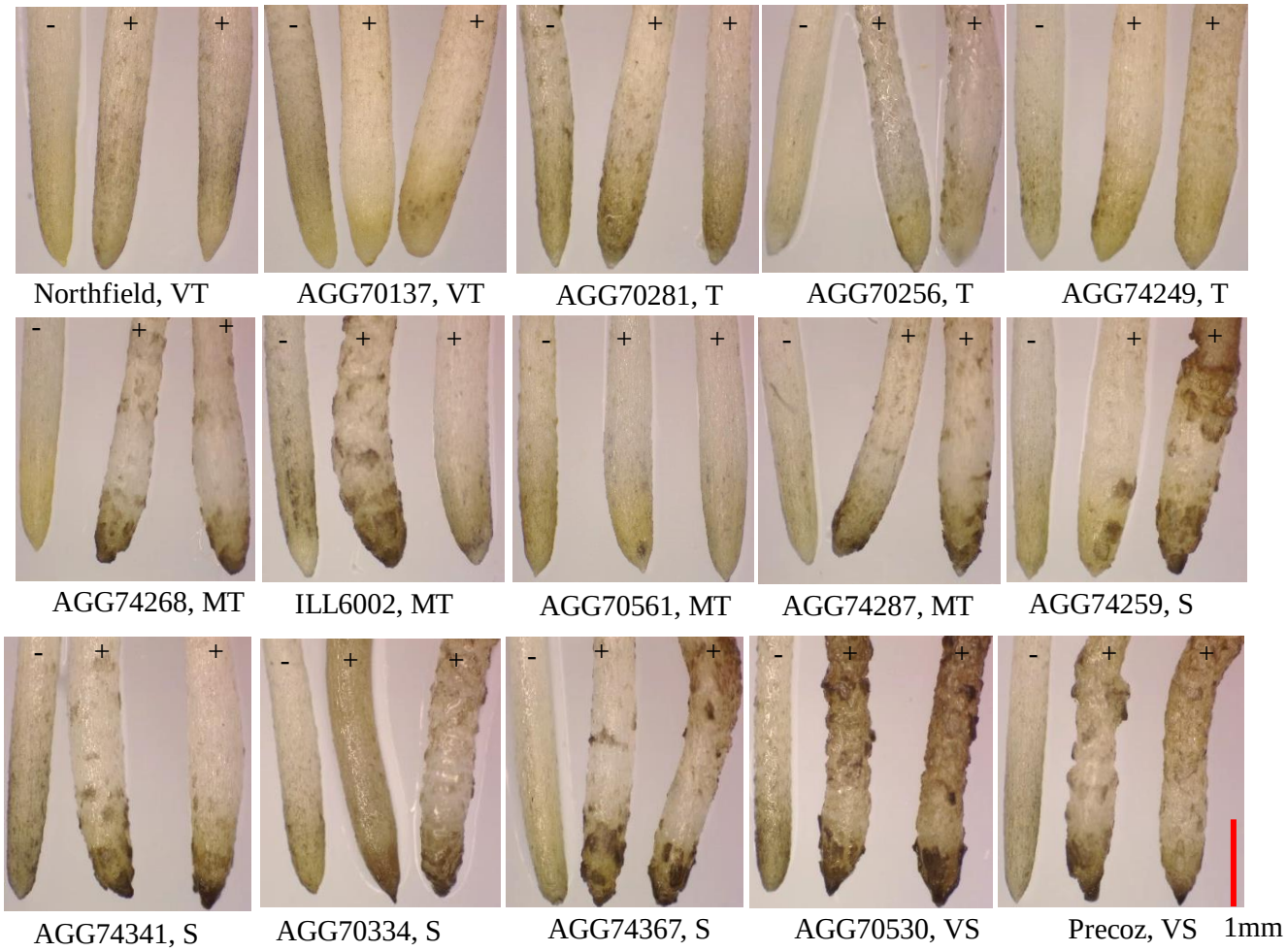


Figure 3.4. Stereomicroscopic observation of selected 15 accessions for Haematoxylin stain after 3 days of Al treatment.

+ 5 µM Al treatment, - Control, tolerance classes: VT - Very tolerant, T - Tolerant, MT - Moderately tolerant , S - Sensitive and VS - Very sensitive

Table 3.1. Tolerance classification of 111 lentil accessions from four sets of hydroponics screen with mean change in root length (ΔRL) of control, Al treatment and mean relative root growth (RRG%) along with total accessions in each class

[a] Tolerance class	Control ΔRL(mm)	Al treatment ΔRL(mm)	RRG%	Total accessions (%) and accessions name
0-21; VS	74.2 ± 1.3	12.5 ± 0.3	17.5 ± 0.3	(23.4%), ILL7537, AGG74327, AGG74358, AGG74331, AGG74329, AGG74453, AGG74357, AGG74364, AGG74356, AGG74346, AGG74363, AGG74371, AGG74434, AGG74330, AGG75305, AGG74436, AGG74370, AGG74348, AGG74369, AGG74359, **Precoz**, AGG74295, AGG74360, AGG74354, **AGG70530**, AGG74343
22-31; S	68.2 ± 1.8	18.2 ± 0.8	26.9 ± 0.4	(34.2%), AGG74298, AGG70335, AGG74335, AGG74288, AGG74273, AGG74362, AGG74300, AGG70527, AGG74324, **AGG74367**, AGG74257, AGG74290, AGG74310, AGG74311, AGG74328, AGG74258, AGG74373, **AGG70334**, AGG74299, AGG74302, AGG74251, AGG70249, AGG70465, AGG74294, AGG70526, AGG70566, AGG70023, AGG70024, AGG70418, **AGG74341**, AGG74301, AGG74267, AGG70942, AGG74286, AGG74293, AGG74250, AGG70085, **AGG74259**
32-41; MT	75.1 ± 2.0	26.9 ± 0.9	36.2 ± 0.5	(27.9%), AGG74266, Indian head, AGG70084, AGG70138, AGG74297, **AGG74287**, AGG70247, AGG70255, AGG70951, AGG70568, **AGG70561**, AGG70419, AGG74309, AGG74308, AGG74265, AGG70336, AGG70273, AGG74306, Digger, **ILL6002**, AGG74307, AGG70954, AGG70297, AGG74285, AGG74252, ILL6788, AGG70337, AGG75392, AGG74305, **AGG74268**, AGG70145
42-51; T	76.4 ± 3.2	36.7 ± 0.8	45.7 ± 0.9	(9%), AGG70949, AGG70163, **AGG74249**, PBA Ace, **AGG70256**, Boomer, **AGG70281**, AGG70338, AGG74325, AGG70940
>52; VT	76.0 ± 1.9	47.6 ± 3.2	63.4 ± 3.8	(5.4%), AGG70164, 07H062L-08HS2004, AGG70340, Cassab, **AGG70137**, **Northfield**

Accessions in **Bold text** are the subset of 15 accessions selected for acid soil screening, histochemical and biochemical analyses. Average values are considered from two hydroponic experiments for these accessions. a - Tolerance classes are based on average relative root growth (RRG%) from different set of hydroponic experiments. VS - very sensitive, S - sensitive, MT - moderately tolerant, T - tolerant, VT - very tolerant, values are means of each tolerance class ± standard error of mean (SEM)

Table 3.2. Root length, lateral roots and fresh root weight of the subset of 15 accessions grown in acid and lime control, along with their relative (as% of limed) performance

Accessions	**RL (mm/plant)**				**LRs (counts/plant)**				**FRW (g/plant)**			
	Acid soil	Lime control	Mean	RRL %	Acid soil	Lime control	Mean	RLRs %	Acid soil	Lime control	Mean	RFRW %
Northfield	164.5	166.2	165.4 *	98.9	9.1	8.9	9.0	102.2*	0.0788	0.0885	0.0836	89
AGG70137	160.2	153.1	156.6	104.6*	6.8	6.1	6.4 *	111.4*	0.0829	0.0762	0.0795	108.7*
AGG70281	134.3	119.6	126.9 *	112.3*	9.1	9.4	9.2	96.8	0.0674	0.0747	0.071	90.2
AGG70256	140.6	152.8	146.7	92.0	8.1	9.5	8.8 *	85.3	0.0719	0.0823	0.0771 *	87.3
AGG74249	133.8	131.7	132.7	101.6	6.5	8.9	7.7	73.0*	0.0522	0.0574	0.0548 *	90.9
AGG74268	145.9	151.8	148.8	96.1	9.9	10.6	10.2	93.3	0.0711	0.0765	0.0738	92.9
ILL6002	143.7	152.4	148.0	94.3	11.5	14.8	13.1	77.0*	0.1066	0.1181	0.1123	90.2
AGG70561	137.9	136.7	137.3	100.9	6.1	7.8	6.9	78.2*	0.0588	0.0596	0.0592	98.6
AGG74287	130.7	131.2	130.9	99.6	7.0	8.1	7.5	86.4	0.0539	0.0594	0.0566	90.7
AGG74259	135.4	140.7	138.0	96.2	6.7	9.4	8.0	71.2*	0.0624	0.0683	0.0653 *	91.3
AGG74341	132.8	140.5	136.6	94.5	5.7	8.6	7.1	66.2*	0.0559	0.0596	0.0577	93.7
AGG70334	141.6	150.8	146.2	93.8	6.9	9.1	8.0	75.8*	0.0615	0.0756	0.0685	81.3*
AGG74367	139.1	145.1	142.1 *	95.8	9.3	12.7	11.0 *	73.2*	0.0753	0.0834	0.0793	90.2
AGG70530	133.2	142.5	137.8	93.4	8.2	9.4	8.8	87.2	0.0597	0.0748	0.0672	79.8*
Precoz	152.6	164.9	158.7	92.5	9.4	17.4	13.4 *	54.0*	0.1008	0.1387	0.1197 *	72.6*
Mean	141.7	145.3 *			8.0	10 *			0.0706	0.0795 *		
p -value (LSD, *p* = 0.05)												
Treatment	0.017 (2.5)				0.009 (1.1)				0.026 (0.0071)			
Accessions	<0.001 (12.7)			<0.001 (10.6)	<0.001 (1.7)			<0.001 (30)	<0.001 (0.009)			0.015 (22.3)
Treatment x accessions	NS (17.5)				0.002 (2.5)				0.012 (0.0134)			

*In the mean indicates the significant difference in acid and lime control and between main effects of accessions at $p<0.05$ by LSD test. *In the relative% indicates significant difference between accessions. For RRL, AGG70137 and AGG70281 are significantly different than other accessions. For RLRs, Northfield and AGG70137 are significantly different than other accessions. Root length (RL), Relative Root length (RRL), Lateral roots (LR), Relative Lateral roots (RLR), Fresh root weight (FRW), Relative Fresh root weight (RFRW)

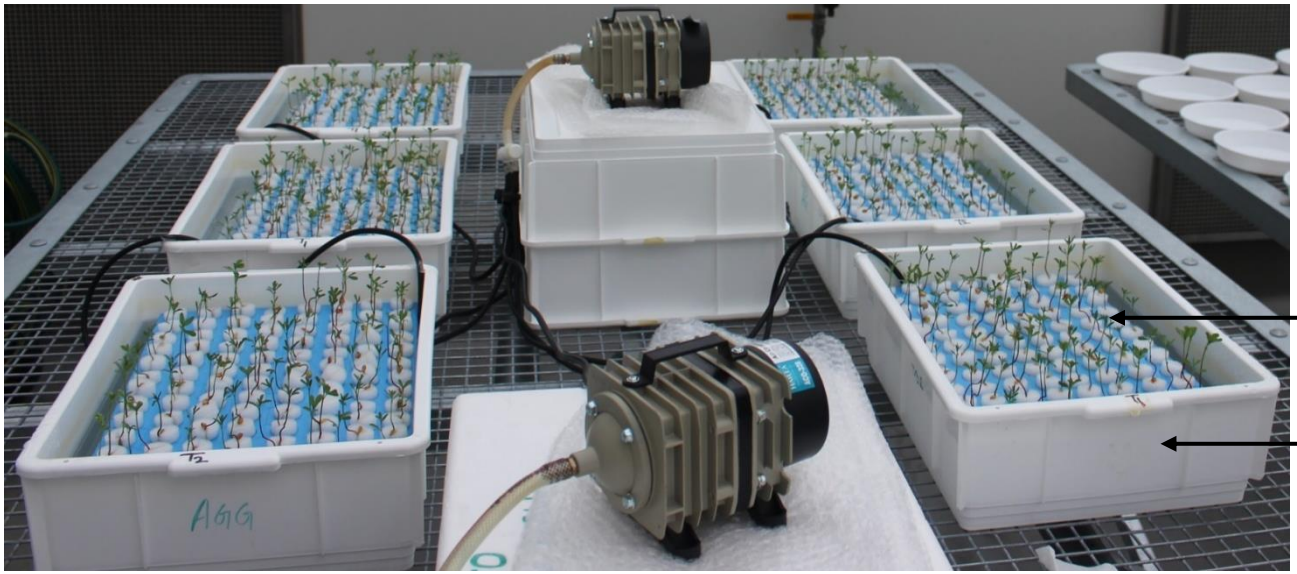
Table 3.3. Relative root growth (RRG) % and selection index of the subset of 15 accessions in the hydroponic and soil screening

		Hydroponics		Soil screening	
[a] Tolerance class	Accessions	[b] Relative root growth %	Ranks	Selection index	Ranks
VT	Northfield	77.5	1	531.9	3
VT	AGG70137	69.3	2	636.7	1
T	AGG70281	46.3	3	536.2	2
T	AGG70256	45.7	4	352.9	7
T	AGG74249	43.8	5	281.7	10
MT	AGG74268	35.4	6	441.6	4
MT	ILL6002	37.9	7	294.4	9
MT	AGG70561	34	8	325.0	8
MT	AGG74287	32.9	9	393.3	5
S	AGG74259	31.4	10	244.8	13
S	AGG74341	30.1	11	193.5	14
S	AGG70334	26.7	12	276.1	11
S	AGG74367	24.7	13	260.8	12
VS	AGG70530	20.6	14	376.2	6
VS	Precoz	19.2	15	76.3	15

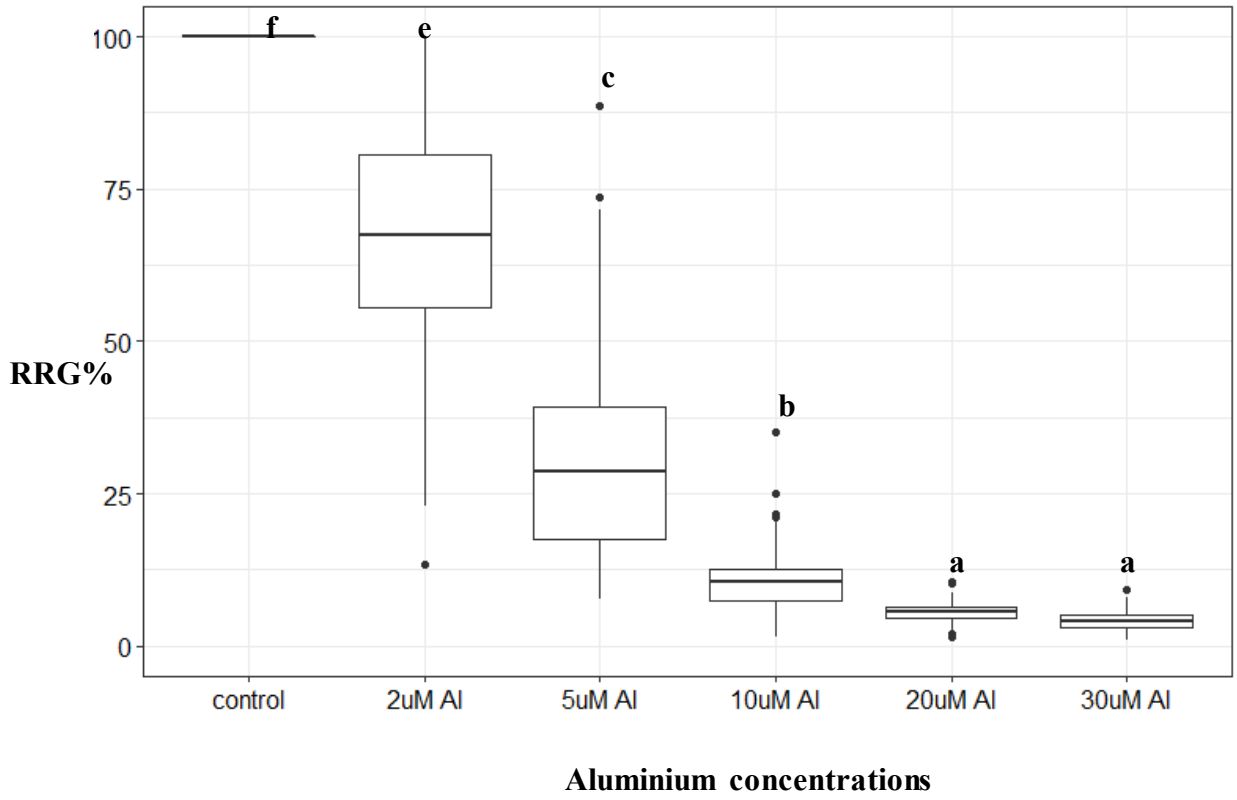
[a] Tolerance classification based on RRG% from set1-4: VS = Very sensitive (RRG% 0-21), S = Sensitive (RRG% 22-31%), MT = Moderately tolerant (RRG% 32-41), T = Tolerant (RRG% 42-51) and VT = Very tolerant (RRG% >52). [b] Indicates the average from two independent experiments.

3.5 Online resources of the manuscript

Online Resource 2

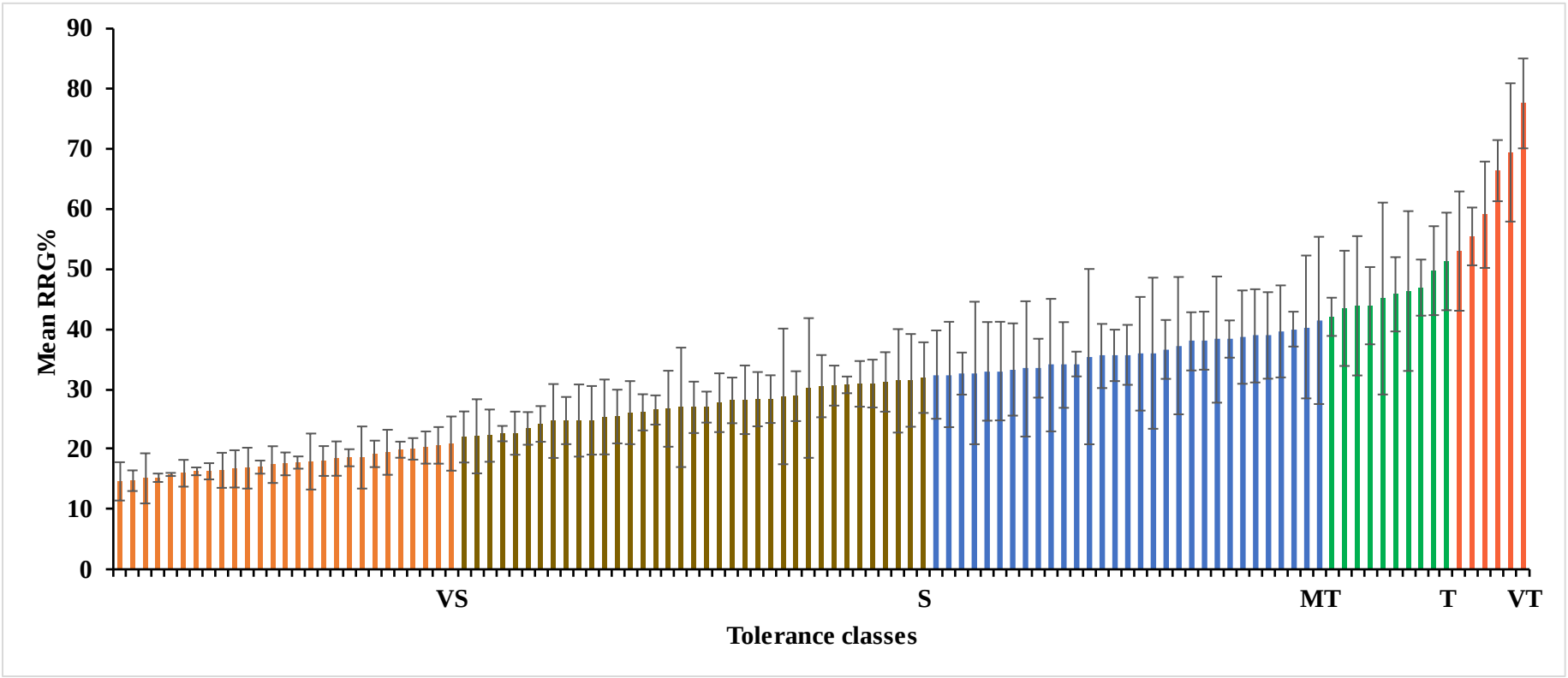


Online Resources Figure 1. Hydroponic setup used in the lentil aluminium toxicity tolerance screening arranged in three replications and two treatments.



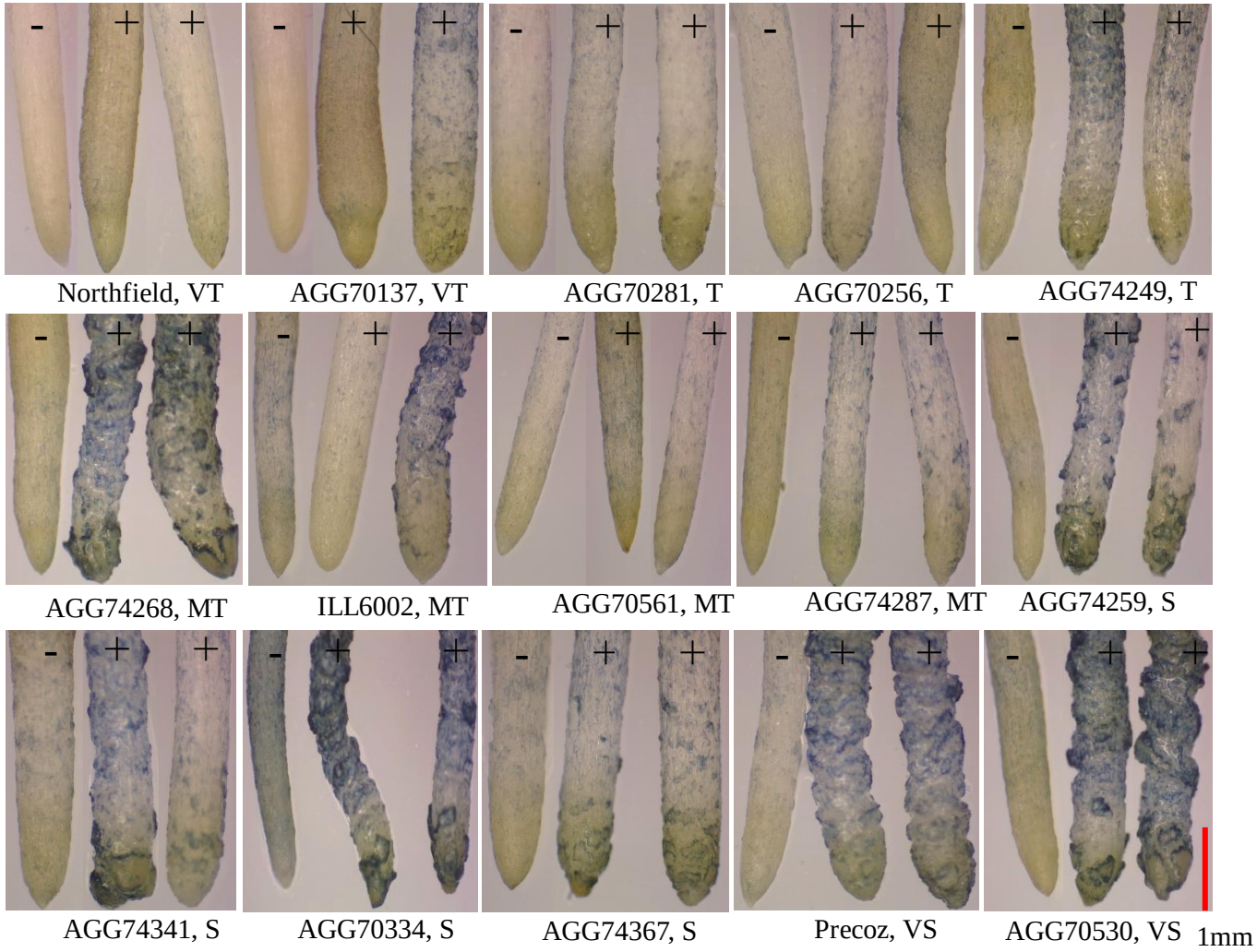
Online Resources Figure 2. Relative root growth (RRG%) at different aluminium concentrations.

Different letters indicate the significance by Fisher protected test. Size of the box shows the variation in the RRG%, line inside the box is the mean, line below and above the box are the minimum and maximum data points, black dots are the outliers. The 22 lentil accessions (AGG70305, AGG70455, AGG71377, AGG71438, AGG71501, AGG71512, AGG71717, AGG72372, AGG74390, ILL7537, Boomer, Nugget, PBA Blitz, CIPAL1301, PBA Jumbo2, PBA Bolt, PBA Greenfield, PBA Ace, 07H062L-08HS2004, AGG70281, AGG70024, ILL6002) used in the pilot experiments.



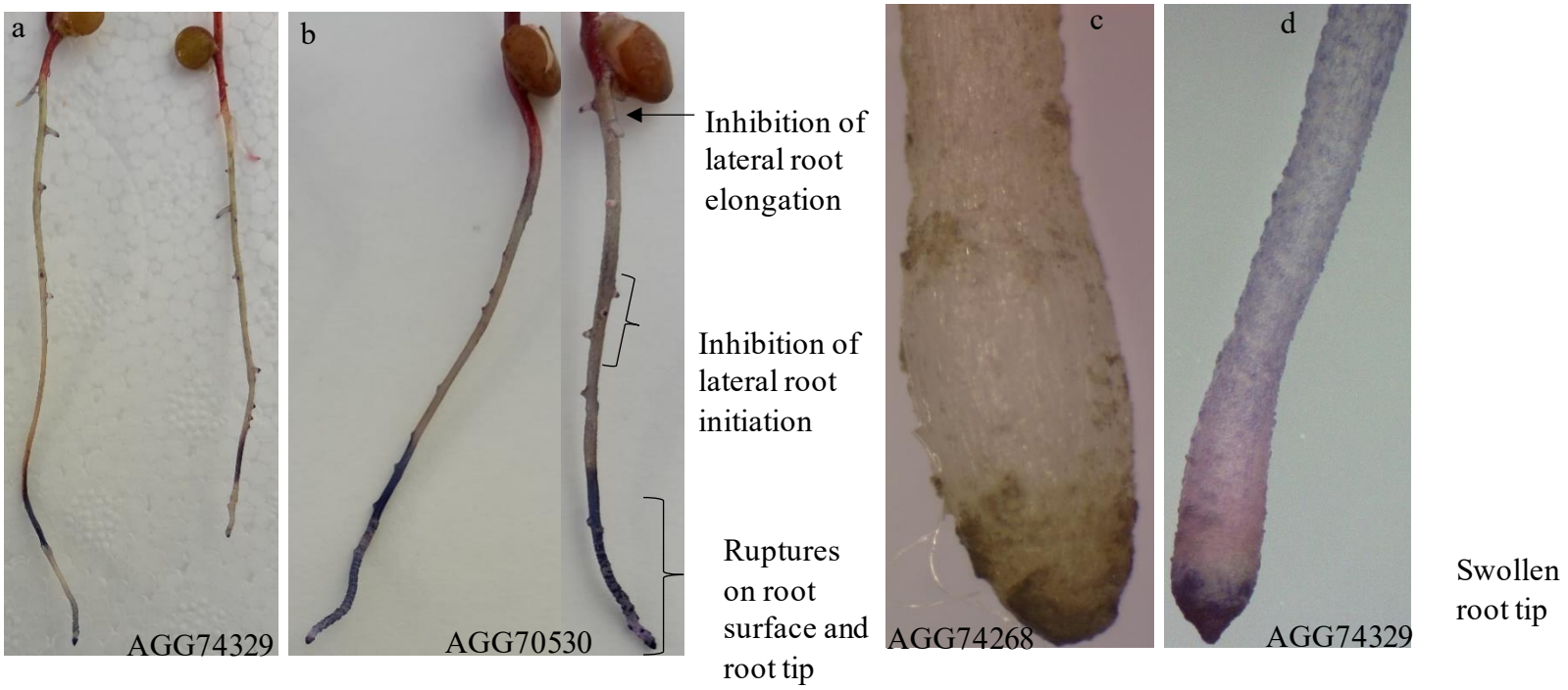
Online Resources Figure 3. Tolerance class of 111 lentil accessions based on relative root growth (RRG%).

Tolerance classes: VT - Very tolerant, T- Tolerant, MT - Moderately tolerant, S - Sensitive and VS - Very sensitive, values are mean ± standard error of mean (SEM), based on three replications



Online Resources Figure 4. Stereomicroscopic observation of selected 15 accessions for Evans blue stain after 3 days of Al treatment.

+ = 5 µM Al, - = Control, Mean relative root growth (RRG%) tolerance classification: VT - Very tolerant, T- Tolerant, MT - Moderately tolerant, S - Sensitive and VS - Very sensitive



Online Resources Figure 5. Haematoxylin stained lentil seedlings showing aluminium toxicity symptoms.

Lentil accessions (AGG74329 and AGG70530 are VS class, AGG74268 is MT class) showing aluminium treatment symptoms such as (a and b) ruptures on root surface and root tip, inhibition of lateral root initiation and elongation, (c and d) swelling of the root tips. VS - very sensitive, MT – moderately tolerant class

Online Resource 1

Online Resources Table 1a. List of the lentil accessions along with country of origin, level of improvement, FIGS information, mean change in root length (Δ RL) of control and Al treatment, and relative root growth (RRG)% in set 1 hydroponic experiment

Accessions	Country of origin	Level of improvement	Belongs to FIGS	Control mean Δ RL	Al treatment mean Δ RL	Mean RRG%
07H062L-08HS2004	Australia	Breeding line	N	75.92	41.67 *	55.41
AGG70023	Ethiopia	Landrace	Y	77.83	21.67 *	28.82
AGG70024	Afghanistan	Landrace	N	85	24.74 *	29.25
AGG70085	Morocco	Landrace	Y	95.5	31.33 *	31.37
AGG70137	Lebanon	Landrace	Y	72	61.08 NS	87.51*
AGG70138	Turkey	Landrace	Y	76.67	23.50 *	32.68
AGG70145	Morocco	Landrace	Y	84.31	33.67 *	41.43
AGG70163	Tunisia	Landrace	Y	91.92	38.75 *	43.44
AGG70164	Tunisia	Landrace	Y	72	37.08 *	52.97
AGG70247	Afghanistan	Landrace	Y	90.42	28.67 *	33.26
AGG70249	Ethiopia	Landrace	Y	70.58	19.00 *	27.73
Boomer	Australia	Advanced Cultivar	N	78.25	34.83 *	45.07
Cassab	Australia	Advanced Cultivar	N	79.67	52.33 *	66.36*
Digger	Australia	Advanced Cultivar	N	70.14	37.75 *	37.5
ILL 7537	Syria	Landrace	N	79.83	11.58 *	14.64*
ILL6002	Syria	Landrace	N	72.21	29.13 *	40.7
ILL6788	Syria	Landrace	N	76.39	26.40 *	39.49
Indian head	Canada	Advanced Cultivar	N	77.67	24.17 *	32.43
Northfield	Jordan	Advanced Cultivar	N	85.81	65.33 *	76.2*
PBA Ace	Australia	Advanced Cultivar	N	87.25	38.25 *	43.88
Precoz	Argentina	Advanced Cultivar	N	65.56	8.78 *	13.49*
p -value, (LSD, $p = 0.05$)						
Al treatment				0.013, (19.8)		
Accessions				<0.001, (15.7)		<0.001, (24.4)

*Indicates the significant mean difference between control and Al treatment for Δ RL, and significant mean difference between ILL6002 with other accessions for RRG% at $p < 0.05$ by Fisher's LSD test.

Online Resources Table 1b. List of the accessions along with country of origin, level of improvement, FIGS information, mean change in root length (Δ RL) of control and Al treatment, and relative root growth (RRG)% in set 2 hydroponic experiment

Accessions	Country of origin	Level of improvement	Belongs to FIGS	Control mean Δ RL	Al treatment mean Δ RL	Mean RRG%
AGG70255	Afghanistan	Landrace	Y	89.78	28.11 *	33.36*
AGG70256	Afghanistan	Landrace	Y	72	35.67 *	51.23
AGG70273	Afghanistan	Landrace	Y	85.44	31.67 *	35.97
AGG70281	Ethiopia	Landrace	N	84.33	38.22 *	45.43
AGG70297	Lebanon	Landrace	Y	86.44	33.56 *	38.65
AGG70334	Nepal	Landrace	Y	62.56	11.22 *	17.58*
AGG70335	Nepal	Landrace	Y	84.33	18.89 *	22.14*
AGG70336	Nepal	Landrace	Y	92	33.44 *	35.88
AGG70337	Nepal	Landrace	Y	75.22	28.89 *	39.6
AGG70338	Nepal	Landrace	Y	71	33.67 *	46.89
AGG70340	Nepal	Landrace	Y	81.56	47.22 *	59.02
AGG70418	Nepal	Landrace	Y	79.44	32.11 *	29.79*
AGG70419	Nepal	Landrace	Y	62.22	21.33 *	34.17*
AGG70465	Ethiopia	Landrace	Y	68.33	19.00 *	28.12*
AGG70526	Ethiopia	Landrace	Y	55.33	16.44 *	28.32*
AGG70527	Ethiopia	Landrace	Y	71.44	16.89 *	24.2*
AGG70530	Ethiopia	Landrace	Y	66.11	10.78 *	16.6*
AGG70568	Morocco	Landrace	Y	74.44	25.33 *	33.99*
AGG70940	Turkey	Landrace	Y	69.17	36.33 *	51.25
AGG70942	Pakistan	Landrace	Y	85.78	26.44 *	30.7*
AGG70949	Lebanon	Landrace	Y	85.44	35.78 *	42.03
AGG70951	Bulgaria	Landrace	Y	83.56	27.89 *	33.48*
AGG70954	Spain	Landrace	Y	96.33	37.67 *	38.24
AGG74249	Nepal	Unknown	Y	70.78	33.44 *	47.43
AGG74250	Nepal	Unknown	Y	67.72	21.56 *	31.19*
AGG74252	Nepal	Unknown	Y	61.67	24.33 *	38.93
AGG74299	Nepal	Unknown	Y	41.33	10.17 *	26.96*
AGG74300	Nepal	Unknown	Y	41.33	14.17 *	24.07*
AGG74305	Nepal	Unknown	Y	69.44	27.56 *	40.34
AGG74325	Nepal	Unknown	Y	84.44	42.11 *	49.71
AGG74328	Nepal	Unknown	Y	76.67	20.22 *	26.08*
ILL6002	Syria	Landrace	N	73.22	37.00 *	51.18
p -value, (LSD, $p = 0.05$)						
Al treatment				0.005, (16.1)		
Accessions				0.014, (14.6)		<0.001, (15.6)

*Indicates the significant mean difference between control and Al treatment for Δ RL, and significant mean difference between ILL6002 with other accessions for RRG% at $p < 0.05$ by Fisher's LSD test.

Online Resources Table 1c. List of the accessions along with country of origin, level of improvement, FIGS information, mean change in root length (Δ RL) of control and Al treatment, and relative root growth (RRG)% in set 3 hydroponic experiment

Accessions	Country of origin	Level of improvement	Belongs to FIGS	Control mean Δ RL	Al treatment mean Δ RL	Mean RRG%
AGG70084	Morocco	Landrace	Y	76.44	25.00 *	32.58
AGG70561	Pakistan	Landrace	Y	71.78	21.33 *	29.92
AGG70566	Pakistan	Landrace	Y	63.89	17.78 *	28.33
AGG74251	Nepal	Unknown	Y	71	19.22 *	27
AGG74257	Nepal	Unknown	Y	48.78	12.00 *	24.76
AGG74258	Nepal	Unknown	Y	66.56	17.44 *	26.13
AGG74259	Nepal	Unknown	Y	67.56	20.33 *	30.06
AGG74265	Nepal	Unknown	Y	71	25.22 *	35.7
AGG74266	Nepal	Unknown	Y	67.67	21.44 *	32.41
AGG74267	Nepal	Unknown	Y	68.44	20.89 *	30.58
AGG74268	Nepal	Unknown	Y	77.33	22.22 *	28.98
AGG74273	Nepal	Unknown	Y	60.33	13.56 *	22.66
AGG74285	Nepal	Unknown	Y	77.33	29.67 *	38.86*
AGG74286	Nepal	Unknown	Y	79.33	24.44 *	30.87
AGG74287	Nepal	Unknown	Y	68.33	19.67 *	29.21
AGG74288	Nepal	Unknown	Y	68.44	15.33 *	22.59
AGG74290	Nepal	Unknown	Y	73.67	18.67 *	24.8
AGG74293	Nepal	Unknown	Y	79.33	24.44 *	30.92
AGG74294	Nepal	Unknown	Y	69.67	20.00 *	28.22
AGG74295	Nepal	Unknown	Y	60.11	11.44 *	19.48
AGG74297	Nepal	Unknown	Y	66.89	21.67 *	32.95
AGG74298	Nepal	Unknown	Y	59.11	13.00 *	22.03
AGG74301	Nepal	Unknown	Y	59.89	18.33 *	30.49
AGG74302	Nepal	Unknown	Y	60.11	16.44 *	26.96
AGG74306	Nepal	Unknown	Y	66.11	24.00 *	36.6
AGG74307	Nepal	Unknown	Y	69.44	26.56 *	38.06
AGG74308	Nepal	Unknown	Y	51.33	18.00 *	35.61
AGG74309	Nepal	Unknown	Y	72.56	25.78 *	35.51
AGG74310	Nepal	Unknown	Y	66.78	16.78 *	25.36
AGG74311	Nepal	Unknown	Y	59.78	14.56 *	25.43
AGG74324	Nepal	Unknown	Y	76.11	18.89 *	24.69
ILL6002	Syria	Landrace	N	83.78	23.66 *	28.5
p -value, (LSD, *p* = 0.05)						
Al treatment				0.002, (9.5)		
Accessions				0.006, (8.0)		0.021, (10.1)

*Indicates the significant mean difference between control and Al treatment for Δ RL, and significant mean difference between ILL6002 with other accessions for RRG% at $p < 0.05$ by Fisher's LSD test.

Online Resources Table 1d. List of the accessions along with country of origin, level of improvement, FIGS information, mean change in root length (Δ RL) of control and Al treatment, and relative root growth (RRG)% in set 4 hydroponic experiment

Accessions	Country of origin	Level of improvement	Belongs to FIGS	Control mean Δ RL	Al treatment mean Δ RL	Mean RRG%
AGG70137	Lebanon	Landrace	Y	81.22	35.22 *	43.1*
AGG70530	Ethiopia	Landrace	Y	66.22	10.89 *	16.96*
AGG74327	Nepal	Unknown	Y	71.56	10.56 *	14.75*
AGG74329	Nepal	Unknown	Y	75.89	12.00 *	15.79*
AGG74330	Nepal	Unknown	Y	64.89	11.22 *	17.55*
AGG74331	Nepal	Unknown	Y	79	12.00 *	15.24*
AGG74335	Nepal	Unknown	Y	66.33	14.56 *	22.25
AGG74341	Nepal	Unknown	Y	87.33	9.56 *	14.07*
AGG74343	Nepal	Unknown	Y	82.17	13.78 *	20.93
AGG74346	Nepal	Unknown	Y	70.11	11.44 *	16.72*
AGG74348	Nepal	Unknown	Y	74.78	13.78 *	18.43
AGG74354	Nepal	Unknown	Y	73.67	14.89 *	20.27
AGG74356	Nepal	Unknown	Y	74.22	12.00 *	16.48*
AGG74357	Nepal	Unknown	Y	78.56	12.78 *	16.31*
AGG74358	Nepal	Unknown	Y	76.33	11.33 *	15.14*
AGG74359	Nepal	Unknown	Y	71.89	13.11 *	18.61
AGG74360	Nepal	Unknown	Y	77.78	15.67 *	20.05
AGG74362	Nepal	Unknown	Y	63.78	15.00 *	23.45
AGG74363	Nepal	Unknown	Y	77.78	13.00 *	16.84*
AGG74364	Nepal	Unknown	Y	73.89	12.11 *	16.32*
AGG74367	Nepal	Unknown	Y	81.33	10.22 *	12.66*
AGG74369	Nepal	Unknown	Y	67.44	12.44 *	18.57
AGG74370	Nepal	Unknown	Y	66	12.00 *	18.03
AGG74371	Nepal	Unknown	Y	76.33	13.00 *	17.01*
AGG74373	Nepal	Unknown	Y	67.33	17.78 *	26.52
AGG74434	Uzbekistan	Landrace	Y	92.11	16.00 *	17.43*
AGG74436	Uzbekistan	Landrace	Y	78	13.89 *	17.94
AGG74453	Russian Federation	Landrace	Y	74.89	12.00 *	15.99*
AGG75305	France	Landrace	Y	84.67	15.11 *	17.77*
AGG75392	Syria	Breeding line	Y	96.33	38.33 *	39.98*
ILL6002	Syria	Landrace	N	90.89	23.67 *	26.07
Northfield	Jordan	Advanced Cultivar	N	87	53.67 *	61.83*
p -value, (LSD, *p* = 0.05)						
Al treatment				<0.001, (7.9)		
Accessions				<0.001, (7.4)		0.001, (8.2)

*Indicates the significant mean difference between control and Al treatment for Δ RL, and significant mean difference between ILL6002 with other accessions for RRG% at p <0.05 by Fisher's LSD test. a = Indicates average RRG% was made from different sets to define tolerance classification, Y = yes, N = No, Tolerance classification based on RRG% from set 1-4: VS = Very sensitive (RRG% 0-21), S = Sensitive (RRG% 22-31%), MT = Moderately tolerant (RRG% 32-41), T = Tolerant (RRG% 42-51) and VT = Very tolerant (RRG% >52), accessions, ILL6002, Northfield, AGG70137 and AGG70530 were used repeatedly in different sets.

Online Resources Table 1e. Mean change in root length (Δ RL) and relative root growth (RRG)% of the subset of 15 lentil accessions under hydroponic screening

Accessions	Control mean Δ RL	Al treatment mean Δ RL	Mean RRG%
Northfield	69.45	56.50 NS	86.07*
AGG70137	61.61	41.02 NS	73.47*
AGG70281	57.12	23.86 *	47.2*
AGG70256	56.38	19.19 *	38.34
AGG74249	54.03	15.69 *	40.29
AGG74268	56.02	18.83 *	52.02*
ILL6002	70.88	22.50 *	39.28*
AGG70561	56.6	18.80 *	38.11
AGG74287	50	15.27 *	36.77
AGG74259	53.36	15.19 *	32.84
AGG74341	50.9	17.75 *	46.29*
AGG70334	59.85	18.80 *	35.87
AGG74367	65.53	18.88 *	36.86
AGG70530	55.9	12.91 *	24.48*
Precoz	69.86	15.55 *	24.93*
p -value, (LSD, p = 0.05)			
Al treatment		0.014, (25.7)	
Accessions		<0.001, (6.5)	<0.001, (16.6)

*Indicates the significant mean difference between control and Al treatment for Δ RL, and significant mean difference between accessions with respect to Precoz and AGG70530 for RRG at p <0.05 LSD test

Online Resources Table 2. Fold change in Al treatment compared to control for Haematoxylin, Evans blue and malondialdehyde content in subset of 15 lentil accessions

[a] Tolerance class	Accessions	control fold change	Fold change for Hematoxylin	Fold change for Evans blue	Fold change for MDA
VT	Northfield	1	1.6	1.5	1
VT	AGG70137	1	2.5 *	1.3	1.1
T	AGG70281	1	3.3 *	1.3	0.7
T	AGG70256	1	3.2 *	1.6	1.4
T	AGG74249	1	3.0 *	2.2 *	1.4
MT	AGG74268	1	3.5 *	3.5 *	1.5
MT	ILL6002	1	3.4 *	2.6 *	1.3
MT	AGG70561	1	2.2 *	1.9	1.2
MT	AGG74287	1	3.4 *	2.5 *	0.7
S	AGG74259	1	3.5 *	2.9 *	1.2
S	AGG74341	1	4.0 *	2.7 *	1.5 *
S	AGG70334	1	3.9 *	4.6 *	1.9 *
S	AGG74367	1	3.9 *	3.5 *	2.2 *
VS	AGG70530	1	4.9 *	4.8 *	2.0 *
VS	Precoz	1	4.1 *	4.3 *	3.6 *
p -value (LSD, p = 0.05)					
	Al treatment		0.011 (1.0)	<0.001 (1.1)	0.002 (0.8)

*Indicates significant difference between control and Al treatment at $p < 0.05$ LSD test

[a] Tolerance classification based on RRG% from set 1-4: VS = Very sensitive (RRG% 0-21), S = Sensitive (RRG% 22-31%), MT = Moderately tolerant (RRG% 32-41), T = Tolerant (RRG% 42-51) and VT = Very tolerant (RRG% >52)

CHAPTER 4: Understanding the genetic diversity and population structure in lentil enables the trait dissection for Aluminium toxicity tolerance

Chapter preface

This Chapter uses the established high throughput hydroponic screening method for Al toxicity tolerance screening from the Chapter 2 and phenotyped larger number of diverse accessions for Al toxicity tolerance. These accessions were genotyped by GBS technology that identified high-quality SNP markers and enabled the identification of population structure, genetic diversity and genomic differentiations in the phenotyped lentil collection. Such information is helpful for breeders to select genetically diverse parental lines for crossing and germplasm characterisation. This Chapter also identifies the Al tolerance linked markers by GWAS which could help in MAS and Al tolerance breeding.

Publication details

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Statement of authorship

Statement from co-author confirming the contribution of the PhD candidate:

"As co-author of the manuscript 'Understanding the Genetic Diversity and Population Structure in Lentil Enables the Trait Dissection for Aluminium Toxicity Tolerance

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Dr Tim Sawbridge 16/11/2020

Abbreviation: AGG, Australian grains genebank; FDR, false discovery rate; FIGS, Focused Identification Germplasm Strategy; GBS, Ggenotyping-by-sequencing; GD, gene diversity; GWAS**,** genome wide-association study; LD, linkage disequilibrium; MAF, maximum allelic frequency; MLM, mixed linear model; NJ, neighbour-joining; PIC, polymorphism information content; QTL, quantitative trait loci; SNP, single nucleotide polymorphism

Abstract

Cultivated lentil (*Lens culinaris* Medik.) is one of the important grain legumes grown worldwide with a relatively narrow genetic base which limits the crop productivity. Assessment of genetic diversity, population structure, and genomic differentiation in cultivated gene pool of lentil helps breeders to select elite germplasm for crop improvement. This information offers multiple opportunities for downstream analysis such as selecting accessions for training sets to be used in genomic prediction or genome wide association studies (GWAS) to dissect the genetic basis of key agronomic traits, and biotitic and abiotic stress tolerance traits. In the present study, a diverse lentil collection of 386 accessions majorly comprising of landraces was genotyped using genotyping by sequencing approach, that resulted in 65,874 high confidence Single Nucleotide Polymorphism (SNP) markers. These SNPs were distributed across seven chromosomes with an average of 9,410 SNPs per chromosome. Genetic diversity and population structure analysis highlighted presence of six subpopulations. Geographical origin of the accessions was the main determining factor in clustering of these accessions into subgroups. Highest genetic differentiation was observed between accessions of Ethiopian origin and accessions originating from Asia (mainly from Nepal). The genetic differentiations in terms of fixation index (Fst) at individual SNP loci allowed the identification of distinctive, subpopulation specific alleles as molecular keys for assigning the germplasm to specific groups. This diverse collection was further used to perform GWAS for Aluminium toxicity tolerance in lentil using the relative root growth phenotypes obtained from hydroponic assays. A compressed Mixed Linear Model (MLM)

model identified potential Quantitative Trait Loci (QTL) region on chromosome 6 with nine significant SNPs associated to Al tolerance traits. A total of 16 candidate genes related to Al tolerance were identified in the vicinity of these SNP loci. The potential QTL region detected four major haplotypes in our population, among which Hap3 and Hap4 were only present in the landraces and associated with the higher Al tolerance. Our findings provide valuable information for selecting lentil accessions with different haplotypes for Al toxicity tolerance to be used in breeding programmes. It has also provided insights into the genetic control of Al tolerance, and the genetic and genomic resources developed here could be used to accelerate development of Al tolerant lentil cultivars.

4.1 Introduction

Lentil belongs to the genus *Lens* of the Leguminosae family and is a diploid (2n-2x=14), self-pollinating crop with a genome size of c. 4 Gbp (Arumuganathan & Earle, 1991). It is one of the earliest domesticated pulse crops, domesticated in Southwest Asia and the Mediterranean region (Dhuppar et al., 2012; Cokkizgin & Shtaya, 2013). Lentil is a high-value, nutritious annual crop cultivated worldwide due to its rich source of protein, minerals (K, P, Fe and Zn) and vitamins (Bhatty, 1988). Lentil is cultivated globally as a rainfed crop in more than 52 countries, with world production of around 7.59 Mt from 6.58 Mha (FAOSTAT, 2017).

Assessments of genetic diversity and the relationships among/within different species of lentil are of great importance for facilitating reliable documentation and utilization of new genetic resources for crop improvement (Hamwieh et al., 2009). This is particularly valuable in cultivated lentil species where a narrow genetic base is reported (Khazaei et al., 2016). Lentils domestication has not only reduced the genetic diversity compared to their wild relatives (Alo et al., 2011) but also decreased the level of resistance to biotic and abiotic stresses (Wong et al., 2015; Malhotra et al., 2019). Hence, there is a critical need to broaden the genetic base of cultivated lentil by introgression of diverse genes available in adapted

landraces or distantly related wild *Lens* taxa. Successful attempts have been made to transfer desirable traits/genes from wild lentils belonging to the primary gene pool (*L. culinaris, L. orientalis, and L. tomentosus*) into the background of cultivated lentils (Singh et al., 2018b). In such crosses, the fertility of the hybrids varied with the final chromosome pairing and chromosome arrangement within themselves (Ladizinsky, 1979; Singh et al., 2018b). However, limitations related to the cross compatibility and fertility of the progeny can be overcome by using landraces, as crosses made with them will be less cumbersome and most likely improve the resistance and the genetic base without greatly affecting the fertility levels of the progeny (Dissanayake et al., 2020). Thus, identification of favorable genes/alleles from landraces which can be introgressed into elite germplasm sources provide an opportunity for genetic improvement (Khazaei et al., 2016) and genetic gain in terms of vigor and yield (Fu et al., 2014).

Natural agro-biodiversity stored in germplasm collections such as gene banks can make a significant contribution towards successful breeding and the genetic improvement of crop species. The Australian Grains Genebank (AGG) holds numerous lentil accessions (5,254) from different parts of the world including wild species (4.0%) and landraces (54%) (Singh et al., 2018b), along with small proportions of breeding lines (10.0%) advanced cultivars (5.0%) and other unknown types (26.0%). Landraces are accessions of cultivated plants that did not undergo formal crop improvement and predate current cultivars, but are locally adapted, diverse and heterogenous populations (Villa et al., 2005). Lentil landraces have the representative variation of the cultivated species (Toklu et al., 2009; Cristobal et al., 2014) and are a good source of novel genes/traits, that are linked to stress tolerances and nutritional quality (Nadia et al., 2019). For example, lentil landraces from Morocco (Nadia et al., 2019), Greece (Tsanakas et al., 2018) and the Mediterranean region (Singh et al., 2016) represent enormous morphological diversity for seed type and seed nutritional contents. Furthermore, landraces from different agro-environments carry potential genes for tolerance to drought,

heat and cold (Idrissi et al., 2015) and have great potential in breeding for stress tolerant cultivars. Similarly for the biotic stresses, an Ethiopian landrace (IG 207) showed higher and stable resistance to ascochyta blight as compared to the resistant cultivar ILL 7537 from Jordan, indicating the potential use of such landraces in crossing programmes for resistance breeding (Dadu, 2018). The landraces from southeast Anatolia, Turkey were used for single plant selection to develop cultivars (Kafkas and Ozbek), with high yield and high level of winter-hardiness (Aydoğan et al., 2007; Aydoğan et al., 2008). This again emphasizes the crucial role of and in-situ germplasm resources especially landraces in current and future lentil breeding.

Over the years, many DNA-based markers have been used to assess genetic diversity and phylogenetic relationships in the genus *Lens* (Lombardi et al., 2014; Wong et al., 2015; Khazaei et al., 2016; Duygu, 2019; Mbasani-Mansi et al., 2019; Pavan et al., 2019; Dissanayake et al., 2020). Among different markers, SNPs are the most abundant markers across all genomes (Agarwal et al., 2008) and with the recent advances in next-generation sequencing platforms, they have become the preferred choice (Singh et al., 2018b). Among different approaches, genotyping-by-sequencing (GBS) has been proven quite successful for large genomes (Malmberg et al., 2018). Transcriptome-based complexity reduction approach as shown in legume crops such as chickpea (Hiremath et al., 2011) and lentil (Malmberg et al., 2018) were shown to be highly successful. The SNP markers from GBS technology, have also been used to study the population structure in diverse germplasm collections. This data complements knowledge of the origin, pedigree, and breeding histories of the germplasm (Khazaei et al., 2016). Furthermore population structure analysis of the Mediterranean gene pool which holds the largest part of lentil diversity (Toklu et al., 2009; Lombardi et al., 2014; Khazaei et al., 2016), showed that the genetics based clustering closely reflected the geographic patterns and phenotypic traits (Pavan et al., 2019). Knowledge of population structure and genetic diversity of germplasms collections

determined using high density SNP markers from GBS approaches, could further enable and contribute to detailed genome-wide association studies (GWAS) and genomics-assisted breeding.

With the increased availability of genetic resources including a reference genome (Bett, 2016), GWAS has gained more attention in lentil to identify genomic regions of interest for multiple traits. Several GWAS studies have been recently published to dissect important traits in lentil such as seed Fe and Zn content, seed dimensions (Khazaei et al., 2017; Khazaei et al., 2018; Ma et al., 2020) and Aphanomyces root rot resistance (Ma et al., 2020). Even though such studies are relatively new in lentil, they have been a proven method of choice for trait dissection in other crops. GWAS has also been used in field pea (Gali et al., 2019; Beji et al., 2020; Dissanayaka et al., 2020; Jha et al., 2020; Tafesse et al., 2020) and chickpea (Upadhyaya et al., 2015; Upadhyaya et al., 2016a; Upadhyaya et al., 2016b; Parida et al., 2017; Li et al., 2018) for various traits including agronomic, quality and abiotic stress tolerances. Aluminium (Al) toxicity tolerance was well studied by using GWAS approach in many plant species such as winter wheat (Zhou et al., 2007; Cai et al., 2008; Navakode et al., 2014), barley (Zhou et al., 2016), rice (Famoso et al., 2011; Zhang et al., 2016; Zhao et al., 2018; Zhang et al., 2019) and Arabidopsis (Nakano et al., 2020). Lentils are sensitive to Al toxicity which hinders the root growth and hence reduces the water and nutrient uptake, which ultimately limits the crop yield. Development of tolerant varieties is an economic way to overcome this (Al toxicity) abiotic stress. The large genetic and phenotypic variation present within the lentil landrace collections offer unprecedented opportunities to explore genetic diversity and beneficial genes for lentil breeding. Therefore, it is worthwhile to utilise these collections to perform genetic association studies such as GWAS to identify genomic regions associated with key traits of interest.

In this study, a collection of 386 lentil accessions sourced from diverse geographical origins, was characterised using SNP markers to study underlying genetic diversity followed by an

association mapping analysis to assess the potential of this resource as a GWAS panel. Accessions were screened for Al toxicity tolerance traits and used in association mapping analysis. The objectives of the study were: 1) to estimate genetic diversity and population structure 2) to identify molecular signatures of divergence and selection in lentil accessions, and 3) to identify marker-trait associations for Al toxicity tolerance using GWAS.

4.2 Materials and methods

4.2.1 Plant material

A total of 386 lentil (*L. culinaris ssp. culinaris*) accessions, originally collected from 35 different countries, were sourced from the Australian Grains Genebank (AGG), Horsham, Victoria, Australia. (Supplementary Table 4.1). Among these accessions, 282 were landraces mostly from Mediterranean and Asian regions, 22 were advanced cultivars, 2 breeder's lines and 80 were of unknown breeding history. The majority of the accessions (291) were selected based on the seed availability and their geographic origin while the remaining 95 were selected from a Focused Identification Germplasm Strategy (FIGS) for putative acid tolerance (screened for Al toxicity tolerance in Chapter 3).

4.2.2 RNA extraction and sequencing

RNA extraction was performed from 8-10 days old lentil seedlings using the RNeasy plant mini kit (QIAGEN, Hilden, Germany) protocol following manufacturer's instructions. The concentration and quality of RNA was confirmed using a NanoDrop UV-Visible spectrophotometer (Thermo-Scientific, Wilmington, DE, United States) at the wavelengths A260/230 and A260/280 nm. Following the manufacturer's guidelines, the integrity of RNA samples was evaluated using TapeStation 2200 platform with the RNA ScreenTape System (Agilent Technologies, Santa Clara, CA, United States). Paired-end multiplexed library preparation was carried out using SureSelect Strand-Specific mRNA library preparation kit (Agilent Technologies, Santa Clara, CA), following manufacturer's instructions. Libraries were evaluated using highly sensitive D1000 ScreenTape assay (Agilent Technologies),

pooled and quantified using Nanodrop and Qubit (Life Technologies) system. Sequencing data was generated using Illumina (San Diego, CA) Hiseq 3000 (2x 150 bp) system.

4.2.3 Read mapping, SNP calling and filtering

Initial raw sequence data (fastq) was trimmed for adaptor sequences as well as low-quality reads and bases (Q ≤20) using a custom perl script followed by cutadapt v1.4.1 (Martin, 2011). Reads with 3 consecutive unassigned nucleotides (N) were also trimmed and finally any reads shorter than 50 bp in length were removed from the final set. The trimmed high quality sequence data were then aligned to the reference genome of CDC Redberry, Lcu.2RBY (Ramsay et al., 2019) cultivar using STAR aligner (Dobin et al., 2013). SNP calling was performed using SAMtools-mpileup (Li et al., 2009) and VCF tools (Danecek et al., 2011) were further used to identify high-quality SNPs. The SNPs were called while ignoring indels and further filtered for depth (DP ≥ 5), maximum allelic frequency (MAF = 0.05), maximum missing data (60%) and base quality (Q30). Any SNPs with heterozygous sites were also removed from the final set. The basic genetic properties such as gene diversity (GD), polymorphism information content (PIC) and minor allele frequencies were calculated for the final set of SNP markers.

4.2.4 Population structure, genetic diversity analysis and genetic differentiation

Population structure was investigated by using Bayesian model based ADMIXTURE (Alexander et al., 2009) programme. As the ADMIXTURE model assumes independence of SNPs (Zheng & Weir, 2016), markers in strong LD (50Kb window size and r > 0.1) were pruned by using PLINK 1.9 programme (Purcell et al., 2007) that resulted in 28,727 SNPs. Population structure analysis was carried out with this pruned dataset for a number of hypothetical subpopulations (parameter K) ranging from 1 to 10. The optimal K value was obtained by setting the cross-validation parameter to 10 and block bootstrap to 2,000 iterations. The most probable number of subpopulations (optimal K) was inferred by plotting the mean delta K and mean loglikelihood values against the different K values. Admixture

proportions were visualized by using R package, Pophelper version 2.3 (Francis, 2017). Individual accessions were assigned to each subpopulation when the corresponding membership coefficient (Q) value was equal to or higher than 0.6, (Taranto et al., 2020) otherwise they were classified as admixed. Genetic distances for each lentil accession were calculated using Nei's method within the StAMPP package (Pembleton et al., 2013) and mean genetic distance for each subpopulation was calculated as inferred by ADMIXTURE, using Nei's coefficient values. A phylogenetic tree was constructed using the unweighted neighbour-joining (NJ) method, as implemented in the DARwin-6.0.17 (Perrier, 2006).

VCF tools (Danecek et al., 2011) were used to calculate genetic differentiation (Fst) (Weir & Cockerham, 1984) among different subpopulations defined by population structure after filtering the admixtured accessions (Q value <0.6) from each subpopulation. Also, Fst estimates of each subpopulation were compared to the entire population (remaining subpopulations) to evaluate divergent genetic loci (selection signature). Genome wide distributions of these loci were visualized by plotting Fst against chromosome positions. The threshold was set at 0.01% of the top Fst value.

4.2.5 Linkage disequilibrium

The linkage disequilibrium (LD) between all pairs of SNPs was estimated by using squared Pearson's correlation coefficient (r^2) in PLINK. The r^2 values were calculated for a window size of 2,000 kb. Genome wide LD decay analysis was conducted by using 1kb binned average r^2 values within 2000 kb region and visualised using R programme. Similarly, LD decay was estimated for individual chromosomes.

4.2.6 Phenotyping for Al toxicity tolerance

The Al toxicity tolerance response from 291 accessions was examined in hydroponic screening in a split plot design with three replicates and two treatments (control and 5 µM Al treatment) as described in Chapter 3 (for 95 FIGS accessions). In this Chapter, 291

accessions were screened in 12 different hydroponic sets with five check lines (Northfield and AGG70137 as tolerant, ILL6002 as moderately tolerant, and Precoz and AGG70530 as sensitive) in each set of the experiment. These accessions have performed consistently similar in all the experiments by maintaining the tolerance rank. Each set had the 32 accessions with four technical replicates of the seedlings per accession in each replication X treatment combination. Root growth (RG), the measure of change in root length (ΔRL) of the seedling after three days of treatment, was calculated in both control and Al treatment by considering pre and post treatment RL of the seedling. The main root length of pre and post treatment seedlings were measured with ruler in mm. The RG in Al treatment is the combination of Al tolerance and root vigour which is the indirect estimate of Al tolerance (Hede et al., 2002), whereas relative root growth (RRG%) is the relative measurement which is a measure of Al tolerance alone. The RG and RRG% were calculated as follows:

RG = Change in root length (ΔRL) = Post treatment RL – Pre treatment RL

Relative root growth (RRG%) = (RG in Al treatment/ RG in control) x100.

The combined data from 291 accessions and 95 FIGS accessions from this Chapter and from Chapter 4 respectively were analysed in Asreml-R package (version 4) (Butler et al., 2018) by fitting a liner mixed model (LMM). In the model accessions and treatments were set to fixed effect and replicates as random effects, and the statistical significance of fixed and random effects were assessed using Wald's test (Wald, 1943). The resulting predicted mean performance of the accessions for RG in control as well as Al treatment was used to calculate the RRG% which were then used for GWAS analysis of Al tolerance. Accessions were classified as sensitive (0-15%), moderately tolerant (16-30%) and tolerant (>31%) based on their RRG value (Supplementary Table 4.1).

4.2.7 Genome-wide association study and favourable allele identification

The GWAS for Al toxicity tolerance was performed by using 386 lentil accessions and 65,

874 SNPs in GAPIT R-package (version 3.0) (Lipka et al., 2012). The CMLM model was used with PCA and kinship matrix. The optimal number of PCs for each trait were determined through model selection using the Bayesian Information Criterion (BIC), with a maximum of ten PCs tested. Among the ten PCA tested, the first two PC's that explained maximum variation were plotted. Kinship was calculated by Vanraden algorithm and used in GWAS model that accounted for the cryptic relationship between the accessions within subpopulations. The significant threshold of p-value for assessing marker-trait association was calculated based on false discovery rate (FDR = p/average number of SNP) at $p = 0.05$. The SNP allele with positive effect was defined as favourable allele, which increased the Al tolerance. The phenotypic effect (a_i) of the significant SNP loci was estimated by comparing the average phenotypic value of accessions with favourable alleles and without favourable alleles (Su et al., 2019).

4.2.8 Haplotype blocks and searching candidate genes

The LD block on chr6 with significant SNPs was constructed by considering ±2 Mb region on either side of SNPs (Gabriel et al., 2002) using Haploview V4.2 (Barrett et al., 2005).

Occurrence of the different haplotypes and their association with Al tolerance was assessed in lentil subpopulations. These LD blocks ±2 Mb flanking region were searched for the annotation in the lentil reference genome version2 (GFF file) to find the probable set of candidate genes for Al tolerance.

4.3 Results

4.3.1 Genome-wide SNP discovery and distribution

A total of 4,391,469,486 paired end reads were obtained from sequencing 386 lentil accessions with an average of 11,376,863 reads per accession. The raw sequencing data was then filtered to remove adaptor sequences and low quality or short reads, resulting in a high-quality set of over 4,275,841,926 paired end reads. The high-quality sequences were then

mapped to the lentil reference genome v2.0 using STAR aligner that identified 2,401,873 SNPs corresponding to the seven chromosomes of lentil. These SNPs were further filtered on sequencing depth (DP ≥ 5), leaving a set of 136,115 SNPs. The next stage of filtering for quality score (Q ≥ 30), maximum missing data (60%; 124, 463) and minor allele frequency (MAF = 0.05), resulted in final set of 65,874 SNPs. These SNPs were distributed across seven chromosomes with an average of 9,410 SNPs per chromosome and an average marker density of 19.2 SNPs per Mb of the genome. Plotting SNP density and distribution across all chromosomes (Figure 4.1) showed that SNPs are more abundant in the telomeric regions of the chromosome arms compared to the pericentromeric regions. The highest number of SNPs were mapped to chromosome 4 (17.8%, 11768) and chromosome 3 (16.8%, 11088), whereas lowest number were mapped to chromosome 1 (11.7%, 7761) and chromosome 7 (11.9%, 7845). The GD values calculated as expected heterozygosity (He) in the population varied from 0.1 (4,266 SNPs) to 0.5 (9,633 SNPs) with an average of 0.31, while PIC values varied from 0.1 (1,669 SNPs) to 0.4 (15,512 SNPs) with an average of 0.25 (Supplementary Figure 4.1a and b). Most of the markers (14,316 SNPs) had a minor allele frequency in the range of 0.06 to 0.1 (Supplementary Figure 4.1c).

4.3.2 Population structure and genetic diversity

The hierarchical population structure was estimated using model-based ADMIXTURE analysis with a subset of 28,727 SNPs that were obtained after SNP pruning based on LD r2 values. Structure analysis revealed possible sub-populations at K = 2 and K = 6 based on ΔK (Figure 4.2a) and loglikelihood values (Supplementary Figure 4.2). However, the high peak value of ΔK = 6 supported the presence of six major subpopulations (Figure 4.2b).

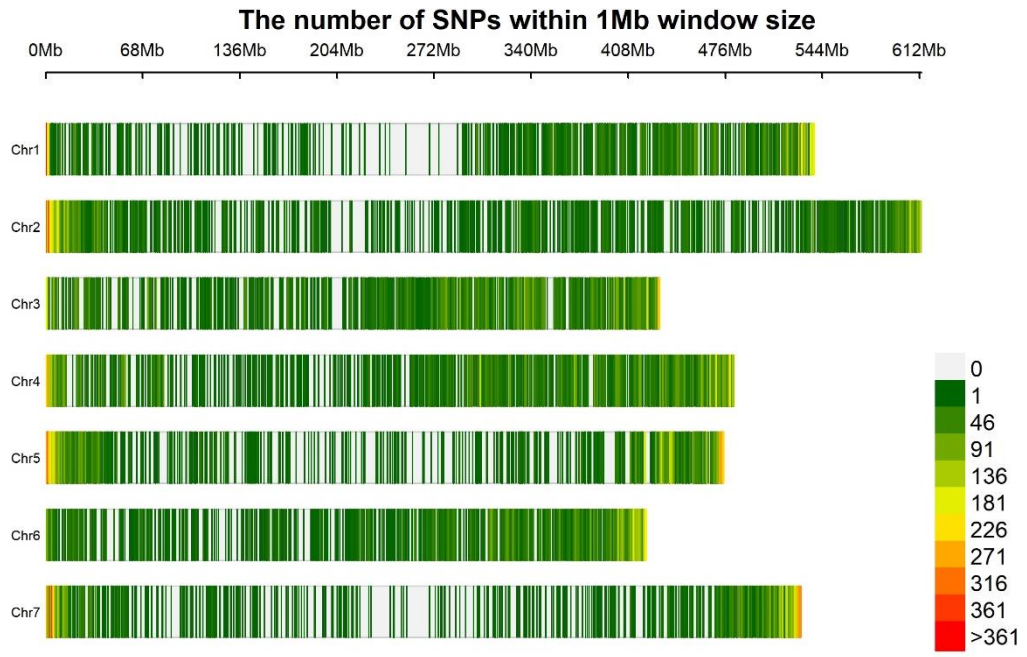


Figure 4.1. Genomic distribution of 65,874 SNP markers across seven lentil chromosomes.

The two subpopulations observed at ΔK = 2, mainly separated the South Asian (India, Nepal, Pakistan and a few from Afghanistan) accessions in subpop2 (n=110) from accessions of other origins in subpop1 (n=228) (Supplementary Table 4.1). Among the six subpopulations observed at ΔK = 6 (Figure 4.2b), five of them were quite distinct in terms of geographical origin. The subpop4 (SA) had a total of 102 accessions that mainly originated from South Asia (India, Nepal and Pakistan) and subpop2 (MED) mostly spanned the Mediterranean region (Jordan, Lebanon and Morocco) with 94 accessions. The subpop5 (ETH) had 27 accessions mainly from Ethiopia, most of the accessions (44) from Afghanistan grouped in subpop3 (AFG) and subpop1 (TUR) represented 60 accessions mainly from Turkey (Supplementary Table 4.1). However, subpop6 (MIX) comprised a total of 59 accessions that were from different geographical origins including Mediterranean, Middle East and Temperate regions. In total, 83% of the accessions were assigned to specific subpopulations and 17% showed admixed ancestry, in which ETH (3%) and SA (6%) had lower admixtures

and TUR had the highest admixture (43%) accessions. The subpopulations were assessed for genetic variation to Al tolerance in the form of RRG% value, in which MED and AFG showed significantly (p <0.001) higher Al tolerance than SA, ETH and MIX (Supplementary Figure 4.3) subpopulations.

Principal components analysis (PCA) was performed to assess population subdivisions. First two PC's (PC1 and PC2) accounted for 22.5% and 10.1% of the variation, respectively and were plotted (Figure 4.2c). PCA revealed that regional adaptation was the main factor contributing to the population structure. PC1 mainly separated SA and AFG accessions from others, while PC2 separated ETH and MED accessions from the rest. Genetic distance (Nei's coefficient) calculated among the accessions ranged from 0.00052 to 0.69356 with mean of 0.38512. The most divergent pair was an accession from Iraq (AGG70286) to another accession from Nepal (AGG74341) with Nei's coefficient value of 0.69356, whereas two accessions (AGG74299 and AGG74302) both originating from Nepal, exhibited the lowest genetic distance (Nei's coefficient value 0.00052). The genetic diversity within subpopulation was highest in MED subpopulation (average genetic distance of 0.2797) followed by TUR (0.2679), while the lowest (0.0569) was observed in ETH (Table 4.1). Further unweighted NJ dendrogram constructed with genetic distance supported the population structure results (Figure 4.3).

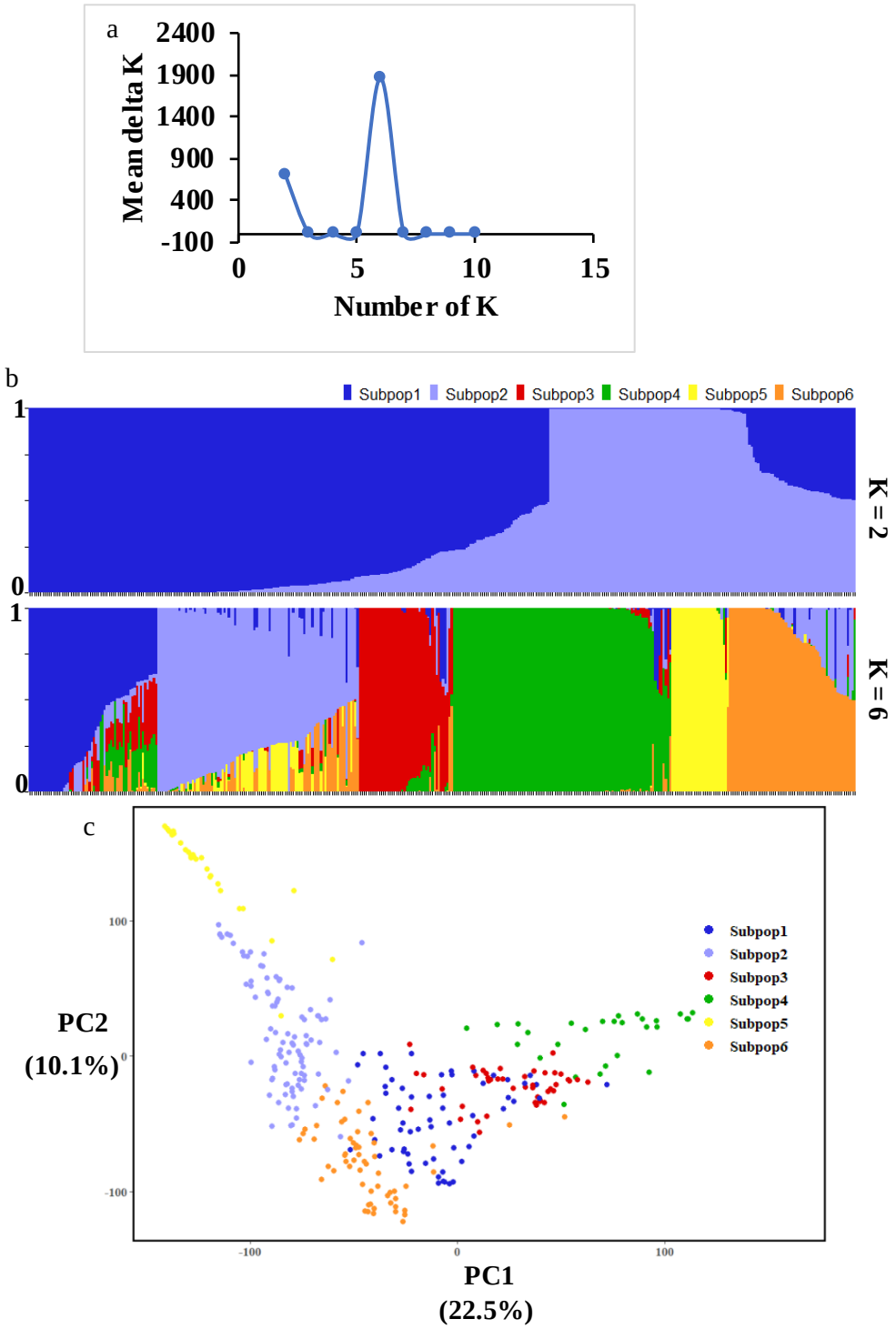


Figure 4.2. Population structure of 386 lentil accessions.

a- Mean delta (Δ)K for different number of subpopulations (K) presenting likely number of subpopulations; b- Estimated subpopulations at K= 2 and K = 6, represented by different colours. The colour of the vertical bar on the x-axis represents the proportion of membership of each accession in each subgroup; c- Principal Components Analysis (PCA) showing spatially distributed accessions in relation to the first two main components. Values in parentheses indicate the percentage of variation explained by each main component and colour code is based on ADMIXTURE population.

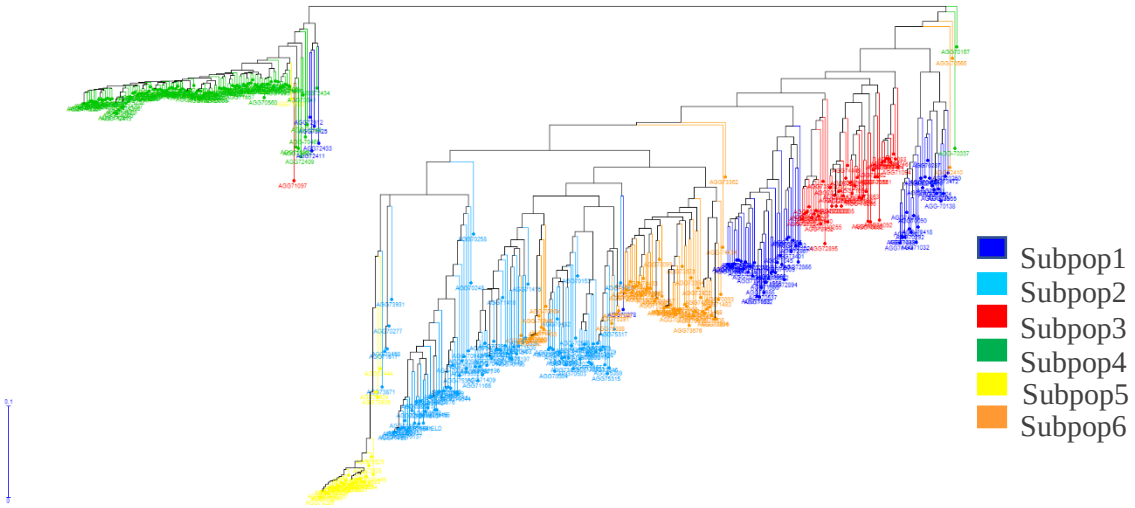


Figure 4.3. Weighted neighbour joining dendrogram created based on genetic distance (Nei's coefficient) calculation from StAMPP in R.

The colour code presents the subpopulations from ADMIXTURE population analysis.

Table 4.1. Pairwise Fst distance matrix, number of SNPs above threshold, and range and mean of the genetic distance (Nei's coefficient) of each subpopulation

	TUR	MED	AFG	SA	ETH	MIX	SNP above threshold	Range and mean of genetic distance
TUR	0						268	(0.0239 - 0.4201) 0.2679
MED	0.26494	0					267	(0.0025 - 0.4645) 0.2797
AFG	0.24925	0.30588	0				306	(0.0066 - 0.3533) 0.2006
SA	0.44472	0.43085	0.33799	0			290	(0.0005 - 0.3197) 0.0616
ETH	0.48569	0.28219	0.49463	0.60579	0		243	(0.0031 - 0.3724) 0.0569
MIX	0.19874	0.23909	0.29649	0.43481	0.46665	0	280	(0.0066 - 0.3533) 0.2196

Subpopulations: TUR (Turkey) = Subpop1, MED (Mediterranean) = Subpop2,
AFG (Afghanistan) = Subpop3, SA (South Asia) = Subpop4, ETH (Ethiopia) = Subpop5,
MIX (Mix of Mediterranean and temperate) = Subpop6

4.3.3 Genome-wide selection signatures and LD decay

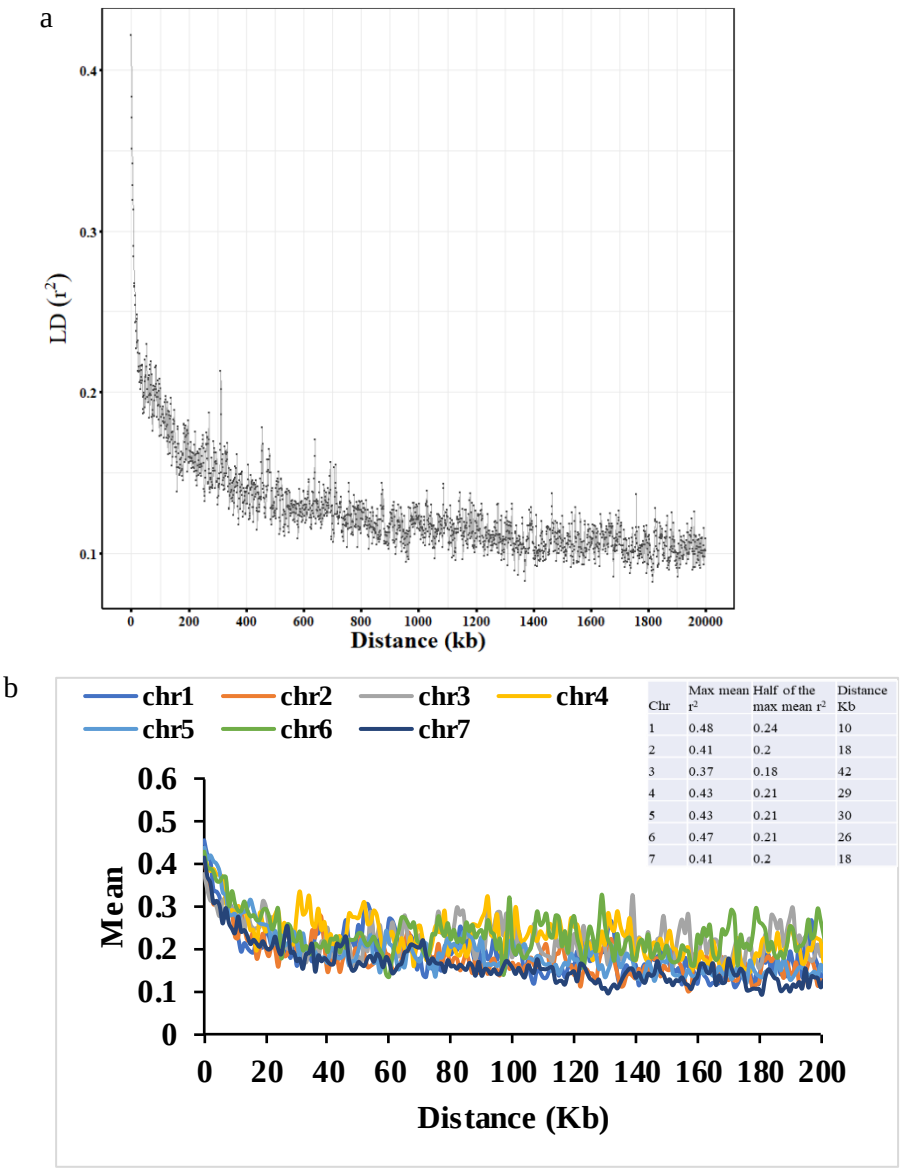
Genetic differentiation was assessed among different subpopulations as well as between each subpopulation and the remaining subpopulations. Genome wide Fst between subpopulations ranged from 0.19874 to 0.60579, with the highest being between ETH and SA and the lowest between TUR and MIX subpopulations (Table 4.1). Similarly, when each subpopulation was independently compared with the remaining subpopulations, the ETH had the highest Fst (0.28665) followed by SA (0.16535), whereas nearly similar level of Fst was observed for AFG (0.13076) and MIX (0.13553) followed by TUR (0.12939) and MED (0.12839). The

SNPs falling within the top 0.01% Fst values were considered as significant and indicators of genomic regions with selection signatures, distributed across the genome (Supplementary Figure 4.4). The largest number of SNP loci (306) with Fst values above the threshold value was detected for AFG, followed by SA subpopulation (290), whereas lowest (243) was detected for the ETH subpopulation (Table 4.1).

The binned r^2 values (LD values) were mapped against the physical distance and defined the LD decay threshold at the distance at which the average of r^2 dropped to half of the maximum values (Lekklar et al., 2019). The average LD for SNPs at 1kb distance from each other was 0.4214 (r^2), that decayed to its half value (~0.210) at around 25 kb (Figure 4.4a). Similarly, LD decay was also estimated for each chromosome which showed most rapid LD decay rate on chr1, 2 and 7, while the slowest rate was observed on chr3 and 5 (Figure 4.4b).

4.3.4 Al toxicity tolerance in diverse lentil collection

We screened this collection for Al tolerance by measuring RG and RRG% as a response to Al toxicity tolerance using hydroponic screening. The control RG displayed the normal distribution (Figure 4.5a) with mean value of 75 mm, whereas Al RG and RRG% showed nearly normal distributions (Figure 4.5b, c) with mean values of 20 mm and 26%. The broad-sense heritability for control and Al RG was 0.4 and 0.59, respectively. In the total lentil collection, nearly 51.5% of the accessions (majority of FIGS set) showed moderate (16 – 30% RRG) levels of Al toxicity tolerance (Supplementary Table 4.1).



Chr	Max mean r²	Half of the max mean r²	Distance Kb
1	0.48	0.24	10
2	0.41	0.2	18
3	0.37	0.18	42
4	0.43	0.21	29
5	0.43	0.21	30
6	0.47	0.21	26
7	0.41	0.2	18

Figure 4.4. Linkage disequilibrium (LD) decay in lentil collection.

a-The whole genome r^2 values from PLINK were first sorted by r^2 values, and then divided into bins of 1kb. The r2 value in each bin were averaged and plotted against the physical distance; b-Chromosome wise LD decay

4.3.5 Genome-wide association study and marker trait effect

The CMLM model used for association mapping, accounted for the population structure and relatedness among the accessions by using the PCA and Kinship matrix (Supplementary Figure 4.5) to reduce false positives. Performance of CMLM model for all the traits was

evaluated by checking quantile plots and comparing the observed p value to the expected p values (Figure 4.6a, c and Supplementary Figure 4.6a).

A total of 10 significant associations for RG in Al treatment and RRG% were identified by the CMLM model that were distributed on chr3 and chr6 (Figure 4.6b, d). Among these, nine SNPs on chr6 were common between RRG% and RG in Al treatment (Supplementary Table 4.2). One significant SNP on chr3 was observed for trait Al RG, which was also observed in RRG% but at a lower *p* value (-log(*p)* = 4.577). However, the CMLM did not detect any significant SNPs for RG in the control treatment (Supplementary Figure 4.6b). The total phenotypic variation explained by common significant SNPs on chr6 for Al RG range from 6.0 to 8.3%, while for RRG% it ranged from 5.2 to 8.4%. Among the common SNPs, SLCU.2RBY.CHR6_42316385 explained the highest phenotypic variation of around 8.4% (Supplementary Table 4.2).

The favourable SNP alleles exhibited significantly different trait means when compared with the unfavourable alleles. The favourable alleles of common SNPs on chr6, significantly increased the Al tolerance to an average of 12.7 RRG%, with highest positive phenotypic effect of around 14% observed by SLCU.2RBY.CHR6_42316382 and SLCU.2RBY.CHR6_42316391 (Figure 4.7, Supplementary Table 4.3). Similarly, increased in Al RG to an average of 12.8 mm was observed with favourable alleles and information about marker trait effect of each SNPs were detailed in Supplementary Table 4.3.

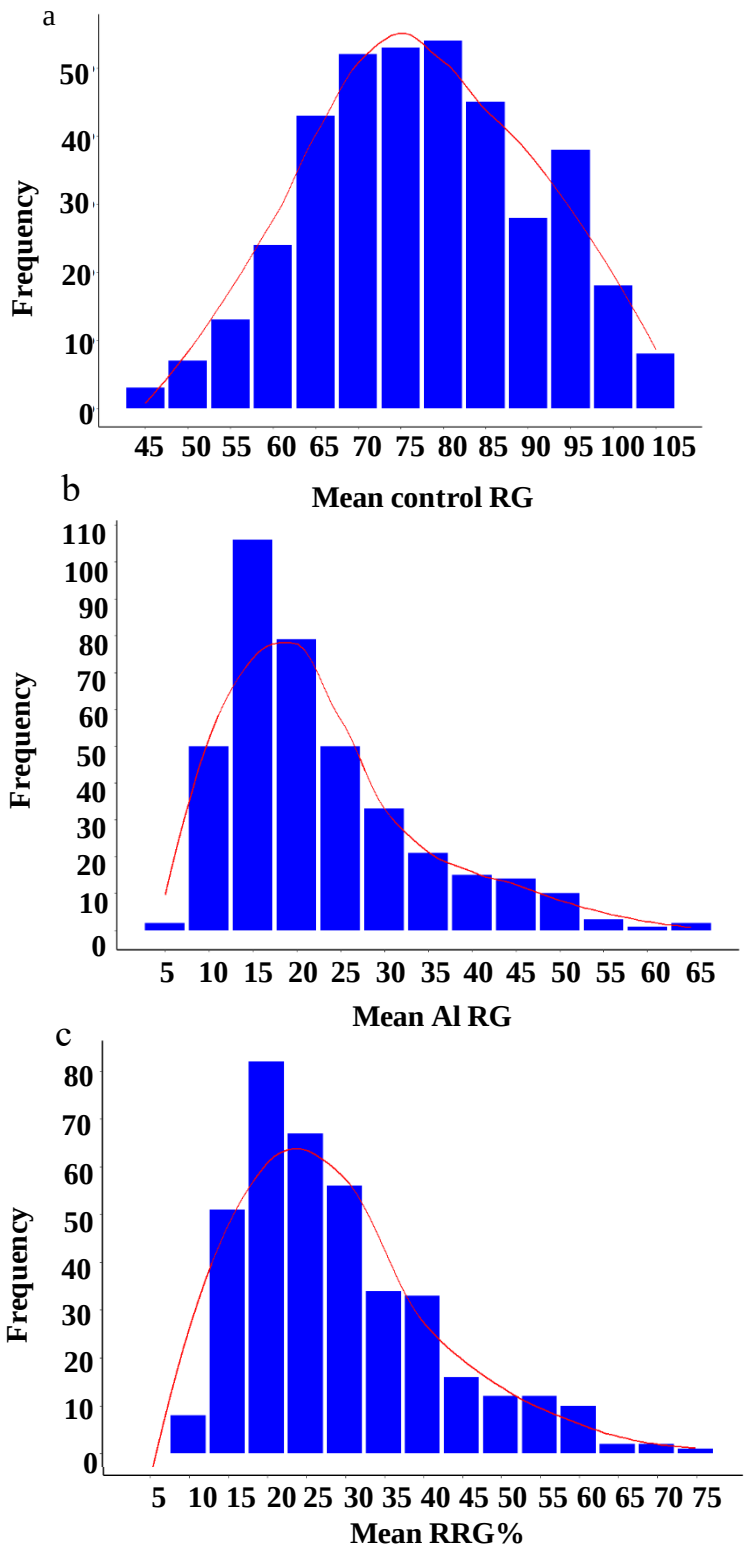


Figure 4.5. Frequency distribution of root growth (RG) and relative root growth (RRG%) of 386 lentil accessions.

a- mean control RG, b- mean Al RG and c- mean RRG%

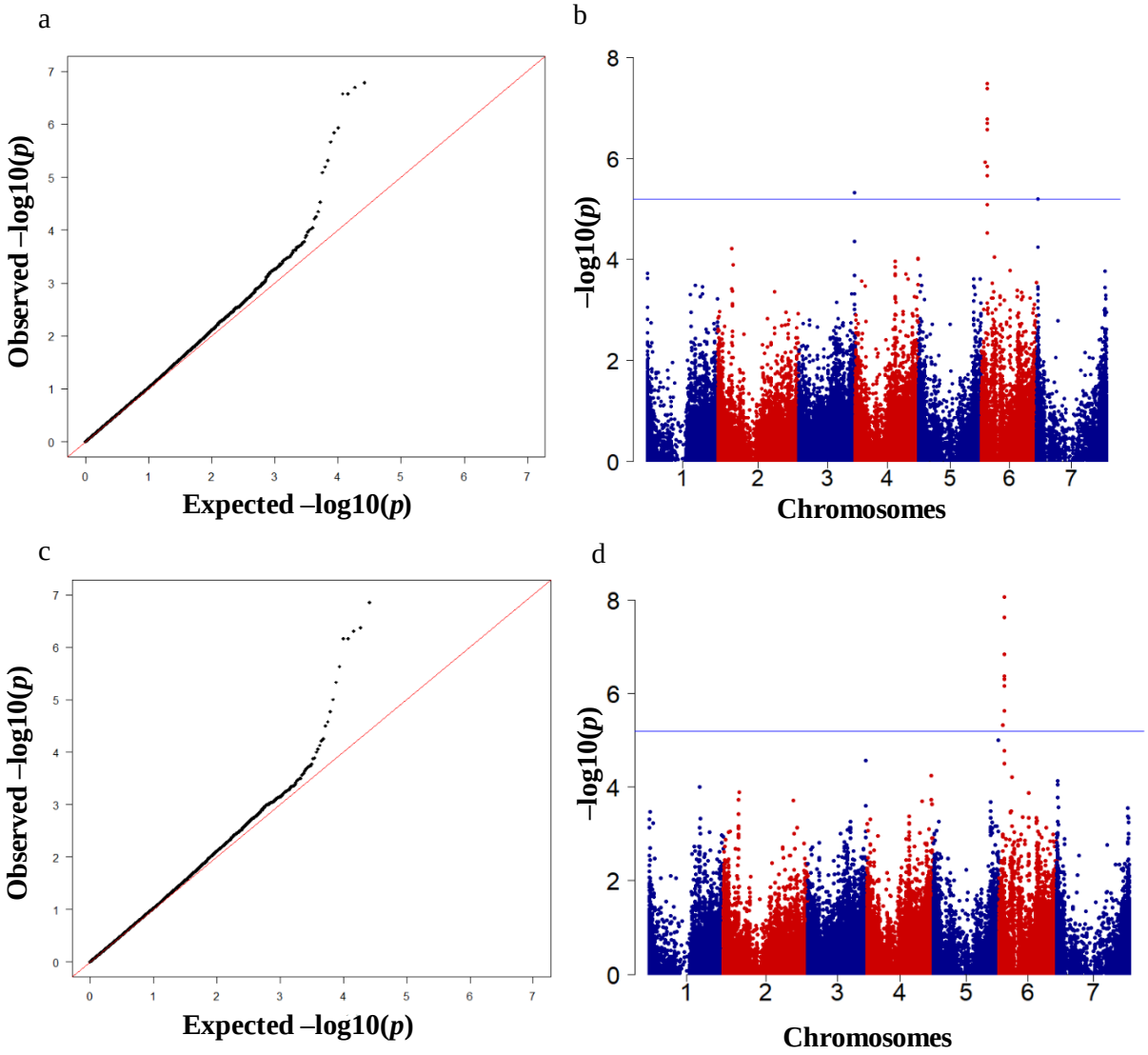


Figure 4.6. Quantile-quantile (QQ) and Manhattan plots of Compressed MLM (CMLM) model.

a, b - RG in Al treatment and c, d - RRG%, Blue line in Manhattan plot shows the FDR = 5.2

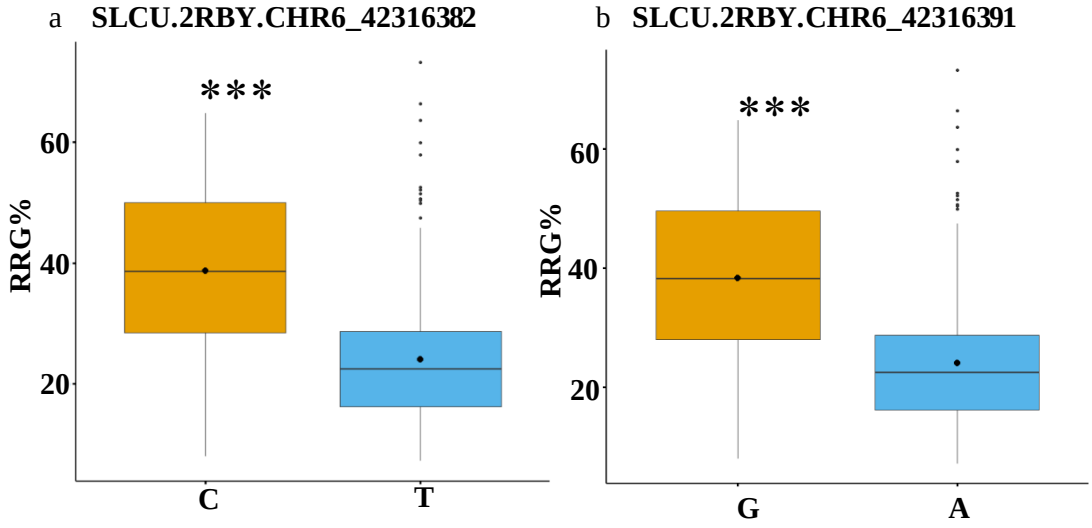


Figure 4.7. Highest allelic effect of significant SNP that are common between Al RG and RRG% on chr6.

For each SNP population was divided into unfavourable (blue) and favourable (orange) allele types which are presented on X axis and RRG% phenotype was presented on y axis. The mean values of each group are indicated by black circles. ***differ at $P < 0.001$ as calculated by Student's t test. RRG% = Relative root growth

4.3.6 Haplotype blocks and haplotypes

Haplotype block analysis showed the presence of six blocks on chr 6 (Figure 4.8a). The eight SNPs that were shown to be associated to both traits Al RG and RRG% were present in a single haplotype block (block 5), covering a 4kb region (Figure 4.8b). A total of four different haplotypes were detected in this region (Figure 4.8c). Hap1 had the unfavourable alleles at all the positions, while Hap2 had all favourable alleles. Hap3 and Hap4 have combinations of favourable and unfavourable alleles with only difference at the SNP, SLCU.2RBY.CHR6_42316385. Hap1 was observed frequently (n = 300 accessions) in all the subpopulations, followed by Hap2 (n = 27) being present in all subpopulations except AFG and ETH. Hap3 (n = 14) and Hap4 (n = 5) were only found in MED and TUR subpopulations, respectively (Table 4.2). The 71% and 60% of the accessions carrying Hap3

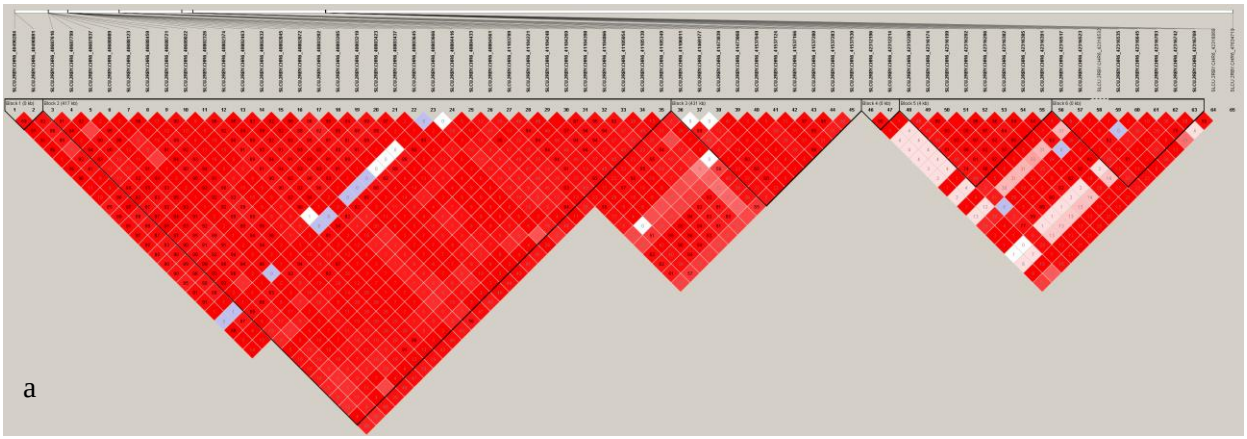
and Hap4 respectively were tolerant and sensitive accessions did not have these haplotypes. Although 21% and 62% of the accessions carrying Hap1 and Hap2 respectively were tolerant, some accessions (24% and 3.7%) with these haplotypes were also sensitive to Al toxicity. Hap3 had significantly higher Al tolerance (as RRG%) than the other haplotypes (Figure 4.9). The reported haplotype block 5 with significant SNPs can be a potential QTL (QTL_AL RG/RRG%) for locating candidate genes for Al toxicity tolerance.

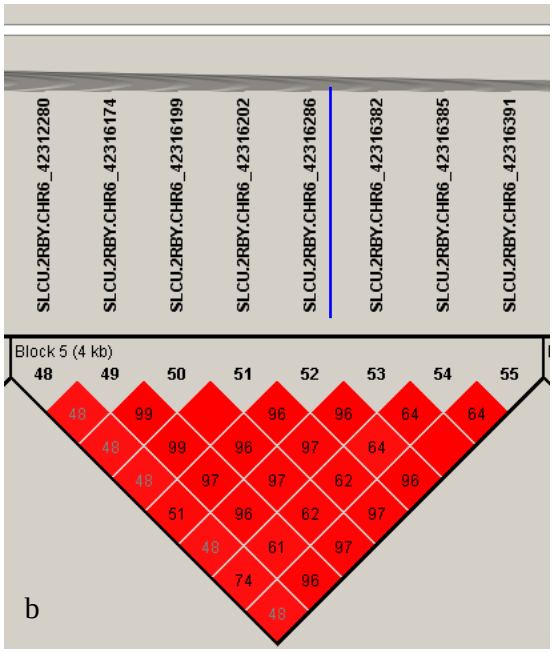
Table 4.2. Observed haplotypes, their total frequency, and occurrence in each tolerance class and subpopulation

Haplotypes	**Frequency**	**S**	**MT**	**T**	**Subpopulations (% accessions having haplotypes)**
Hap1	300	73	163	64	TUR (56.6), MED (63.8), AFG (93.1), SA (95.0), ETH (88.8) and MIX (74.5)
Hap2	27	1	9	17	TUR (21.6), MED (6.3), SA (0.9) and MIX (11.8)
Hap3	14	0	4	10	MED (14.8)
Hap4	5	0	2	3	TUR (6.6)

Tolerance classes ; S = sensitive, MT = moderately tolerant, T = tolerant

Subpopulations: Subpop1 = TUR (Turkey), Subpop2 = MED (Mediterranean), Subpop3 = AFG (Afghanistan), Subpop4 = SA (South Asia), Subpop5 = ETH (Ethiopia), Subpop6 = MIX (Mix of Mediterranean and temperate)





c

	Significant SNP positions							
Haplotypes	42312280	42316174	42316199	42316202	42316286	42316382	42316385	42316391
Favourable alleles	T	T	C	T	T	C	A	G
Hap1	C	C	T	C	A	T	C	A
Hap2	T	T	C	T	T	C	A	G
Hap3	C	T	C	T	T	C	C	G
Hap4	C	T	C	T	T	C	A	G

Figure 4.8. Haplotype block analysis of chromosome 6 and details of haplotypes.

(a) the six haplotype blocks on the whole chromosome, (b) the haplotype block5 containing the 7 significant SNPs that are common between Al RG and RRG%, blue underlined SNP is not significant but present with low *p* (-log (*p*) = 5.0) value, (c) four different haplotypes observed in haplotype block5 along with favourable alleles at each SNP

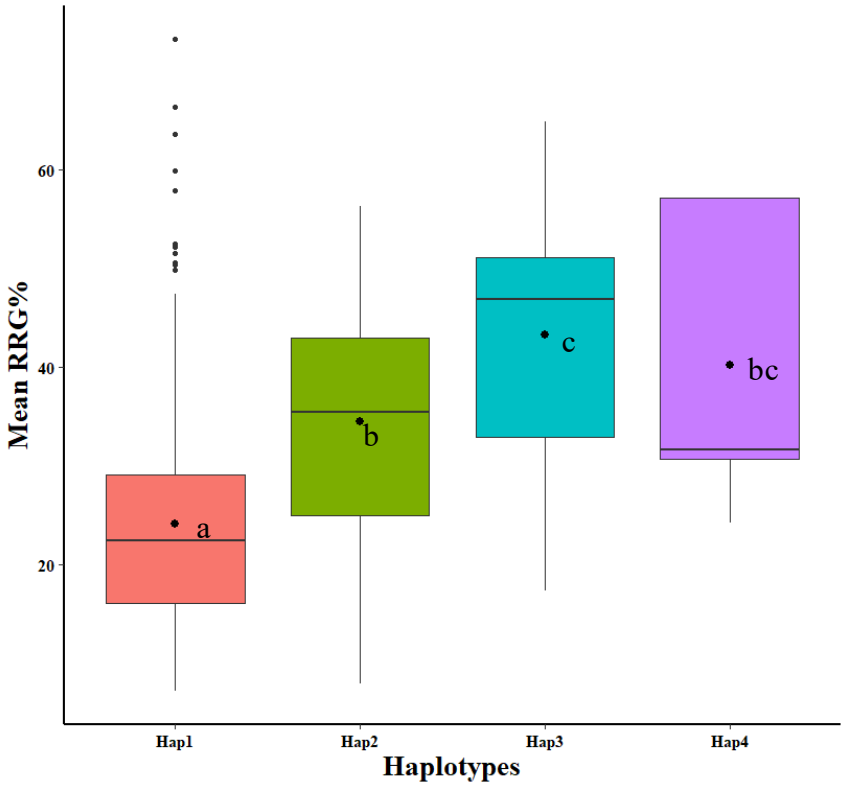


Figure 4.9. Al tolerance as relative root growth (RRG%) of different haplotypes.

Different letters indicate the significant mean RRG% difference between the haplotypes by Fisher protected test (after ANOVA analysis), black dot inside the box plot presents the mean value.

4.3.7 Candidate genes underlying QTLs/SNP

We predicted candidate genes in the vicinity of loci (±2 Mb) that were found to be significantly associated with the trait. The most common type of genes identified were, transmembrane protein, Serine/Threonine kinase, PPR containing plant-like protein, different DUF family proteins and different types of zinc finger proteins (RING, CCCH and GRF). The QTL_Al RG/RRG% on chr6 has around 16 potential candidate genes and on the QTL region on chr3 for Al RG also caries few important which were detailed in Supplementary Table 4.4.

4.4 Discussion

4.4.1 Detection of SNP markers for genetic characterisation

Identification of SNPs using GBS method is a cost-effective approach, that has the ability to identify a large number of high confidence SNPs. Fewer and simpler amplification and clean up steps, and efficient barcoding method compared to other technology has made GBS a unique tool for genomic assisted breeding in crops (Chung et al., 2017; Dissanayake et al., 2020). Using transcriptome based GBS, we identified 65,874 high quality SNPs from 386 diverse lentil collections, that were distributed across seven chromosomes with an average marker density of 19.2 SNPs per Mb of the genome. The observed regions without SNP coverage are generally in the large pericentromeric regions, where repetitive DNA makes it difficult to identify unique flanking regions around SNPs (Otyama et al., 2019; Serba et al., 2019). This could also be due to the nature of the GBS, as it avoids sequencing of the repetitive regions (Wong et al., 2015) resulting in some gaps in such sites. The GD and PIC values are extremely helpful for evaluating level of polymorphisms and usefulness of these markers in assessing genetic diversity in populations. The average GD and PIC for the lentil collection were 0.31 and 0.25, respectively. This indicates the diverse nature of this collection which is likely due to the accessions originating from different geographic regions. The majority of the accessions in this collection predates green revolution related crop improvement and are categorised as landraces which are known to be diverse and heterogenous. The observed GD and PIC values were comparable to an earlier genetic diversity study (GD of 0.4 and PIC of 0.3) that was performed on 394 landraces using 384 map based highly informative SNPs (Lombardi et al., 2014).

4.4.2 Population structure and genetic diversity

Based on the ΔK value obtained from population structure analysis, six potential subpopulations at K = 6 were considered that coincides with PCA and NJ-tree results. Results from these analyses could efficiently explain the sub-clustering in the population is

mostly based on geographical origin of the accessions. Some exceptions were observed in the MIX subpopulation with accessions grouping from Mediterranean as well as Temperate regions. This could be due to a sampling issue as a relatively small number of accessions were sampled from a particular country of origin. Similar to earlier studies (Erskine et al., 1989; Lombardi et al., 2014; Khazaei et al., 2016; Pavan et al., 2019), these six subpopulations highlight the occurrence of geographic stratification between the MED (Jordan, Lebanon and Morocco) and SA (India, Nepal and Pakistan) accessions, suggesting distinct genetic drift events that followed the diffusion of lentil cultivation in easterly and western directions from the centre of origin. Along with this, specific selection pressures due to the differing agro-climatic zones of the Mediterranean and Asian regions might have caused the genetic differentiation (Khazaei et al., 2016). However, in contrast to these reports, in the present study, ETH and AFG accessions were grouped separately from MED and SA subpopulations respectively (Lombardi et al., 2014; Khazaei et al., 2016; Pavan et al., 2019). This separation suggests further specific adaptation of ETH and AFG accessions to their local environmental conditions.

Further, Nei's coefficient value of 0.6935 obtained from the current study, was comparable to an earlier report on genetic diversity assessment from different gene pools of *Lens* (Dissanayake et al., 2020) (Nei's coeff 0.71163). Accessions from ETH and SA subpopulation (mainly Nepalese accessions) showed higher diversity for example single accessions, AGG74341 showed greater distances to multiple (AGG70249, AGG71087 and AGG70250) accessions. The high divergence between ETH and SA subpopulation accessions was also supported by the PCA plot where they formed the distinct clusters. It has been demonstrated that accessions with moderate levels of genetic distance can be easily crossed to improve the traits of interest, as shown in case of Indianhead and Northfield (~0.691 Nei's coefficient) that were crossed to improve the Ascochyta blight resistance (Rodda et al., 2017). The high genetic diversity (mean Nei's coefficient 0.27974 and

0.267955) observed in MED and TUR subpopulations is similar to that reported earlier (Lombardi et al., 2014; Khazaei et al., 2016; Pavan et al., 2019; Dissanayake et al., 2020). This variation could be because of the lentil domestication that has occurred in the eastern Mediterranean in which non-domesticated *Lens* species occur more frequently (Zohary, 1972). Exploring the landraces from MED and TUR subpopulations in the current lentil breeding may be useful as these populations contain high genetic diversity as well as variation to the Al tolerance trait.

4.4.3 Selection signatures and LD

The selection signatures were evaluated based on Fst values. The highest Fst between ETH and SA subpopulations indicated higher genomic differentiation, which could be contributed by their adaptation to the different climatic zones. In agreement with this, accessions from these regions showed marked differences for the pod traits (pod apex and rudimentary tendrils) and seed size in an earlier study (Barulina, 1930; Erskine et al., 1998). This higher differentiation between ETH and SA as compared to AFG and SA, also suggests that introduction of lentil into SA region might be via (Ferguson et al., 1998) Central Asia and Afghanistan. The ETH subpopulation also showed relatively higher genomic differentiation when compared with the rest of the subpopulations. This suggests that migratory and domestication routes for ETH accessions might be different from rest of the accessions. The differing genome-wide pattern of SNP variation (0.01% of top Fst) within each subpopulation signifies a selection signature in that particular subpopulation, indicating which alleles were under adaptive selection. Identification of these targets harbouring selection signatures from the SNP polymorphism data would play an important role in understanding adaptation response to stress, gene discovery and MAS (Serba et al., 2019). Several selection signatures were detected across multiple genomic regions for different subpopulations, ranging from 243 to 306 SNPs. This indicates the possibility to develop molecular tools to assign lentil germplasm to a specific subpopulation, which are of great

significances for germplasm characterisation in genebanks. The highly divergent genetic loci of the ETH subpopulation had more extreme Fst values (>0.80), that could be associated with either natural or artificial selection pressures (Taranto et al., 2020). The search for these molecular selection signatures could be beneficial as generally, loci under selection are associated with useful agronomic traits as reported in durum wheat (Taranto et al., 2020).

The rapid LD decay (~25kb) observed in the population studied might be due to the presence of large numbers of landraces from diverse origins. A similar rapid LD decay rate was also reported in a soybean population containing accessions of diverse geographic origins (Jaiswal et al., 2019; Li et al., 2019). In other lentil studies, landraces from ICARDA/Pullman and Turkey showed rapid LD decay (< 5cM) (Fedoruk, 2013; Duygu, 2019). Landraces often display faster LD decay as these populations have gone through very little selection pressure. Such populations also tend to have more allelic diversity per locus because they did not undergo genetic bottlenecks of selection and domestication (Sharpe et al., 2013). The LD decay pattern in the present lentil collection suggested the potential for high mapping resolution with high density SNP markers. The decay of LD over the genetic distance is an important parameter for determining the number and density of molecular markers that are appropriate for GWAS and selection strategies (Mather et al., 2007).

4.4.4 Genome-wide association of Al toxicity tolerance and candidate genes identification

The evaluation of 386 lentil accessions for Al tolerance in terms of RG and RRG%, classified 59.6% of the accessions as highly Al tolerant (RRG% ≥ 24.8) relative to the known tolerant line (ILL6002), suggesting this collection as potential source for Al tolerance. The observed normal to slightly skewed distribution of the trait data and medium broad-sense heritability (H^2 = 0.4 and 0.5) indicates the quantitative, complex inheritance of the trait. Similar level of broad-sense heritability (0.4 – 0.3) was also reported for net root growth in case of the maize (Krill et al., 2010) for Al tolerance.

We have used a CMLM model which accounted for population structure (Q) and kinship relationship to minimize spurious associations and detected significant marker-trait associations (FDR at $P < 0.05$) mainly on chr6. The identified nine common significant SNPs between Al RG and RRG%, are on ch6 with eight being observed in same haplotype block spanning a 4kb region. This is also a novel region for Al toxicity tolerance, as in an earlier lentil study two QTLs were mapped on linkage group (LG)1 for root regrowth (qAlt_rrg) and fluorescence signals (qAlt_fs) (Singh et al., 2018a). None of the previously reported Al tolerance QTL in lentils overlapped with this region. Abiotic stress tolerance studies in lentils using landraces were very few and mostly using very limited number of accessions F. Majority of the accessions used in current study were not previously used for any genetic analysis and are of diverse geographical origins and might harbour new QTL and alleles of interest. The difference in the detected QTL loci could be because of the differences in the accessions used and/or phenotyping approach.

The phenotypic variation explained by these common SNPs ranged from 5.2 to 8.4% indicating a low to medium level of inheritance, suggesting that the trait might be affected by numerous alleles with minor effects. It is common to detect QTL with low effects in GWAS due to the stringent models used where in majority of the variation is explained by the model and also the model assumes and partitions remaining variation among all several thousands of SNPs used in the analysis. However, surprisingly in our studies we only detected two QTL and they showed moderate effects on phenotype. Similar levels of low to medium inheritance of Al tolerance was also reported in barely (9.7%) (Cai et al., 2013) and rice (11%) (Zhang et al., 2016; Zhao et al., 2018; Zhang et al., 2019), where Al tolerance was assessed as relative longest root growth and relative root elongation.

A number of candidate genes were found in the vicinity of common significant SNPs such as, different transcription factors (Zinc finger types and RWP-RK) which have regulatory roles in development processes along with plant defence responses including Al tolerance

(Yanhui et al., 2006; Daspute et al., 2017). Similar candidate genes such as, PPR proteins, transmembrane, and serine/threonine kinase genes were also reported in Arabidopsis (Tiwari et al., 2014) and Medicago (Chandran et al., 2008) for regulating Al stress responses. The observed UNC-93 domain protein, regulates K^+ translocation from roots to shoots in Arabidopsis, and plays a critical role in abiotic stress tolerance and plant growth by maintaining K^+ homeostasis through ABA-dependent signal transduction pathways (Xiang et al., 2018). Further studies, including sequence analysis, map-based cloning and functional studies are needed to confirm the linkage between these SNPs and gene(s) imparting Al toxicity tolerance

4.4.5 Marker-trait effect of significant SNPs and haplotypes in lentil collection

The favourable alleles showed significant increases in Al tolerance in terms of Al RG and RRG% at each of the common significant SNPs on chr6. The examination of favourable SNP alleles or SNPs with higher phenotypic effects are useful for plant breeders to select the germplasm resources for breeding for Al tolerance. However, in recent years advanced methods of analysis like haplotype based GWAS were efficiently used to capture large number of low effect QTL for complex traits (Contreras-Soto et al., 2017; Xiao et al., 2017). Most of the accessions in the studied lentil collection had Hap1, however around 14.8 and 6.6% of the MED and TUR subpopulation accessions showed the Al tolerant Hap3 and 4 respectively, which were specific to these subpopulations. This indicates geographic distribution of these haplotypes could be the result of specific adaptation of the observed landraces. In contrast to the cultivars which only contained Hap1 and 2, landraces showed all four haplotypes. Use of the landraces with Hap3 that presented the significantly higher Al tolerance compared to others could be beneficial for Al tolerance and could be more widely incorporated into the breeding programmes as parents. The information on haplotypes with phenotypic traits might be useful to select for crossing using molecular

markers in order to accumulate the favourable haplotype variants in breeding lines (Qian et al., 2017).

4.5 Conclusion

In the present study SNPs from GBS have revealed a high level of genome variation within the lentil collection studied. Accessions from the highly differentiated subpopulations with high genetic distance (ETH and Nepal accessions) could be of great interest for lentil genomics and breeding programmes for increasing diversity. Identified selection signature (SNPs) specific to each subpopulation can be used as molecular keys for germplasm characterisation in genebank. Further rapid LD decay in diverse landraces and high marker densities enabled this lentil collection to identify the association of genomic regions related to Al toxicity tolerance by GWAS. The GWAS identified a potential QTL region on chr6 for Al tolerance and identified useful candidate genes that are involved in abiotic stresses/Al tolerances. The landraces with Al tolerant Hap3 and 4 having favourable alleles could be worthwhile to use for the improvement of Al toxicity tolerance in breeding lines. Taken together, this genetically characterised lentil collection can be used for evaluating other agronomic traits which can be further utilised for genomic section programmes. The identified SNP markers for Al toxicity tolerance could be evaluated in new lentil collections to validate these results, with aim to use them in routine lentil breeding programmes.

4.6 References

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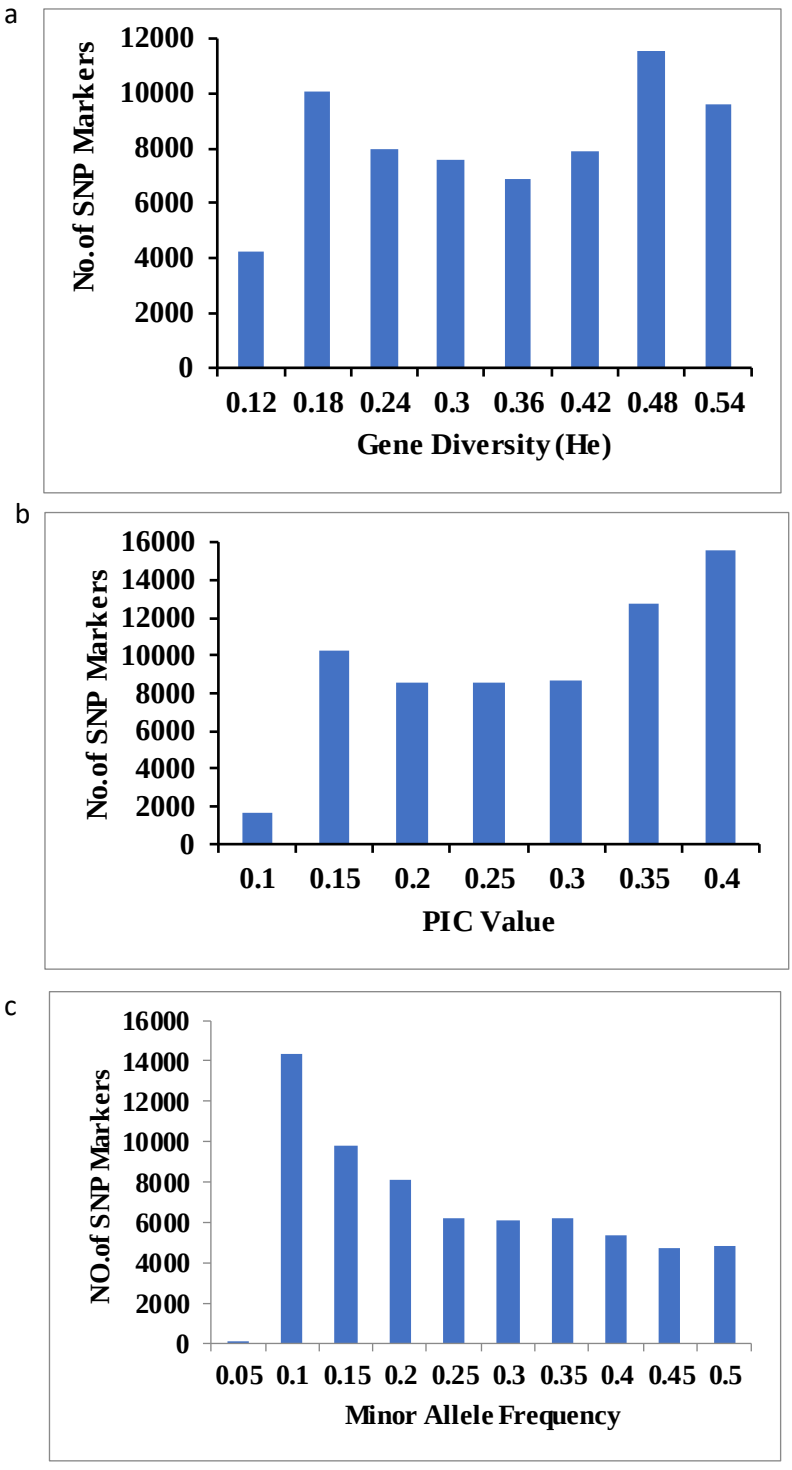
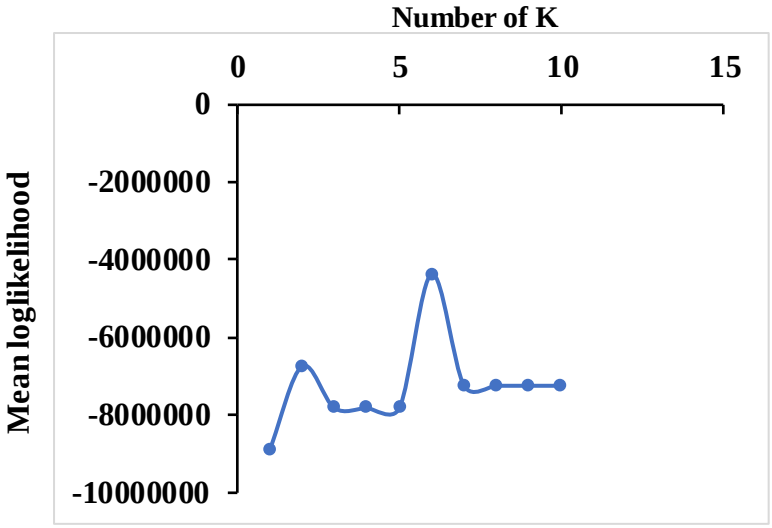
4.7 Supplementary data

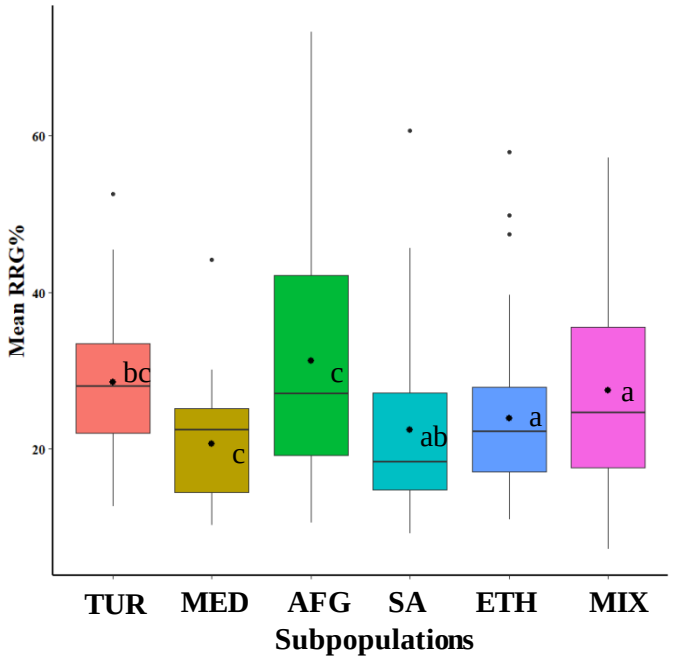


Supplementary Figure 4.1. Frequency distribution of 65,874 SNP markers in 386 lentil accessions.

a- Gene diversity (He); b- Polymorphic information content(PIC); and c- Minor allele frequency

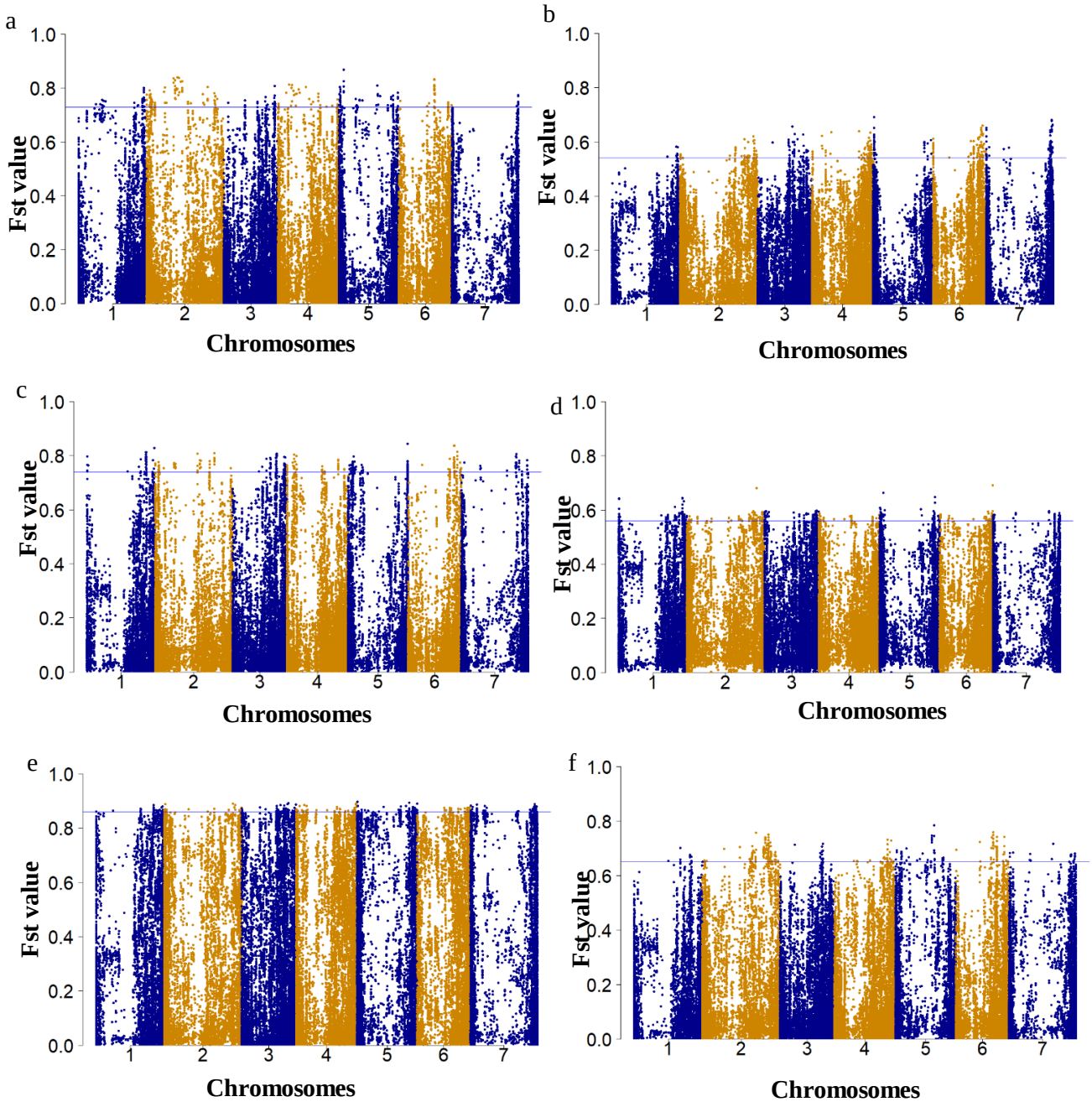


Supplementary Figure 4.2. The mean marginal likelihood value for different subpopulations presenting likely number of subpopulations.



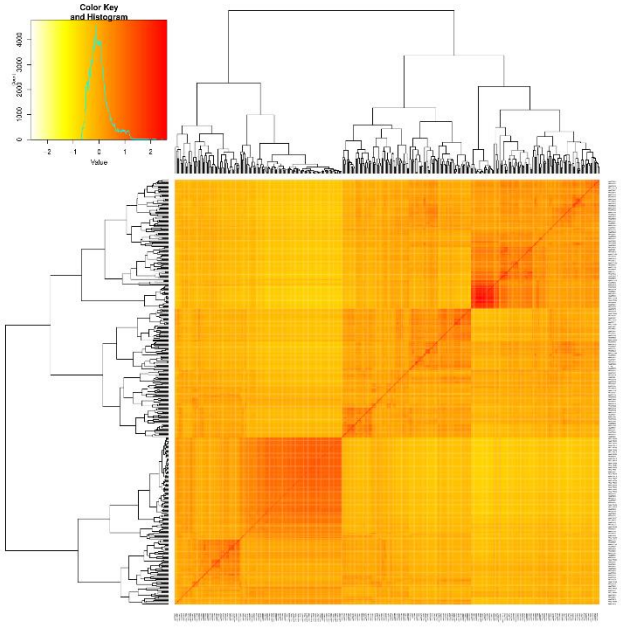
Supplementary Figure 4.3. Al tolerance as relative root growth (RRG%) among the subpopulations from ADMIXTURE analysis.

Different letters indicate the significant mean RRG% difference between the subpopulations by Fisher protected test (after ANOVA analysis), black dots inside the box plot presents the mean value.

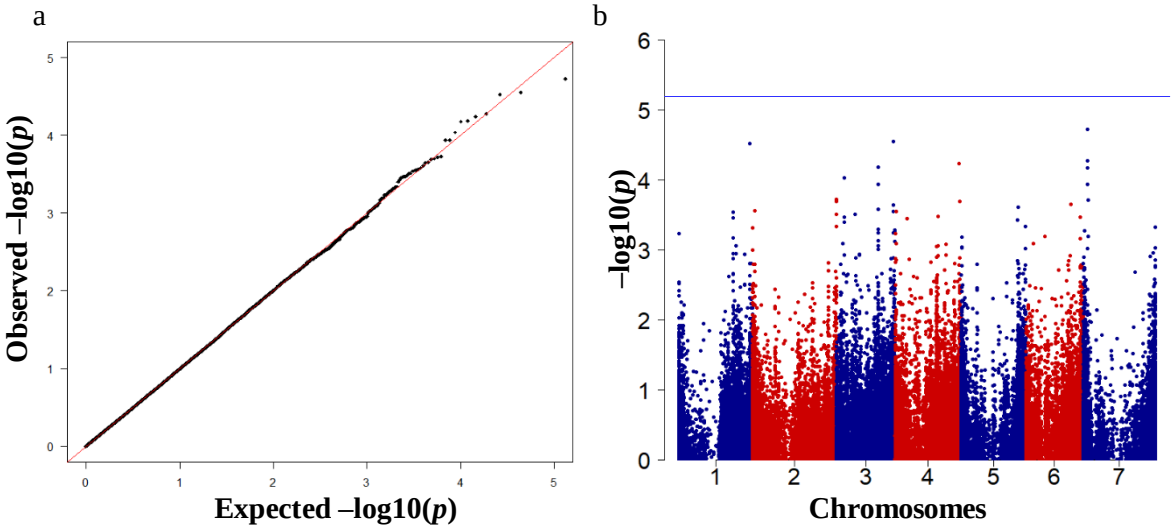


Supplementary Figure 4.4. Genome wide differentiation between the subpopulations calculated from Weir and Cockerham's Fst method and plotted against the chromosomes.

Each point represents the SNP marker and blue horizontal line is threshold line based on top 0.01% Fst vales. a – TUR, b – MED, c - AFG, d – SA, e – ETH, f – MIX



Supplementary Figure 4.5. Kinship matrix of the 386 lentil accessions estimated using SNP data and presented in a heat map.



Supplementary Figure 4.6. Compressed MLM (CMLM) model for control RG.

a - Quantile-quantile (QQ) and b - Manhattan plot. Blue line in Manhattan plot shows the FDR = 5.2

Supplementary Table 4.1. Details of the accessions used in the study along with country of origin, level of improvement (LI), population structures at K = 2 and 6, relative root growth (RRG%), tolerance class and haplotypes

Accessions	Origin	LI	K = 2	K = 6	RRG%	Tolerance	Haplotypes
AGG70001	Argentina	AC	Subpop1	Admixture	19.35	MT	NA
AGG70008	Mexico	L	Subpop1	Subpop6	12.22	S	Hap1
AGG70009	Russian	L	Subpop1	Subpop6	14.22	S	Hap1
AGG70015	Germany	U	Subpop1	Subpop6	22.47	MT	Hap1
AGG70023	Ethiopia	L	Subpop1	Subpop5	27.35	MT	NA
AGG70024	Afghanistan	L	Admixture	Admixture	23.18	MT	NA
AGG70025	Afghanistan	L	Admixture	Admixture	20.52	MT	NA
AGG70034	Egypt	AC	Admixture	Subpop3	25.31	MT	Hap1
AGG70037	Macedonia	L	Subpop1	Subpop6	14.12	S	Hap1
AGG70038	Macedonia	AC	Subpop1	Subpop6	27.9	MT	NA
AGG70044	Lebanon	L	Subpop1	Subpop2	20.42	MT	NA
AGG70045	Tunisia	L	Subpop1	Subpop2	22.62	MT	Hap1
AGG70081	Iraq	L	Admixture	Admixture	17.7	MT	Hap1
AGG70084	Morocco	L	Subpop1	Subpop6	32.7	T	Hap1
AGG70085	Morocco	L	Subpop1	Subpop2	32.81	T	NA
AGG70090	Afghanistan	L	Subpop1	Admixture	21.47	MT	Hap1
AGG70091	Afghanistan	L	Admixture	Admixture	16.72	MT	Hap1
AGG70092	Afghanistan	L	Subpop1	Subpop3	15.93	S	Hap1
AGG70093	Afghanistan	L	Subpop1	Subpop6	24.9	MT	Hap1
AGG70095	Afghanistan	L	Subpop1	Subpop6	17.59	MT	Hap1
AGG70096	Pakistan	L	Subpop2	Subpop4	13.65	S	Hap1
AGG70097	Pakistan	L	Subpop2	Subpop4	18.26	MT	Hap1
AGG70098	Pakistan	L	Subpop1	Subpop2	29.88	MT	Hap3
AGG70099	Egypt	L	Subpop1	Subpop6	24.46	MT	NA
AGG70107	Peru	L	Subpop1	Admixture	29.91	MT	Hap1
AGG70117	Lebanon	L	Subpop1	Subpop2	47.11	T	Hap3
AGG70118	Lebanon	L	Subpop1	Subpop2	29.2	MT	Hap1
AGG70120	Mexico	L	Subpop1	Subpop6	10.51	S	Hap1
AGG70121	Mexico	L	Subpop1	Subpop6	14.74	S	Hap1
AGG70130	Turkey	L	Subpop1	Subpop1	20.65	MT	Hap1
AGG70137	Lebanon	L	Subpop1	Subpop2	52.88	T	Hap3
AGG70138	Turkey	L	Admixture	Admixture	30.65	MT	Hap1
AGG70145	Morocco	L	Subpop1	Subpop6	39.93	T	Hap1
AGG70153	Lebanon	L	Subpop1	Subpop2	17.22	MT	NA
AGG70154	Algeria	L	Subpop1	Admixture	30.82	MT	Hap1
AGG70155	Algeria	L	Subpop1	Subpop2	22.8	MT	Hap1
AGG70156	Algeria	L	Subpop1	Subpop2	17.68	MT	Hap1

Accessions	Origin	LI	K = 2	K = 6	RRG%	Tolerance	Haplotypes
AGG70157	Algeria	L	Subpop1	Subpop2	18.76	MT	Hap1
AGG70163	Tunisia	L	Subpop1	Subpop2	42.16	T	Hap1
AGG70164	Tunisia	L	Subpop1	Subpop2	51.5	T	Hap1
AGG70167	Pakistan	L	Subpop2	Admixture	15.4	S	Hap1
AGG70205	Iran	L	Subpop1	Subpop2	19.09	MT	Hap1
AGG70215	Iran	L	Admixture	Subpop3	29.31	MT	NA
AGG70246	Afghanistan	L	Subpop2	Subpop3	12.64	S	Hap1
AGG70247	Afghanistan	L	Subpop1	Admixture	26.01	MT	Hap1
AGG70248	Afghanistan	L	Subpop1	Admixture	22.47	MT	NA
AGG70249	Ethiopia	L	Subpop1	Subpop5	26.92	MT	Hap1
AGG70250	Ethiopia	L	Subpop1	Subpop5	25.85	MT	Hap1
AGG70255	Afghanistan	L	Admixture	Admixture	31.31	T	Hap1
AGG70256	Afghanistan	L	Admixture	Subpop3	42.56	T	Hap1
AGG70257	Afghanistan	L	Admixture	Subpop3	30.51	MT	Hap1
AGG70258	Afghanistan	L	Subpop1	Admixture	24.87	MT	Hap1
AGG70260	Afghanistan	L	Admixture	Admixture	17.74	MT	Hap1
AGG70266	Afghanistan	L	Admixture	Subpop3	22.58	MT	Hap1
AGG70267	Afghanistan	L	Admixture	Subpop3	26.52	MT	Hap1
AGG70268	Afghanistan	L	Admixture	Subpop3	25.49	MT	Hap1
AGG70269	Afghanistan	L	Subpop1	Subpop3	33.41	T	NA
AGG70272	Afghanistan	L	Subpop1	Subpop1	15.28	S	Hap1
AGG70273	Afghanistan	L	Subpop1	Subpop2	37.06	T	Hap1
AGG70276	Morocco	L	Subpop1	Subpop6	14.7	S	Hap1
AGG70277	Morocco	L	Subpop1	Subpop2	12.45	S	Hap1
AGG70278	Morocco	L	Subpop1	Admixture	24.26	MT	Hap4
AGG70286	Iraq	L	Subpop1	Subpop5	16.98	MT	Hap1
AGG70287	Iraq	L	Admixture	Admixture	14.04	S	Hap1
AGG70291	Pakistan	L	Subpop1	Subpop1	23.49	MT	Hap1
AGG70292	Pakistan	L	Subpop1	Admixture	15.54	S	Hap1
AGG70293	Pakistan	L	Subpop1	Subpop1	7.88	S	Hap1
AGG70297	Lebanon	L	Admixture	Subpop3	38.82	T	Hap1
AGG70299	Lebanon	L	Subpop1	Subpop2	17.33	MT	Hap3
AGG70304	Afghanistan	L	Admixture	Subpop3	16.86	MT	Hap1
AGG70334	Nepal	L	Subpop1	Subpop5	24.64	MT	Hap1
AGG70335	Nepal	L	Subpop1	Subpop5	22.4	MT	Hap1
AGG70336	Nepal	L	Subpop1	Admixture	36.35	T	Hap1
AGG70337	Nepal	L	Admixture	Admixture	38.4	T	Hap1
AGG70338	Nepal	L	Subpop2	Subpop4	47.42	T	Hap1
AGG70340	Nepal	L	Subpop2	Subpop4	57.9	T	Hap1
AGG70375	Jordan	L	Subpop1	Subpop2	10.93	S	Hap1
AGG70376	Syria	L	Subpop1	Subpop2	16.11	MT	Hap1

Accessions	Origin	LI	K = 2	K = 6	RRG%	Tolerance	Haplotypes
AGG70384	Syria	L	Subpop1	Admixture	17.75	MT	Hap1
AGG70398	Syria	L	Subpop1	Subpop2	15.25	S	Hap1
AGG70406	Chile	L	Admixture	Admixture	10.64	S	Hap1
AGG70418	Nepal	L	Subpop1	Admixture	40.42	T	Hap1
AGG70419	Nepal	L	Subpop2	Subpop4	34.29	T	Hap1
AGG70444	Yemen	L	Subpop1	Admixture	23.82	MT	Hap1
AGG70445	Yemen	L	Subpop1	Subpop5	23.39	MT	Hap1
AGG70457	Yemen	L	Subpop1	Subpop6	9.75	S	Hap1
AGG70458	Yemen	L	Subpop1	Subpop2	10.55	S	Hap1
AGG70463	Czech	AC	Subpop1	Admixture	15.48	S	Hap1
AGG70465	Ethiopia	L	Subpop2	Admixture	25.37	MT	Hap1
AGG70470	Jordan	L	Subpop1	Subpop2	45.76	T	Hap1
AGG70472	Jordan	L	Subpop1	Subpop2	20.4	MT	Hap1
AGG70476	Jordan	L	Subpop1	Subpop2	10.73	S	Hap1
AGG70479	Jordan	L	Subpop1	Subpop2	20.95	MT	Hap3
AGG70480	Jordan	L	Subpop1	Subpop2	21.91	MT	Hap1
AGG70481	Jordan	L	Subpop1	Subpop2	13.69	S	Hap1
AGG70482	Jordan	L	Subpop1	Subpop2	17.86	MT	Hap1
AGG70484	Jordan	L	Subpop1	Subpop2	14.51	S	Hap1
AGG70489	Jordan	O	Subpop1	Subpop2	19.49	MT	Hap1
AGG70490	Iraq	O	Admixture	Admixture	8.5	S	Hap1
AGG70491	Morocco	O	Subpop1	Admixture	13.88	S	Hap1
AGG70497	Jordan	O	Subpop1	Subpop2	13.91	S	Hap1
AGG70498	Jordan	O	Subpop1	Subpop2	42.17	T	Hap2
AGG70500	Iraq	O	Subpop1	Subpop2	15.76	S	Hap1
AGG70502	Jordan	O	Subpop1	Subpop2	23.02	MT	NA
AGG70503	Jordan	O	Subpop1	Subpop2	14.61	S	NA
AGG70504	Jordan	O	Subpop1	Subpop2	14.36	S	Hap1
AGG70526	Ethiopia	L	Subpop1	Subpop5	25.89	MT	Hap1
AGG70527	Ethiopia	L	Admixture	Subpop3	23.64	MT	Hap1
AGG70528	Ethiopia	L	Subpop1	Subpop5	16.7	MT	Hap1
AGG70529	Ethiopia	L	Subpop1	Subpop5	30.07	MT	Hap1
AGG70530	Ethiopia	L	Subpop1	Subpop5	15.92	S	NA
AGG70531	Ethiopia	L	Subpop1	Subpop5	22.47	MT	Hap1
AGG70533	Ethiopia	L	Admixture	Subpop3	20.21	MT	Hap1
AGG70534	Ethiopia	L	Subpop1	Subpop5	25.3	MT	Hap1
AGG70536	Cyprus	L	Subpop1	Subpop2	10.76	S	Hap1
AGG70537	Cyprus	L	Subpop1	Subpop1	38.85	T	Hap2
AGG70540	Pakistan	L	Subpop2	Subpop4	12.5	S	Hap1
AGG70541	Pakistan	L	Subpop2	Subpop4	13.91	S	Hap1
AGG70542	Pakistan	L	Subpop2	Subpop4	15.18	S	Hap1

Accessions	Origin	LI	K = 2	K = 6	RRG%	Tolerance	Haplotypes
AGG70543	Pakistan	L	Subpop2	Subpop4	11.97	S	Hap1
AGG70544	Pakistan	L	Subpop2	Subpop4	14.92	S	Hap1
AGG70545	Pakistan	L	Subpop2	Subpop4	16.32	MT	Hap1
AGG70546	Pakistan	L	Subpop2	Subpop4	14.75	S	Hap1
AGG70547	Pakistan	L	Subpop2	Subpop4	12.23	S	Hap1
AGG70548	Pakistan	L	Subpop2	Subpop4	17.54	MT	NA
AGG70549	Argentina	L	Subpop1	Admixture	14.9	S	Hap1
AGG70550	Argentina	L	Subpop1	Admixture	16.53	MT	Hap1
AGG70559	Pakistan	L	Subpop2	Subpop4	17.79	MT	Hap1
AGG70560	Pakistan	L	Subpop2	Subpop4	17.89	MT	Hap1
AGG70561	Pakistan	L	Admixture	Subpop3	31.2	T	Hap1
AGG70562	Pakistan	L	Admixture	Subpop3	37.05	T	Hap1
AGG70564	Pakistan	L	Subpop1	Subpop2	23.93	MT	Hap1
AGG70565	Pakistan	L	Subpop2	Subpop4	21.21	MT	Hap1
AGG70566	Pakistan	L	Admixture	Admixture	27.83	MT	NA
AGG70568	Morocco	L	Subpop1	Subpop2	34.03	T	Hap1
AGG70781	Pakistan	L	Admixture	Admixture	13.15	S	NA
AGG70940	Turkey	L	Admixture	Subpop3	52.53	T	Hap1
AGG70942	Pakistan	L	Subpop1	Admixture	24.01	MT	Hap2
AGG70949	Lebanon	L	Subpop1	Admixture	41.87	T	Hap1
AGG70951	Bulgaria	L	Subpop1	Subpop6	25	MT	Hap1
AGG70954	Spain	L	Subpop1	Admixture	39.1	T	Hap1
AGG70977	Guatemala	L	Subpop1	Subpop2	37.3	T	Hap1
AGG70986	Turkey	L	Subpop1	Subpop1	30.68	MT	Hap4
AGG70991	Turkey	L	Subpop1	Subpop6	9.19	S	Hap1
AGG70994	Turkey	L	Subpop1	Subpop1	15.05	S	Hap1
AGG71009	Turkey	L	Subpop1	Subpop1	35.5	T	NA
AGG71018	Turkey	L	Subpop1	Subpop1	41.31	T	Hap2
AGG71024	Turkey	L	Subpop1	Subpop1	25.11	MT	Hap2
AGG71032	Syria	L	Subpop1	Admixture	25.08	MT	Hap1
AGG71037	Turkey	L	Subpop1	Subpop6	9.55	S	Hap1
AGG71039	Turkey	L	Admixture	Subpop3	19.41	MT	Hap1
AGG71041	Turkey	L	Subpop1	Subpop1	31.16	T	Hap1
AGG71045	Turkey	L	Subpop1	Subpop1	18.89	MT	Hap1
AGG71046	Turkey	L	Subpop1	Subpop1	27.81	MT	NA
AGG71067	Lebanon	L	Subpop1	Subpop2	39.38	T	NA
AGG71081	Ethiopia	L	Subpop1	Subpop6	15.35	S	Hap1
AGG71082	Ethiopia	L	Subpop1	Subpop5	18.89	MT	NA
AGG71083	Ethiopia	L	Subpop2	Subpop3	31.56	T	Hap1
AGG71084	Ethiopia	L	Subpop1	Subpop5	25	MT	Hap1
AGG71087	Ethiopia	L	Subpop1	Subpop5	14.42	S	Hap1

Accessions	Origin	LI	K = 2	K = 6	RRG%	Tolerance	Haplotypes
AGG71091	Afghanistan	L	Admixture	Subpop3	32.76	T	Hap1
AGG71092	Afghanistan	L	Admixture	Subpop3	22.49	MT	Hap1
AGG71093	Afghanistan	L	Subpop1	Subpop1	32.27	T	Hap2
AGG71094	Afghanistan	L	Subpop1	Admixture	26.87	MT	Hap1
AGG71095	Afghanistan	L	Subpop2	Admixture	13.76	S	Hap1
AGG71096	Afghanistan	L	Admixture	Subpop3	14.21	S	Hap1
AGG71097	Afghanistan	L	Subpop2	Subpop3	19.66	MT	Hap1
AGG71099	India	L	Subpop1	Subpop6	13.63	S	Hap1
AGG71100	India	L	Subpop2	Subpop4	18.38	MT	Hap1
AGG71105	Pakistan	L	Subpop2	Subpop4	11.01	S	Hap1
AGG71106	Pakistan	L	Subpop2	Subpop4	25.42	MT	Hap1
AGG71123	Pakistan	L	Subpop2	Subpop4	38.98	T	Hap1
AGG71165	Greece	L	Subpop1	Subpop1	18.09	MT	Hap1
AGG71166	Greece	L	Subpop1	Subpop2	23.86	MT	Hap1
AGG71167	Greece	L	Subpop1	Subpop2	32.05	T	Hap1
AGG71182	Greece	L	Subpop1	Subpop1	24.19	MT	Hap1
AGG71184	Greece	L	Subpop1	Subpop2	32.04	T	Hap1
AGG71195	Greece	L	Subpop1	Subpop6	13.78	S	NA
AGG71196	Greece	L	Subpop1	Subpop2	25.43	MT	Hap1
AGG71197	Greece	L	Subpop1	Subpop2	23.58	MT	Hap1
AGG71222	France	L	Subpop1	Subpop6	16.22	MT	Hap1
AGG71224	Peru	L	Subpop1	Subpop2	17.72	MT	Hap1
AGG71408	Chile	L	Subpop1	Admixture	27.28	MT	Hap1
AGG71409	Chile	L	Subpop1	Subpop2	24.49	MT	Hap1
AGG71410	Chile	L	Subpop1	Admixture	30.32	MT	NA
AGG71415	Chile	L	Subpop1	Admixture	50.6	T	Hap1
AGG71416	Chile	L	Subpop1	Subpop2	20.08	MT	Hap1
AGG71437	Chile	L	Admixture	Subpop3	23.46	MT	Hap1
AGG71438	Chile	L	Subpop1	Subpop6	25	MT	Hap1
AGG71440	Chile	L	Subpop1	Subpop2	21.73	MT	NA
AGG71443	Syria	L	Subpop1	Admixture	43.61	T	Hap1
AGG71444	Syria	L	Subpop1	Admixture	36.3	T	Hap1
AGG71449	Lebanon	L	Subpop1	Subpop2	57.7	T	Hap3
AGG71450	Lebanon	L	Subpop1	Subpop2	51.22	T	Hap3
AGG71451	Lebanon	L	Subpop1	Subpop2	45.52	T	Hap3
AGG71452	Lebanon	L	Subpop1	Subpop2	64.82	T	Hap3
AGG71453	Lebanon	L	Subpop1	Subpop2	50.99	T	Hap3
AGG71456	Lebanon	L	Subpop1	Subpop2	28.72	MT	Hap1
AGG71468	Mexico	L	Subpop1	Subpop6	17.09	MT	Hap1
AGG71471	Mexico	L	Subpop1	Subpop6	18.38	MT	Hap1
AGG71472	Mexico	L	Subpop1	Subpop6	22.65	MT	Hap1

Accessions	Origin	LI	K = 2	K = 6	RRG%	Tolerance	Haplotypes
AGG71483	Bulgaria	L	Subpop1	Subpop6	26.59	MT	Hap1
AGG71529	Turkey	L	Subpop1	Subpop1	56.96	T	NA
AGG71530	Turkey	L	Subpop1	Subpop1	57.16	T	Hap4
AGG71531	Turkey	L	Subpop1	Subpop1	49.57	T	Hap2
AGG71532	Turkey	L	Subpop1	Subpop1	57.2	T	Hap4
AGG71533	Turkey	L	Subpop1	Subpop1	49.11	T	NA
AGG71538	Turkey	L	Subpop1	Subpop2	53.2	T	Hap2
AGG71557	Turkey	L	Subpop1	Subpop1	37.5	T	Hap1
AGG71558	Turkey	L	Subpop1	Subpop1	56.33	T	Hap2
AGG71559	Turkey	L	Subpop1	Subpop1	7.19	S	Hap1
AGG71561	Turkey	L	Subpop1	Subpop1	54.79	T	Hap2
AGG71562	Turkey	L	Subpop1	Subpop1	35.44	T	Hap2
AGG71563	Turkey	L	Subpop1	Subpop1	43.86	T	Hap2
AGG71565	Turkey	L	Subpop1	Subpop1	35.9	T	NA
AGG71575	Russian	L	Subpop1	Subpop6	12.41	S	Hap1
AGG71615	Morocco	L	Subpop1	Subpop6	38.48	T	Hap1
AGG71616	Morocco	L	Subpop1	Subpop2	50.13	T	Hap3
AGG71617	Morocco	L	Subpop1	Subpop2	24.05	MT	Hap1
AGG71618	Morocco	L	Subpop1	Admixture	28.12	MT	Hap1
AGG71619	Morocco	L	Subpop1	Admixture	16.67	MT	NA
AGG71636	Jordan	L	Subpop1	Subpop2	27.7	MT	Hap1
AGG71638	Jordan	L	Subpop1	Subpop2	26.78	MT	Hap1
AGG71640	Jordan	L	Subpop1	Subpop2	59.13	T	NA
AGG71641	Jordan	L	Subpop1	Subpop2	42.18	T	Hap3
AGG71642	Jordan	L	Subpop1	Subpop2	27.97	MT	Hap3
AGG71649	Pakistan	L	Subpop2	Subpop4	17.08	MT	Hap1
AGG71657	Russian	L	Subpop2	Subpop4	26.22	MT	Hap1
AGG71664	Uzbekistan	L	Subpop2	Subpop4	27.18	MT	Hap1
AGG72380	Iran	L	Admixture	Subpop3	19.19	MT	Hap1
AGG72382	Iran	L	Admixture	Subpop3	20.55	MT	Hap1
AGG72402	Brazil	L	Subpop1	Subpop6	14.54	S	Hap1
AGG72404	Brazil	AC	Subpop1	Subpop6	16.69	MT	Hap1
AGG72409	India	L	Admixture	Admixture	12.86	S	Hap1
AGG72410	India	L	Admixture	Subpop6	26.14	MT	Hap1
AGG72411	India	L	Admixture	Admixture	18.08	MT	Hap2
AGG72412	India	L	Subpop2	Admixture	20.02	MT	Hap1
AGG72433	India	L	Subpop2	Admixture	13.78	S	Hap1
AGG72434	India	L	Subpop2	Admixture	18.97	MT	Hap2
AGG72595	India	L	Subpop2	Subpop4	25.24	MT	Hap1
AGG72602	India	L	Subpop2	Subpop4	28.01	MT	Hap1
AGG72603	India	L	Subpop2	Subpop4	29.87	MT	Hap1

Accessions	Origin	LI	K = 2	K = 6	RRG%	Tolerance	Haplotypes
AGG72646	India	L	Subpop2	Subpop4	13.73	S	Hap1
AGG72647	India	L	Subpop2	Subpop4	10.99	S	Hap1
AGG72648	India	L	Subpop2	Subpop4	17.14	MT	Hap1
AGG72654	India	L	Subpop2	Subpop4	17.6	MT	Hap1
AGG72655	India	L	Subpop2	Subpop4	16.86	MT	Hap1
AGG72656	India	L	Subpop2	Subpop4	26.97	MT	Hap1
AGG72657	India	L	Subpop2	Subpop4	25.4	MT	Hap1
AGG72661	India	L	Subpop2	Subpop4	26.45	MT	NA
AGG72865	Iran	L	Subpop2	Subpop4	20.06	MT	Hap1
AGG72866	Iran	L	Subpop1	Subpop1	8.01	S	Hap2
AGG72867	Iran	L	Subpop1	Subpop1	24.8	MT	Hap2
AGG72894	Iran	L	Subpop1	Subpop1	12.06	S	Hap1
AGG72895	Iran	L	Subpop1	Admixture	19.35	MT	Hap1
AGG72901	Iran	L	Admixture	Subpop3	29.95	MT	Hap1
AGG73215	Spain	AC	Subpop1	Subpop6	24.43	MT	Hap1
AGG73218	Spain	L	Subpop2	Subpop4	25.3	MT	Hap1
AGG73362	China	L	Subpop1	Admixture	13.04	S	Hap1
AGG73363	China	L	Admixture	Subpop3	19.17	MT	Hap1
AGG73373	Bulgaria	AC	Subpop1	Subpop6	35.71	T	NA
AGG73375	Bulgaria	L	Subpop1	Subpop6	20.07	MT	Hap1
AGG73379	Russian	L	Subpop1	Subpop2	43.98	T	NA
AGG73381	Bulgaria	L	Subpop1	Subpop6	11.68	S	Hap1
AGG73382	Bulgaria	L	Subpop1	Subpop6	36.52	T	Hap2
AGG73384	Russian	L	Subpop1	Subpop6	23.21	MT	Hap1
AGG73392	Russian	L	Subpop1	Admixture	66.4	T	Hap1
AGG73393	Russian	L	Subpop1	Admixture	35.65	T	Hap1
AGG73394	Russian	L	Subpop1	Subpop6	60.65	T	NA
AGG73395	Russian	L	Admixture	Subpop3	45.4	T	Hap1
AGG73401	Bulgaria	L	Subpop1	Subpop1	24.48	MT	Hap1
AGG73402	Bulgaria	L	Subpop1	Subpop6	27.76	MT	Hap2
AGG73403	Bulgaria	L	Subpop2	Admixture	18.94	MT	NA
AGG73404	Bulgaria	L	Subpop2	Subpop3	37.93	T	Hap1
AGG73425	Pakistan	L	Admixture	Subpop1	31.62	T	Hap4
AGG73427	Spain	L	Admixture	Subpop3	27.74	MT	Hap1
AGG73439	Morocco	L	Subpop1	Subpop2	25.49	MT	Hap1
AGG73443	Morocco	L	Subpop1	Subpop2	52.14	T	Hap1
AGG73452	Morocco	L	Admixture	Subpop3	35.85	T	NA
AGG73456	Morocco	L	Subpop1	Subpop2	46.72	T	Hap3
AGG73468	Pakistan	L	Subpop2	Subpop4	37.41	T	Hap1
AGG73469	Pakistan	L	Subpop2	Subpop4	33.89	T	Hap1
AGG73471	Spain	AC	Subpop1	Subpop2	50.39	T	Hap1

Accessions	Origin	LI	K = 2	K = 6	RRG%	Tolerance	Haplotypes
AGG73472	Spain	AC	Admixture	Admixture	16.27	MT	Hap1
AGG73652	Afghanistan	L	Subpop2	Subpop3	32.8	T	Hap1
AGG73676	Czech	AC	Subpop1	Subpop6	28.55	MT	Hap1
AGG73691	Afghanistan	L	Subpop2	Subpop3	29.27	MT	Hap1
AGG73692	Afghanistan	L	Subpop2	Subpop3	41.32	T	Hap1
AGG73704	Afghanistan	L	Subpop2	Subpop3	33.7	T	Hap1
AGG73846	Australia	AC	Subpop1	Subpop2	38.26	T	Hap2
AGG73852	Jordan	L	Subpop1	Subpop2	63.63	T	Hap1
AGG73871	Turkey	L	Subpop1	Admixture	20.37	MT	Hap1
AGG73909	Ethiopia	U	Subpop1	Subpop5	24.16	MT	Hap1
AGG73914	Ethiopia	U	Subpop1	Subpop5	12.2	S	Hap1
AGG73915	Ethiopia	U	Subpop1	Subpop5	13.07	S	Hap1
AGG73918	Ethiopia	U	Subpop1	Subpop5	13.01	S	Hap1
AGG73924	Ethiopia	U	Subpop1	Subpop5	14.07	S	Hap1
AGG73925	Ethiopia	U	Subpop1	Subpop5	15.66	S	Hap1
AGG73930	Ethiopia	U	Subpop1	Subpop5	10.28	S	Hap1
AGG73931	Syria	U	Subpop1	Subpop2	20.44	MT	Hap1
AGG73937	Syria	U	Subpop1	Admixture	26.21	MT	Hap1
AGG73947	Ethiopia	U	Subpop1	Subpop5	10.89	S	Hap1
AGG74000	Ethiopia	U	Subpop1	Subpop5	14.52	S	Hap1
AGG74016	Syria	U	Subpop1	Admixture	34.91	T	Hap1
AGG74249	Nepal	U	Subpop2	Subpop4	39.26	T	Hap1
AGG74250	Nepal	U	Subpop2	Subpop4	31.83	T	Hap1
AGG74252	Nepal	U	Subpop2	Subpop4	39.46	T	Hap1
AGG74257	Nepal	U	Subpop2	Subpop4	24.6	MT	Hap1
AGG74258	Nepal	U	Subpop2	Subpop4	26.21	MT	Hap1
AGG74259	Nepal	U	Subpop2	Subpop4	29.11	MT	Hap1
AGG74265	Nepal	U	Subpop2	Subpop4	35.52	T	Hap1
AGG74266	Nepal	U	Subpop2	Subpop4	31.69	T	Hap1
AGG74267	Nepal	U	Subpop2	Subpop4	30.52	MT	Hap1
AGG74268	Nepal	U	Subpop2	Subpop4	30.98	MT	Hap1
AGG74285	Nepal	U	Subpop2	Subpop4	38.36	T	Hap1
AGG74288	Nepal	U	Subpop2	Subpop4	22.4	MT	Hap1
AGG74290	Nepal	U	Subpop2	Subpop4	25.34	MT	Hap1
AGG74295	Nepal	U	Subpop2	Subpop4	19.04	MT	Hap1
AGG74297	Nepal	U	Subpop2	Subpop4	32.39	T	Hap1
AGG74298	Nepal	U	Subpop2	Subpop4	21.99	MT	Hap1
AGG74299	Nepal	U	Subpop2	Subpop4	24.6	MT	Hap1
AGG74300	Nepal	U	Subpop2	Subpop4	34.27	T	Hap1
AGG74302	Nepal	U	Subpop2	Subpop4	27.36	MT	Hap1
AGG74305	Nepal	U	Subpop2	Subpop4	39.68	T	Hap1

Accessions	Origin	LI	K = 2	K = 6	RRG%	Tolerance	Haplotypes
AGG74306	Nepal	U	Subpop2	Subpop4	36.3	T	Hap1
AGG74307	Nepal	U	Subpop2	Subpop4	38.24	T	Hap1
AGG74308	Nepal	U	Subpop2	Subpop4	35.06	T	Hap1
AGG74309	Nepal	U	Subpop2	Subpop4	35.53	T	Hap1
AGG74310	Nepal	U	Subpop2	Subpop4	25.12	MT	Hap1
AGG74311	Nepal	U	Subpop2	Subpop4	24.35	MT	Hap1
AGG74324	Nepal	U	Subpop2	Subpop4	24.82	MT	Hap1
AGG74325	Nepal	U	Subpop2	Subpop4	49.87	T	Hap1
AGG74326	Nepal	U	Subpop2	Subpop4	24.05	MT	Hap1
AGG74327	Nepal	U	Subpop2	Subpop4	14.75	S	Hap1
AGG74328	Nepal	U	Subpop2	Subpop4	26.38	MT	Hap1
AGG74329	Nepal	U	Subpop2	Subpop4	15.81	S	Hap1
AGG74330	Nepal	U	Subpop2	Subpop4	17.29	MT	Hap1
AGG74331	Nepal	U	Subpop2	Subpop4	15.19	S	Hap1
AGG74333	Nepal	U	Subpop2	Subpop4	24.05	MT	Hap1
AGG74335	Nepal	U	Subpop2	Subpop4	21.94	MT	Hap1
AGG74341	Nepal	U	Subpop2	Subpop4	21.4	MT	Hap1
AGG74343	Nepal	U	Subpop2	Subpop4	19.47	MT	Hap1
AGG74346	Nepal	U	Subpop2	Subpop4	16.32	MT	Hap1
AGG74348	Nepal	U	Subpop2	Subpop4	18.42	MT	Hap1
AGG74351	Nepal	U	Subpop2	Subpop4	24.05	MT	NA
AGG74353	Nepal	U	Subpop2	Subpop4	24.05	MT	Hap1
AGG74354	Nepal	U	Subpop2	Subpop4	20.21	MT	Hap1
AGG74356	Nepal	U	Subpop2	Subpop4	16.17	MT	Hap1
AGG74357	Nepal	U	Subpop2	Subpop4	16.27	MT	Hap1
AGG74358	Nepal	U	Subpop2	Subpop4	14.85	S	Hap1
AGG74359	Nepal	U	Subpop2	Subpop4	18.24	MT	Hap1
AGG74360	Nepal	U	Subpop2	Subpop4	20.14	MT	Hap1
AGG74362	Nepal	U	Subpop2	Subpop4	23.52	MT	Hap1
AGG74363	Nepal	U	Subpop2	Subpop4	16.71	MT	Hap1
AGG74364	Nepal	U	Subpop2	Subpop4	16.39	MT	Hap1
AGG74367	Nepal	U	Subpop2	Subpop4	19.89	MT	Hap1
AGG74369	Nepal	U	Subpop2	Subpop4	18.45	MT	Hap1
AGG74370	Nepal	U	Subpop2	Subpop4	18.18	MT	Hap1
AGG74371	Nepal	U	Subpop2	Subpop4	17.03	MT	Hap1
AGG74373	Nepal	U	Subpop2	Subpop4	26.4	MT	Hap1
AGG74432	Uzbekistan	L	Subpop1	Subpop6	26.59	MT	Hap2
AGG74434	Uzbekistan	L	Subpop1	Admixture	17.37	MT	Hap2
AGG74436	Uzbekistan	L	Subpop1	Subpop6	17.81	MT	Hap2
AGG74443	Tajikistan	L	Admixture	Subpop3	27.57	MT	Hap1
AGG74445	Tajikistan	L	Admixture	Admixture	26.11	MT	Hap1

Accessions	Origin	LI	K = 2	K = 6	RRG%	Tolerance	Haplotypes
AGG74450	Russian	L	Subpop1	Subpop6	9.68	S	Hap1
AGG74452	Russian	L	Subpop1	Subpop1	31.42	T	Hap2
AGG74453	Russian	L	Subpop1	Subpop6	16.02	MT	Hap1
AGG74463	Uzbekistan	L	Admixture	Subpop3	28.35	MT	Hap1
AGG74585	Bulgaria	U	Subpop1	Subpop6	15.16	S	Hap1
AGG74879	Australia	AC	Subpop1	Subpop2	51.74	T	Hap2
AGG74955	Morocco	U	Admixture	Admixture	17.22	MT	Hap1
AGG75305	France	L	Subpop1	Subpop6	17.85	MT	Hap1
AGG75315	Australia	AC	Subpop1	Subpop2	35.66	T	NA
AGG75317	Australia	BL	Subpop1	Subpop2	59.47	T	NA
AGG75320	Australia	AC	Subpop1	Subpop5	44.13	T	Hap1
AGG75373	Australia	AC	Subpop1	Subpop2	14.87	S	Hap1
AGG75386	Australia	AC	Subpop1	Subpop6	41.91	T	Hap2
AGG75387	Australia	AC	Subpop1	Subpop2	73.25	T	Hap1
AGG75388	Australia	AC	Subpop1	Admixture	45.6	T	Hap2
AGG75389	Australia	AC	Subpop1	Subpop2	33.85	T	Hap2
AGG75391	Australia	AC	Subpop1	Subpop6	34.79	T	NA
AGG75392	Syria	BL	Subpop1	Subpop2	39.79	T	Hap1
ILL6002	Syria	L	Subpop1	Subpop6	24.87	MT	Hap1
Northfield	Jordan	AC	Subpop1	Subpop2	59.94	T	Hap1
Precoz	Argentina	AC	Subpop1	Admixture	15.85	S	Hap1

Bold text accessions are from to FIGS set, S: Sensitive, MT: Moderately tolerant, T:Tolerant,

Advanced Cultivar = AC, Breeder's Line = BL, Landrace = L, Other = O, Unknown = U

Population at K = 2 includes Subpop1 = Other, Subpop2 = South Asia

Population at K = 6 includes Subpop1 = TUR (Turkey), Subpop2 = MED (Mediterranean),

Subpop3 = AFG (Afghanistan), Subpop4 = SA (South Asia), Subpop5 = ETH (Ethiopia),

Subpop6 = MIX (Mix of Mediterranean and temperate),

NA in haplotypes = Not assigned because of ambiguous (N, M,W, Y and R) nucleotides in one of the loci

Supplementary Table 4.2. Summary of the significant SNPs marker identified by CMLM model in GWAS analysis for Al tolerance assessed as root growth (RG) in Al treatment and relative root growth (RGG)%

SNP	Chr	Position	maf	Al RG $-\log_{10}(p)$	Al RG R^2 %	RRG% $-\log_{10}(p)$	RRG% R^2 %
SLCU.2RBY.CHR3_422388794	3	422388794	0.137	5.31	5.60	4.57	4.36
SLCU.2RBY.CHR6_31129993	6	31129993	0.197	5.93	6.36	5.32	5.20
SLCU.2RBY.CHR6_42312280	6	42312280	0.127	7.38	8.19	7.62	7.89
SLCU.2RBY.CHR6_42316174	6	42316174	0.201	6.78	7.43	6.37	6.41
SLCU.2RBY.CHR6_42316199	6	42316199	0.202	6.57	7.16	6.16	6.16
SLCU.2RBY.CHR6_42316202	6	42316202	0.202	6.57	7.16	6.16	6.16
SLCU.2RBY.CHR6_42316382	6	42316382	0.204	6.69	7.32	6.31	6.34
SLCU.2RBY.CHR6_42316385	6	42316385	0.146	7.48	8.32	8.07	8.42
SLCU.2RBY.CHR6_42316391	6	42316391	0.204	5.84	6.25	5.62	5.55
SLCU.2RBY.CHR6_42316645	6	42316645	0.137	5.66	6.03	6.85	6.97

Bold texted SNPs are common between Al RG and RRG%. Significant threshold (-log10(p) = 5.2) calculated by FDR at p =0.05. The percentage of variation explained (R^2) by the SNP was calculated as the difference between the R^2 of the GAPIT model with and without the strongest associated SNP .

Supplementary Table 4.3. Favourable alleles, their phenotypic effects (ai), and representative accessions of significant common SNPs for Al root growth (RG) and relative root growth (RRG%)

SNP	Position	Allele	Favourable allele	Number of accessions	Al RG			RRG%		
					ai for Al RG	Accessions	Mean	ai for RRG	Accessions	Mean
SLCU.2RBY.CHR6_31129993	31129993	C/T	T	22	13 ***	AGG75317 AGG74879 AGG71558	44.02 44.08 50.8	13.8 ***	AGG71558 AGG71529 AGG75317	56.3 56.9 59.4
SLCU.2RBY.CHR6_42312280	42312280	C/T	T	32	11.7 ***	AGG71533 AGG71538 AGG71558	46 49.8 50.8	5.5 ***	AGG71529 AGG75317 AGG73394	56.9 59.4 60.6
SLCU.2RBY.CHR6_42316174	42316174	C/T	T	60	13.5 ***	AGG71558 AGG71452 AGG71640	50.8 53.4 55.7	14.1 ***	AGG71640 AGG75317 AGG71452	59.1 59.4 64.8
SLCU.2RBY.CHR6_42316199	42316199	T/C	C	60	13.4 ***	AGG71558 AGG71640 AGG71640	50.8 53.4 55.7	14.1 **	AGG71640 AGG75317 AGG71452	59.1 59.4 64.8

SNP	Position	Allele	Favourable allele	Number of accessions	Al RG			RRG%		
					ai for Al RG	Accessions	Mean	ai for RRG	Accessions	Mean
SLCU.2RBY.CHR6_42316202	42316202	C/T	T	60	13.4 ***	AGG71558 AGG71452 AGG71640	50.8 53.4 55.7	14.1 ***	AGG71640 AGG75317 AGG71452	59.1 59.4 64.8
SLCU.2RBY.CHR6_42316382	42316382	T/C	C	58	14 ***	AGG71558 AGG71452 AGG71640	50.8 53.4 55.7	14.7 ***	AGG71640 AGG75317 AGG71452	59.1 59.4 64.8
SLCU.2RBY.CHR6_42316385	42316385	C/A	A	40	11.8 ***	AGG71530 AGG71538 AGG71558	48.3 49.8 50.8	12.2 ***	AGG71530 AGG71532 AGG75317	57.1 57.1 59.4
SLCU.2RBY.CHR6_42316391	42316391	A/G	G	57	13.6 ***	AGG71538 AGG71558 AGG71452	49.8 50.8 53.4	14.3 ***	AGG71449 AGG75317 AGG71452	57.7 59.4 64.8
SLCU.2RBY.CHR6_42316645	42316645	G/A	A	33	11.3 ***	AGG71530 AGG71538 AGG71558	48.3 49.8 50.8	11.7 ***	AGG71530 AGG71532 AGG75317	57.1 57.1 59.4

*** significant ap *p* value, ai is calculated as the difference between favourable and unfavourable allele

Supplementary Table 4.4. The genes in the vicinity of significant SNP that are associated with Al tolerance from lentil reference genome

Trait	SNP on chr6	Position	ID/Gene name	Start	End	Annotation
Al RG	SLCU.2RBY.CHR6_42316199	42316199	Lcu.2RBY.6g006490	41325953	41326302	Uncharacterized protein
Al RG	SLCU.2RBY.CHR6_42316202	42316202	Lcu.2RBY.6g006520	41415208	41416241	Uncharacterized protein
QTL_RG/RRG%	**SLCU.2RBY.CHR6_42312280	42312280	Lcu.2RBY.6g006540	41434656	41435023	Serine/Threonine kinase family protein
QTL_RG/RRG%	**SLCU.2RBY.CHR6_42316174	42316174	Lcu.2RBY.6g006550	41436248	41436598	GRF zinc finger protein
QTL_RG/RRG%	**SLCU.2RBY.CHR6_42316382	42316382	Lcu.2RBY.6g006570	41469030	41475797	Cofactor-independent phosphoglycerate mutase
QTL_RG/RRG%	**SLCU.2RBY.CHR6_42316385	42316385	Lcu.2RBY.6g006590	41795437	41798123	Plant regulator RWP-RK family protein
RRG%	SLCU.2RBY.CHR6_42316645	42316645	Lcu.2RBY.6g006620 Lcu.2RBY.6g006650 Lcu.2RBY.6g006680	42097280 42311727 42571590	42097764 42317117 42572193	Integrase, catalytic region Zinc finger, CCHC-type Peptidase aspartic, catalytic Pmr5/Cas1p GDSL/SGNH-like acyl-esterase family protein Transmembrane protein, putative
			Lcu.2RBY.6g006690	42586796	42588271	Ion channel regulatory protein UNC-93
			Lcu.2RBY.6g006700	42589039	42595211	OSBP(Oxysterol-binding protein)-related protein 4C
			Lcu.2RBY.6g006710	42597199	42597975	Beta-1,4-N-acetylglucosaminyltransferase-like protein
			Lcu.2RBY.6g006730	42787234	42788152	Perchloric acid soluble translation inhibitor-like protein
			Lcu.2RBY.6g006740	42876714	42878444	Ulp1 protease family, carboxy-terminal domain protein
			Lcu.2RBY.6g006750	42900731	42901289	Glucose-methanol-choline (GMC) oxidoreductase family protein
			Lcu.2RBY.6g006760	42984553	42999241	AMP-activated kinase, gamma regulatory subunit, putative

Trait	SNP on chr3	Position	ID/Gene name	Start	End	Annotation
Al RG	SLCU.2RBY.CHR3_422388794	422388794	Lcu.2RBY.3g072660	420706263	420709036	DUF630 family protein
			Lcu.2RBY.3g072680	420737929	420739306	DUF241 domain protein
			Lcu.2RBY.3g072710	420848033	420851457	Transmembrane protein
			Lcu.2RBY.3g072740	420927211	420938963	GDP-fucose O-fucosyltransferase-like protein
			Lcu.2RBY.3g072760	420944129	420948823	DUF760 family protein
			Lcu.2RBY.3g072800	421089020	421092837	PPR containing plant-like protein
			Lcu.2RBY.3g072890	421457548	421461985	Plant regulator RWP-RK family protein
			Lcu.2RBY.3g072920	421543760	421546661	Serine/Threonine kinase family protein
			Lcu.2RBY.3g072940	421617224	421621587	Receptor Serine/Threonine kinase
			Lcu.2RBY.3g072960	421687270	421689590	Glycosyltransferase family 92 protein
			Lcu.2RBY.3g073060	421968914	421972961	MLO family protein;Annotator
			Lcu.2RBY.3g073080	421998853	422006089	S-acyltransferase
			Lcu.2RBY.3g073090	422080123	422083040	MLO family protein;Annotator
			Lcu.2RBY.3g073110	422291391	422294508	Glyceraldehyde-3-phosphate dehydrogenase B
			Lcu.2RBY.3g073130	422314818	422318748	Glutamate receptor
			Lcu.2RBY.3g073170	422402458	422405558	F-box/kelch-repeat plant protein
			Lcu.2RBY.3g073200	422474214	422487628	Calcium-binding EF hand protein
			Lcu.2RBY.3g073250	422760757	422771013	F-box SKIP5-like protein
			Lcu.2RBY.3g073260	422861193	422862976	CUP-SHAPED COTYLEDON3

CHAPTER 5: General discussion

Abbreviations: Al, aluminium; AM, association mapping; CMLM, compressed mixed linear model; FIGS, Focused Identification of Germplasm Strategy; GBS, genotyping-by-sequencing; GEBVs, genomic estimated breeding values; GS, genomic selection; GWAS, genome wide association study; ICP-OES, Inductively coupled plasma - optical emission spectrometry; ICP-MS, Laser Ablation Inductively Coupled Plasma Mass Spectrometry; LD, linkage disequilibrium; LG, linkage group; MAS, marker assisted selection; MLMM, multi-locus mixed models; NGS, next generation sequencing; PPR, pentatricopeptide repeat; QTL, quantitative trait loci; RRG, relative root growth; SNP, single nucleotide polymorphism;

5.1 Background and context of the research

Cultivated lentil (*L. culinaris ssp. culinaris)* is a diploid ($2n=2x=14$) annual crop that offers a great source of dietary protein (22-35%) (Kumar et al., 2015). Lentil seeds are particularly low in fat, high in protein (Iqbal et al., 2006), and are an excellent source of both soluble and insoluble fibre, complex carbohydrates, B vitamins and minerals (such as potassium, phosphorus, calcium, magnesium, copper, iron and zinc) (Yadav et al., 2007). In agriculture, lentil is typically used as a rotational crop due to its ability to fix biological nitrogen as well as breaking disease, insect/pest and weed cycles. Lentil is cultivated in more than 52 countries; equating to world production of approximately 7.59 Mt from 6.58 Mha, with Canada, India, Turkey, USA, Kazakhstan, Nepal, Australia and the Russian Federation being the main contributors (FAOSTAT, 2017). Australia is a significant producer as well as exporter of red lentil (GRDC, 2017) where approximately 95% of production is exported to the Middle East and South Asia (PulseAustralia, 2015).

Around the world, lentil is often cultivated as a rainfed crop under difficult edaphic conditions and is therefore gets exposed to various biotic and abiotic stresses (Kumar et al., 2013; Sharpe et al., 2013). Among the major abiotic stresses, acid soils and its associated aluminium (Al) toxicity, are major concerns to lentil production due to their sensitivity to low pH (Ryan, 2018). In Australia 50 Mha of agricultural soils are affected by surface soil acidity (pH < 5.5), with 23 Mha showing sub surface soil acidity (pH < 4.8) (AACM-International, 1995; NLWRA, 2001). Although the information on economic loss due to acid soils in Australia varies significantly, estimates show that NSW and WA are the most affected states, averaging $90 – 380 M and $500 M annual losses, respectively (ENRC, 2004; Herbert, 2009; Ryan, 2018).

Acid soils, apart from the high concentration of H^+ ions which can reduce plant growth, also cause toxicity and deficiency of other essential elements. However, the primary reason of poor growth on acid soils is the increased concentrations of soluble aluminium cations (Al^{3+}) which inhibit root growth, even at low concentrations (Foy, 1984; Ryan, 2018). The highly toxic cation (Al^{3+}) becomes prevalent below pH~5.5, but this also depends on other soil characteristics as well as plant species (Foy, 1984; Ryan, 2018). Soil acidity can primarily be managed by applying lime and gypsum to help raise soil pH. However, this is only effective on surface soils, as when sub soil acidity is present, these ameliorations become more difficult and expensive to apply. Therefore, the development of cultivars/varieties with greater tolerance to acid soils and Al toxicity is critically important to maintain long term production and profitability, and expansion of cultivation area on problematic soils.

Given the background discussed above, the major aims of this study were;

1) To establish a screening method suitable to evaluate lentil for acidity and Al toxicity tolerance in sufficient numbers to enable genome wide association study (GWAS).

2) Phenotype putative acid tolerant Focused Identification of Germplasm Strategy (FIGS) accessions and a diverse lentil landrace collection for Al toxicity tolerance to identify tolerant accessions.

3) Validate Al tolerance in selected accessions by biochemical and histochemical analyses, and acid soil screening. Test the tolerance mechanisms in the selected contrasting accessions to gain some insights about tolerance mechanisms in lentil.

4) Genotype the phenotyped lentil lines to understand their genetic diversity, population structure and selection signatures, thus enabling the identification of marker-trait associations for the Al toxicity tolerance trait through GWAS.

The outcomes of this work are; identification of tolerant and genetically diverse accessions that can be used in Al tolerance breeding, and Al tolerance linked markers for marker assisted breeding. This could ultimately help to expand lentil cultivation to include acid soils in Australia.

5.2 Overview of the research

The established hydroponics screening in the present study is a simple and quick method for evaluating lentil accessions for Al toxicity tolerance. It is high throughput in terms of the number of accessions that can be screened in each run, as it can hold 96 individual seedlings for three days of Al treatment in low ionic strength nutrient solution. This high throughput system facilitated the screening of a large number of accessions in a relatively short time, which enabled studies such as GWAS, where success depends on phenotyping a large number of accessions. All the screening Experiments described in this thesis were conducted in the low ionic nutrient (Chapter 2, 3 and 4) with reduced concentrations of other cations. This type of solution is ideal to maintain the activity coefficient of trivalent (Al) ion and increase the likelihood of Al accumulation on negatively charged sites within the root cell wall and root plasma membrane, and thus maintaining Al^{3+} activity to cause toxicity (Famoso et al., 2010).

For screening, at the optimal Al concentrations there is need to consider the level of tolerance of each crop species and genotypes, and whether complete root growth inhibition is desired (Akhter et al., 2009; Xu et al., 2017) to reveal the trait. Among the different Al concentrations (2, 3, 5, 10, 20 and 30 µM) tested (Chapter 2), the 5 µM (1.2 ppm) Al treatment consistently caused the most obvious morphological symptoms of Al toxicity in the sensitive accessions and displayed overall Al tolerance variation (15.7 to 43.9% RRG2%) within the germplasm set. At Al treatments higher than 5 µM, root growth was totally inhibited causing no variation among the lentil accessions for Al tolerance. Similar observations were reported in sorghum (Akhter et al., 2009), an Al sensitive crop, where high Al concentrations (20 µM) did not show variation for the tolerance trait. In contrast to high Al treatments, low Al treatment (2 µM) increased the tolerance (RRG2%) in some accessions (AGG71377 and AGG71438) by promoting the root growth. This was also reported in wheat (Kinraide, 1993) and silver birch (Kidd & Proctor, 2000) where low concentrations of Al stimulated the root growth either in the short, or longer term, by preventing H^+ toxicity and induction of root elongation. The concentration of 5 µM Al is equivalent to 1.2 ppm, which is also the closest concentration to that found in soil Al. Generally the soil Al concentration between 2-5 ppm is considered toxic to sensitive species such as lentil (SoilQuality, 2013) and low Al concentrations are recommended for Al tolerance studies in such species (Akhter et al., 2009). Hence 5 µM Al was selected as the optimal concentration for further screening Experiments in lentil.

Hydroponics screening at 5 µM Al treatment produced the most obvious Al toxicity symptoms including stunted root growth with reduced or absence of lateral roots and thick and brittle root tips with brown discolouration (Mossor-Pietraszewska et al., 1997). These morphological symptoms are the result of complex interactions of Al with apoplasmic (cell wall), plasma membrane, and symplasmic (cytosol) targets (Kochian et al., 2005). In hydroponics, seedlings started to show symptoms by the end of the second day and increased

in severity thereafter. This was supported by ΔRL or RG measurement of the tolerant accessions (Northfield and AGG70137) which showed 3.1 times higher average ΔRL to sensitive accessions (Precoz and AGG70530) after two days of 5 µM Al treatment. This was further increased to 5.2 times after three days of treatment. This indicates that for lentil in this screen two days of Al treatment is enough to differentiate the tolerance.

In the present study, seedlings were assessed for Al toxicity tolerance based on the relative root growth (RRG%). This is a more reliable and reproducible phenotypic index than absolute root length measurement in Al treatment. The RRG% can eliminate genotype-specific differences in root growth and normalizes the comparisons between genotypes (Baier et al. 1995) for better separation of the genotypes for Al tolerance (Xu et al., 2017). The elimination of genotype-specific differences in root growth is necessary when screening the diverse landraces (Chapter 3 and 4) as they present more variation for morphological and adaptive traits such as root vigour or root length. The RRG%, is the relative measurement of the root growth in hydroponic solution, where the root growth or ΔRL of Al treated seedlings were compared to root growth in the control without Al. This is a measure of Al tolerance alone. This is different measure than that used in an earlier lentil study (Singh et al., 2012), where the roots were grown in Al treatment and root regrowth after Haematoxylin staining was used to measure the Al tolerance. This root regrowth measurement is a combination of root vigour (long roots) and Al tolerance. This type of measurement failed to detect Al tolerance in rye genotypes with poor root vigour (Hede et al., 2002). This emphasises the importance of considering the correct analysis of measurements taken in low pH controls for Al tolerance screening.

In nature, acidity (H^+ ion) and Al toxicity stress are difficult to separate as Al is only soluble in acid solutions (Foy, 1984). In Chapter 2, a wide set of accessions including cultivars and landraces were evaluated for the acidity tolerance (RRG1%) and Al toxicity tolerance (RRG2% at 5 and 10 µM Al). There was no correlation between these traits, indicating

independent tolerances to these stresses among the tested accessions. Hence the mechanisms for acidity and Al toxicity tolerance are likely to be different in lentil, as reported in Arabidopsis (Ikka et al., 2007) and fababean (Belachew & Stoddard, 2017). Furthermore, the K-means of clustering of acidity and mean Al tolerance showed separate grouping of acidity tolerant (Digger, AGG70305 and AGG70085) and Al tolerant (Cassab, PBA Jumbo2, Northfield, AGG70164 and AGG70137) accessions (Chapter 2). The differences in their respective tolerances could be contributed by their geographic origins and adaptation to local ecological zones for the landraces, and to some extent the pedigree might have contributed for the cultivars. This phenotypic clustering could suggest the presence of different genetic backgrounds in these clusters. The differences in adaptation of landrace accessions to the nature of the soil material they are collected from plays an important role, as shown in Yorkshire-fog grass and Silver Birch trees, where the accessions from acid organic soils were H^+ tolerant, while those from acid mineral soils were Al^{3+} tolerant but not necessarily H^+ tolerant (Kidd & Proctor, 2001).

The reduction of root growth or ΔRL was observed more when under Al treatment (23%) compared to acidity treatment (low pH or control 1) (71%). Hence, Al toxicity has more adverse effects than the acidity alone, and for acidity tolerance (RRG1%) there was no significant differences between most of the accessions tested, except for a few extreme phenotypes. This implies the hydroponic screening method developed in this study can be used to focus on Al tolerance.

With the newly established hydroponics high throughput screening method for Al treatment in lentil, a large enough number of accessions were screened for tolerance, to enable understanding of the genetic variability and inheritance of the trait. These methods have the potential to identify genetic source(s) of Al toxicity tolerance, and to identify possible tolerance mechanisms. This knowledge is key to allow for Al toxicity tolerance breeding to

develop new Al tolerant varieties/cultivars, which is complementary to a liming acid soil management strategy for improving crop production on acid soils.

The 98 putative acid tolerant accessions used in Chapter 3 were selected using FIGS, where accessions were selected from the genebank with targeted traits and genes in them (Mackay et al., 2004; Bari et al., 2012). It works on the premise that the expression of specific adaptive traits is determined by the selection pressures of the environment in which the population is grown. The lentil acid tolerant FIGS set was developed by considering georeferenced accessions which were further filtered by using the collection site information and environmental data related to acid soil, such as detailed physical and chemical properties of top and subsoil (Street et al., 2016). Under highly acidic soil conditions (pH 5.0), Al solubilizes and becomes phytotoxic (Kochian et al., 2004; Famoso et al., 2010), hence the use of FIGS selected acid tolerant accessions may result in a high frequency of toxic Al tolerant accessions. This acid tolerant FIGS set was made available for the present study, and 13 other accessions (cultivars and landraces) including a known Al tolerant control line were also considered for Al toxicity tolerance screening in Chapter 3. This screening identified nearly 42.3% of the acid tolerant lentil accessions as high Al toxicity tolerant sources, having a RRG value of greater than 32%, that were arbitrarily classified into very tolerant (VT), tolerant (T) and moderately tolerant (MT) classes. Most of the VT and T accessions originated from Afghanistan, Lebanon, Morocco, Tunisia, Turkey, Jordan and Syria indicating their adaptability to these Mediterranean and semi-arid conditions. The well-developed deep root system or high root vigour (Ghanem et al., 2017) in these VT and T accessions could be an adaptive trait for drought tolerance as lentil often faces terminal drought in these areas. This adaptive trait with high root vigour or root growth might have helped to overcome the Al toxicity in these accessions in the present study. Although in this study (Chapter 3) we did not use high pH (6.0) as a control to evaluate acidity tolerance, the relatively higher root growth or ΔRL in the acidic control (pH 4.5) without Al of the very

sensitive (VS) and sensitive (S) accessions, suggests these classes are acid tolerant. These classes showed better root growth in the acidic control with a high average ΔRL (71.6 mm) but failed to grow in Al treatment (average ΔRL of 15.6 mm) indicating their adaptability to only acid conditions but not for toxic Al. These collection sites had acidic soil (Kharal et al., 2018; Mosissa, 2018) but may not have had toxic Al as a selection pressure. This reinforces the fact that low pH is necessary for Al^{3+} toxicity, but not all soils with low pH contain toxic levels of Al^{3+}, especially as its concentration in acid soil also depends on other soil factors (Ryan, 2018). Hence, even though we identified the majority of the FIGS accessions as acid tolerant as shown by high root growth (RG) or ΔRL in acidic control conditions, their tolerance to Al toxicity varied.

In addition to the FIGS germplasm, we also screened (Chapter 4) a very diverse set of lentil accessions (Non-FIGS, 291) that were selected based on multiple geographic origins (35 different countries) and seed availability for screening. When the Al toxicity tolerance of this set was compared and assessed altogether with the FIGS set, it showed that a higher proportion of the FIGS accessions (56.8%) were more Al toxicity tolerant than the known Al tolerant line (ILL6002, 24.8% RRG%), compared to the non-FIGS accessions (43.2% of the accessions). FIGS accessions were also presented the higher mean RRG% of 29 compared to non-FIGS accessions (26% RRG) set. The presence of a high proportion of tolerant accessions in the FIGS set could be a valuable source for Al toxicity tolerance for breeding programmes. Further acid soil field evaluation of all the identified tolerant accessions will help to validate hydroponics Al tolerance and assess the yield potential in Al toxicity conditions. This study also showed the importance of the FIGS approach in selecting a subset of accessions from large genebank collections with a higher likelihood of tolerance traits. This approach has previously been proved successful in wheat for, powdery mildew (Bhullar et al., 2009), sun pest (Bouhssini et al., 2009), Russian wheat aphid (El Bouhssini

et al., 2011), stem rust (Bari et al., 2012) and stripe rust (Bari et al., 2014), and recently in lentils for *Ascochyta* resistance (Dadu et al., 2019).

Following the large-scale hydroponics screen, a subset of 15 accessions from the FIGS hydroponics were selected for acid soil screening, histochemical and biochemical analyses (Chapter 3). The short-term acid soil screening provided a realistic rooting environment in contrast to hydroponics. We observed medium correlation ($r^2 = 0.54$) between the ranks assigned based on hydroponics (average RRG%) and acid soil (selection index) screening, which could be due to the difference in the rooting media. In the hydroponics systems Al^{3+} toxicity was the only root growth limiting factor whereas in soil multiple factors could have affected the root growth. Also, soil is complex in terms of Al distribution and concentration. As such, different screening methods may show different levels of Al^{3+} toxicity effects among the same set of accessions. In the literature, the results of hydroponics screenings are not always in agreement with those of soil based screenings (Mackay et al., 1990; Villagarcia et al., 2001). Low correlations have been observed in barley (Moroni et al., 2010; Ferreira et al., 2017) and in *Medicago truncatula* (Narasimhamoorthy et al., 2007) with inconsistency in Al toxicity tolerance between screening methods. Screening in acid soil also suggested the importance of considering lateral roots along with main root system while evaluating for Al toxicity tolerance, as VT and T class accessions produced significantly more laterals in acid soil whilst the VS and S accessions failed to produce them. Sensitivity of the lateral roots to Al toxicity was reported in wheat and barley genotypes where in acid soil (Al concentration >15 ppm) lateral root length was reduced by more than half compared to the lime treatment, whereas primary root length was marginally or not affected in any of the tested lines (Haling et al., 2010). The importance of lateral roots along with other root types (basal and tap root) was also been noted in Al tolerant maize cv. CMS-36 and soybean PI416937, whose taproots penetrated the Al toxic bottom layer of the acid soil chamber and also initiated large numbers lateral roots in the Al toxic layers compared to Al sensitive

cultivars (Bushamuka & Zobel, 1998). These results also demonstrated the independent responses of the root types to Al toxicity within a plant root system, suggesting differences in Al tolerance mechanisms among root types. Measurement of a single root type may over or underestimate the actual tolerance level, therefore different root types should be considered as distinct entities in Al tolerance evaluation (Bushamuka & Zobel, 1998). Although the formation of lateral roots is a developmental process, it may also be adaptive in response to environmental influences within the rhizosphere (Jung & McCouch, 2013) hence it is important to consider lateral root production in the context of Al tolerance.

Qualitative and quantitative assessment of Haematoxylin and Evans blue stains supported the hydroponics results (RRG%) as shown in the subset of 15 accessions (Chapter 3). The tolerant accessions (Northfield, AGG70137, AGG70281 and AGG70561) from the VT and T class had a low average fold change over control for Al accumulation (2.7), plasma membrane damage (1.5) and oxidative stress (1.1) compared to accessions in the MT, VS and S classes. These tolerant accessions could be used directly in Al toxicity tolerance breeding programmes. This result suggests the use of these stains along with root growth measurements need to be used to screen for Al toxicity tolerance.

As a complement to the screening, two FIGS landraces (Al tolerant in hydroponics: AGG70137 and Al sensitive in hydroponics: AGG70530) and two advanced cultivars (Al tolerant in hydroponics: Northfield and Al sensitive in hydroponics: Precoz) were selected for a Al toxicity tolerance mechanisms study at different Al treatments (5 and 10 µM) and durations. The sensitive accessions prominently showed root surface ruptures and hard root tips, compared to the tolerant accessions, when exposed to both Al treatments. It appeared that most of the Al accumulated in the roots was bound to pectin constituents of the cell walls (Yang et al., 2008). This modification to the cell wall composition has been shown to affect cell wall properties, such as its extensibility (Jones et al., 2006; Ma et al., 2014), which is thought to have contributed to the observed morphologic effects; that are similar to the

symptoms observed in pea (Yamamoto et al., 2001; Motoda et al., 2011; Motoda et al., 2010), cowpea (Kopittke et al., 2008) and maize roots (Jones et al., 2006).

The significant difference between contrasting accessions for root growth, Haematoxylin and Evans blue stain accumulation (in terms of fold increase) was only observed after two days of Al treatment, whereas one day treatment was not enough to cause differences in RG and Evans blue accumulation, indicating Al was not accumulated to a toxic level to reduce the RG and to affect the plasma membrane damage in this time. This supports the view that, screening for Al toxicity tolerance at optimal Al treatment (5 µM) needs two days of treatment in lentils. At the higher 10 µM Al treatment, all the accessions accumulated a similar level of Al (Chapter 2; Figure 2.4, Supplementary Figure 2-5), with the tolerant accessions also showing reduced ΔRL or root growth that suggests Al tolerance is possible at 5 µM, with the tolerance mechanisms overwhelmed at higher concentration. This was also supported by Inductively coupled plasma-optical emission spectrometry (ICP-OES) analysis of root Al content. The tolerant accessions, despite the high root Al content at 5 µM Al after three days of treatment, maintained significantly higher root growth compared to sensitive accessions, which indicates either exclusion or detoxification of Al taken up into the root cells. The tolerant accession, Northfield, did not show any release of organic acids in exclusion tests, however it had a high shoot Al content at high (10 µM) Al concentration. This indicates an internal detoxification mechanism, which might have translocated Al to shoots after a certain amount of Al accumulation in the roots. Rice has been reported to have a similar type of Al uptake with internal detoxification in the variety Modan, where root Al storage capacity was saturated after 24 hour Al exposure, with Al accumulation observed in the shoots after 48 hour exposure (Roselló et al., 2015). In contrast, the other tolerant accession AGG70137 tested released oxalic acid after 1 hour (35.6mg/L with retention time 4.4) and 3 hours (42.3mg/L with retention time 4.2) of 100 µM Al treatment. In addition, this line did not show much shoot Al content under the 10 µM Al treatment (Figure 2.6,

Chapter 2) compared to Northfield, suggesting an exclusion type of tolerance mechanism, largely consistent with the pattern II type. This type of tolerance has been reported in an earlier investigation into lentil Al toxicity tolerance (Singh et al., 2016), in which significantly higher citrate and malate peaks were reported in resistant breeding lines (L-7903, L-4602) and a resistant wild line (ILWL-185), only after 3 hour of the Al treatment; indicating the presence of a lag time between Al exposure and organic acid release.

In the present study the observed release of oxalic acid in AGG70137 as a tolerance mechanism is a surprising result, as generally oxalic acid release was observed in tolerant species like buckwheat (Zheng et al., 1998a, 1998b). Lentil is classified as being a sensitive species in which oxalic acid release is generally not reported, and as such the release of such organic acids needs further investigation. There is great genetic variation, both among and between species of plants to Al resistance, suggesting that Al-resistant species, cultivars or lines possess several mechanisms for detoxifying Al. It was also suggested in maize that, although organic acid release appeared to be the main tolerance mechanism, internal detoxification which allows the root tip to cope with the ongoing Al accumulation, is also likely to be present (Piñeros et al., 2002; Piñeros et al., 2005; Giannakoula et al., 2008). Hence in the present study, the tolerant AGG70137 (FIGS landrace) could have either a mainly exclusion, or a combination of exclusion and internal detoxification tolerance mechanism, which needs further experimental support. These results indicate that the tolerant accessions, Northfield (advanced cultivar) and AGG70137 (FIGS landrace) have different types of tolerance mechanisms. This is supported by the GWAS results (Chapter 4), where these accessions presented different haplotypes observed in the potential quantitative trait loci (QTL) region on chr6. In this region, AGG70137 which indicated the presence of exclusion type of tolerance mechanism had Hap3 which has a combination of favourable and unfavourable alleles of the significant SNPs at the potential QTL region. In Northfield where the presence of an internal detoxification mechanism was demonstrated,

Hap1 which has only unfavourable alleles of the significant SNPs at the QTL region is present. Further presence of different tolerant mechanisms can be confirmed by developing mapping populations by using these tolerant lines in crossing with sensitive lines and evaluating these populations for Al tolerance in terms of RRG%, Haematoxylin staining, organic acid release and Al content in different parts of the plant.

In Chapter 4, a diverse lentil collection of 386 accessions including the FIGS set was genotyped by using transcriptome genotyping by sequencing (GBS) technology, which has been successfully used in an earlier lentil study (Malmberg et al., 2018). This method detected the most reliable sequence polymorphisms and also identified splice variants in field pea (Sudheesh et al., 2016). In the present study 65,874 high quality SNPs were identified which enabled us to investigate population structure, genetic diversity, selection signatures and linkage disequilibrium (LD) in the diverse lentil collection. Estimating the population structure and genetic diversity of cultivated lentil is useful for breeding purpose to widen the genetic base and to understand the crop adaptation in this crop (Khazaei et al., 2016; Tsanakas et al., 2018). The population structure in the lentil collection with six subpopulations closely reflected the accessions' origin and their adaptability to agroclimatic zones. Among the different subpopulations, MED showed high genetic diversity among the accessions and was associated with high Al tolerances. This information guides the choice of appropriate parental lines for Al tolerance breeding. The most divergent pairs were reported between the ETH and SA subpopulations (mainly with Nepalese accessions). Accessions of such divergence can be easily crossed to improve traits of interest, as shown in the literature in the case of an Indianhead and Northfield lentil cross (Rodda et al., 2017). These accessions with reported medium genetic distance (~0.691 Nei's coefficient) have been crossed to improve Ascochyta blight resistance (Rodda et al., 2017). Signatures of selection, evaluated based on Fst values, were detected in all chromosomes at several genomic regions for different subpopulations, ranging from 243 to 306 SNPs (Chapter 4).

These signatures of selection have functional importance in conferring enhanced fitness or productivity (O'Brien et al., 2014). In these regions, alleles have been preferentially increased in frequency and fixed in a population during the period of adaptation. Hence these signatures of selection can be used as molecular keys to assign lentil germplasm to a specific subpopulation, which would be of great significance for ease of germplasm characterisation in genebanks.

GWAS was conducted in the present study by using a diverse lentil collection of 386 accessions and 65,874 high quality SNPs (Chapter 4) to identify the associated markers/SNPs for the Al toxicity tolerance trait (assessed in terms of RG in Al treatment and RRG%). The compressed mixed linear model (CMLM) model with population structure (Q) and Kinship matrix, detected a QTL region (9 SNPs) on chr6, that was common between Al RG and RRG%. Identified common SNPs between the absolute trait (Al RG) and relative (RRG%) root trait indicate the reliability of the measurements which was also supported by their high correlation. One significant SNP was also detected in Al RG on chr3. Different phenotype measurements for Al tolerance could detect different chromosomal regions. This was also reported in barley, where different root indexes influenced the detection of QTL/SNPs by GWAS analysis (Cai et al., 2013).

The potential QTL region on chr6 covered 8 SNPs in haplotype block5 which covered a 4 kb region and presented four different haplotypes in the collection. Among the four haplotypes, Hap3 and Hap4 were only found in MED and TUR subpopulations, respectively and are mainly associated with higher Al tolerance. Selection for these haplotypes could be beneficial for Al tolerance and could be more widely incorporated into the breeding programmes. The information on haplotypes with associated phenotypic traits might be useful to select for crossing using molecular markers in order to accumulate the favourable haplotype variants in breeding lines (Qian et al., 2017).

The significant SNPs in the potential QTL region explained a low to medium level of the phenotypic variation in the range of 5.2 to 8.4%, suggesting that the trait might be affected by numerous alleles with minor effects. Similar levels of inheritance were also reported in barley (9.7%) (Cai et al., 2013) and rice (11%) (Zhao et al., 2018; Zhang et al., 2019a), where Al tolerance was assed as relative longest root growth and relative root elongation respectively. However, the level of variation explained might be improved by phenotyping other traits such as dry root weights or callose accumulation along with RRG, as SNPs for dry root weight explained up to 17% of the phenotypic variation in rapeseed (Gao et al., 2020) and fluorescent signals QTL for callose accumulation explained 52% of the phenotypic variation in lentil (Singh et al., 2018).

In the present study the identified potential region for Al tolerance is novel as in an earlier lentil report (Singh et al., 2018) two QTLs were detected on linkage group (LG1) relate to the root regrowth and fluorescence signals for callose accumulation. These differences could be due to the difference in the materials used (mapping population vs natural population) and phenotypic measurement of Al tolerance (root regrowth after staining and callose accumulation vs relative root growth). Considering the root regrowth only in the Al treatment as in earlier lentil studies (Singh et al., 2012; Singh et al., 2015; Singh et al., 2018) is an indirect estimate of Al tolerance, this phenotype being a combination of Al tolerance (Al-tolerance alleles) and root vigour (Hede et al., 2002), whereas relative root growth (RRG%) is a measure of Al tolerance alone. This points to the potential QTL region from the present study being an important region for Al toxicity tolerance in lentil. Some possible candidate genes related to transcription factors (Zinc finger types and RWP-RK), transmembrane and PPR proteins involved in Al tolerance or abiotic stresses were observed in the vicinity (±2 mb region) of this potential region. However, further studies, including sequence analysis and map-based cloning are needed to confirm the linkage between observed SNPs from potential QTL region and gene(s). The significant markers from this

study can be used for marker assisted selection (MAS) breeding after validation in new lentil collections. Overall, GWAS offered valuable information and insight into the genetic architecture of the Al toxicity tolerance trait, with the possibility of accelerating candidate gene(s)discovery.

5.3 Future directions

The research described in this thesis identifies number of areas for further investigation. In our screening for Al toxicity tolerance, significant symptoms of toxicity were observed after two days of the Al treatment. On this basis future hydroponics screening at optimised Al concentration in lentil can be reduced to two days instead of a three-day period. Along with root length measurement other root traits including lateral roots can also be considered to determine tolerance. Furthermore, florescence signals to detect callose biosynthesis in Al stressed plant roots could be used, in which sensitive accessions show more callose deposition compared to tolerant accessions as shown in rice (Alvim et al., 2012), sorghum (Too et al., 2014) and lentil (Singh et al., 2018).

Peat and perlite growing media can be an alternative for hydroponics solution for Al toxicity tolerance screening. These media provide a solid medium for longer term root growth, along with a clean harvest of roots and easy access to leaves for detection of the stress responses (Belachew & Stoddard, 2017). During long term growth seedlings can be used for the measurement of other root traits (fresh and dry root weight) and morpho-physiological parameters such as leaf gas exchange, photosynthesis rate, chlorophyll concentration and transpiration rate, which have been found to be informative in different crops during the Al treatment (Ohki, 1986; Simon et al., 1994; Misra et al., 2001; Szabó et al., 2015). Although different growth parameters including root and shoot, lengths, dry weights and pods per plant were evaluated in lentil in a 65 day long term experiment in hydroponics (Singh et al., 2012), considering other growing medium and morphophysiological parameters can offer easy handling and provide additional information.

Field based screening is essential to evaluate field Al tolerance, but these are laborious, take a long time, are costly and generally give moderate correlation with hydroponics screenings (Aguilera et al., 2016). These factors place importance on a reliable and fast screening method to save the time and resources. A high-throughput rapid hydroponics or other growing medium screen enables selected accessions identified in these screens to be tested in glass houses in acid soil. These are undertaken mainly using acid soil collected from cultivated fields, and the plants are grown for longer period to evaluate the growth responses of plants with acid soil and Al toxicity.

Al toxicity has lethal effects on many aspects of rhizobia/legume symbiosis. Along with affecting the main host in terms of decreasing root elongation and root hair formation, it also lowers the soil rhizobial population, reduces nodule formation and number, and ultimately impairs the N2 fixation process (Jaiswal et al., 2018). Although it's out of scope of the present study, future lentil studies need to consider this aspect of Al toxicity, as for sustained legume production for increased food security (Unkovich et al., 2008), legumes and rhizobia need to be screened together to identify nodulation Al tolerances for use in Al rich soils (Abdel-Salam et al., 2010).

The release of organic acids is thought to be the main type of external detoxification in plants, based on the literature. We considered whole root system for the release of organic acids, however, the exclusion of the organic acid anions from excised root tips (0-5 mm) is more important than the considering whole root system in response to toxic Al. As demonstrated in wheat (Delhaize et al., 1993b) and maize (Pellet et al., 1995), the mechanisms of Al tolerance are localized to the root apex, where the primary symptoms of Al toxicity are also localized (Ryan et al., 1993). The release of organic acids from excised root tips in response to different Al concentrations and incubation periods can be determined and the concentration can be calculated using liquid chromatography mass spectrometry (LC-MS, HPLC) systems. Along with major organic acids (succinate, malate, citrate and

oxalate), other organic acids such as acetate, lactate, pyroglutamate and pyruvate can also be considered as they were found in pea root exudates in response to toxic Al (Kichigina et al., 2017), although they did not correlate with Al tolerance. Other exclusion mechanism includes: 1) mucilage formation and its binding to Al has been reported in cowpea (Horst et al., 1982) and wheat (Archambault et al., 1996) contributing to the formation of a diffusion barrier to Al, and 2) a pH barrier, was suggested for Al tolerance in wheat, barley, rye, and triticale, where an increased pH of the growth medium was associated with Al tolerance (Foy et al., 1965). These are also other factors that can also be checked in lentils.

An internal detoxification mechanism occurs by chelating Al^{3+} in the cytoplasm with organic acid anions or other organic ligands, and subsequently compartmentalisation of the Al into vacuoles (Aggarwal et al., 2015). Once Al enters the plant, highly Al tolerant species employ multiple genes and mechanisms for Al tolerance at different levels in the roots (Zhang et al., 2019b). Al tolerance mechanisms mainly include call wall modification, uptake and subsequent sequestration of Al, and root to shoot translocation of Al (Kochian et al., 2015). Pectin is a complex polysaccharide in the cell wall and methyl esterification of the carboxylic group of pectin determines the negative charges it carries, and ultimately the quantity of Al it can bind (Schmohl et al., 2000). In maize, low cell wall pectin content in 1-2 mm apical root sections and higher degree of methylation causing lower Al accumulation in the cell wall confirmed the Al resistance (cv ATP-Y) in addition to the release of organic acid anions (Eticha et al., 2005). Similar was also reported in rice resistant cultivar (Nipponbare) (Yang et al., 2008) along with low pectin methyl esterase (PME) activity and polysaccharides (Pectin, hemicellulose 1 and 2). Hence Al content in root apical cell walls, extraction of cell walls and measurement of polysaccharides, PME activity and immunofluorescence for localisation of cell wall pectin are important to consider for Al tolerance study, which can be contributed for genotypic differences in Al resistance.

Several types of transporters have been reported in plants that are involved in Al uptake and subsequent sequestration, and root to shoot translocations of Al. Although most of Al is bound to cell wall, a proportion of Al enters the root cell wall, hence analysis of intracellular Al accumulation by cryosections of root tips and secondary ion mass spectrometry can be used as guide for Al localisation after short (30 min) exposure to Al (Lazof et al., 1994). In some other species (buckwheat, hydrangea, tea and *melastoma malabatbricum*), the role of organic acids has been reported in Al uptake, translocation, accumulation, and internal detoxification (Zhang et al., 2019b), which could also be checked in lentil tolerant line (Northfield) which indicated some internal detoxification. Along with root length measurements, determination of organic acids in root exudates, checking the specificity of organic acid release under Al treatment, location of secretion site in root sections and in intact roots, purification and identification of Al complexes in the cell sap of roots and leaves are essential. Al complexes in purified cell sap can be determined by ^{13}C and ^{27}Al-nuclear magnetic resonance analysis as used in buckwheat (Ma et al., 1998).

An efficient antioxidant defence system to combat Al induced reactive oxygen species (ROS) is also important for providing Al internal resistance, as ROS can cause cellular damage. Some reported enzymatic antioxidants, such as superoxide dismutase (SOD), catalases (CAT) and peroxidases (POD) are important to consider and evaluate in order to detoxify ROS (Apel & Hirt, 2004). Al induced increase in antioxidant enzymes have been reported in many plant species including wheat, rice and pea (Darkó et al., 2004; Sharma & Dubey, 2007; Matsumoto & Motoda, 2012), hence measuring the antioxidant enzymes are an option to consider in lentil tolerant accessions to study internal detoxification. Along with this, checking the elemental uptake of Ca, Mg and K is essential as it suggested that Al toxicity is significantly decreased by increased concentration of these elements in sorghum (Bernal & Clark, 1997) and other plant species (Rengel, 1992; Silva et al., 2001; Kobayashi et al., 2013; Pandey et al., 2013). This is probably due to competition for the binding sites

of Al ions in the apoplast, and these nutrients elements were shown to be important in enhancing Al tolerance. Anatomical and histological study of the root tips of tolerant and sensitive accessions can provide information on Al accumulation and its transport in the root tissues; such as its apoplastic and symplastic movement. In buckwheat, staining of Al with morin has been shown to be a suitable method for qualitatively showing the radial Al distribution along the root tip axis. This was also correlated with total Al distribution determined by Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) method (Klug et al., 2011). These approaches can also be used to further study the tolerance mechanisms of lentil accessions.

We genotyped 386 diverse lentil accessions, that consisted of mainly landraces from different geographic origins, that showed a wide genetic diversity, and high subpopulation differentiations when evaluated with 65,874 high quality SNPs. Such a genotyped lentil collection with valuable accessions is important for phenotyping other agronomic traits such as yield and yield related traits, seed-specific and plant architecture characters, disease related traits, abiotic traits mainly drought and heat stresses. Once phenotyped, such a population can be further used for GWAS for dissecting complex traits, to find the genomic regions associated with the phenotyped traits, or for doing genomic selection (GS).

The present study relied on the single locus CMLM approach of GWAS to identify significant markers based on root traits for Al toxicity tolerance. It has been proposed that using a combination of single and multi-locus models could improve the efficiency of detecting QTLs. This has been reported in other crops for different traits such as, salt tolerance in rice (Cui et al., 2018), fibre quality traits in cotton (Li et al., 2018) and pasting properties in maize (Xu et al., 2018). The use of a combination models has also been suggested in soybean and maize, for the traits differing in heritability (Kaler et al., 2020). Hence the use of a combination of models along with other complex approaches like multi-locus mixed models (MLMM) and models using Bayesian approaches, which have higher

statistical power, are recommended for future studies. Although a number of candidate genes related to, or involved in, Al tolerance was identified, confirmatory candidate gene analysis, that includes sequencing of contrasting haplotypes, expressions analysis and physiological characterization of the genes, is required to confirm their role. The two tolerant accessions suggested the presence of different tolerance mechanisms in the present study which was also supported by the GWAS analysis. Therefore, further study should include these accessions to explain the tolerance mechanisms at the molecular level or gene level.

In the present study with the GBS approach, valuable dense SNP markers were identified throughout the lentil genome. This information can be further utilized in lentil GS breeding programmes. Genomic selection is an alternative to traditional MAS and has enormous potential in improving selection in breeding programs. GS uses a large set of genome wide markers to potentially capture all the QTL underlying the trait (Hayes & Goddard, 2001). Fitting all markers simultaneously avoids multiple testing and the need to identify marker-trait associations based on an arbitrarily chosen significance threshold (Haile et al., 2020). GS uses a training population, that has been phenotyped and genotyped for the traits of interest which will allow the model to predict genomic estimated breeding values (GEBVs) of individuals in the target breeding population (Hayes & Goddard, 2001). GS is currently being applied to a range of crops including maize (Crossa et al., 2014), wheat (Rutkoski et al., 2011) and rice (Spindel et al., 2015). A recent study in lentil (Haile et al., 2020) suggested the applications of GS in lentil breeding programmes to make predictions within-population and across environments. Hence the genome wide SNPs from this study could be used in GS in lentil once the present diverse population is phenotyped for complex traits and evaluated for these traits in multiple environments.

5.3.1 Application of results for breeding programs

The improvement of any trait by plant breeding mainly relies on the presence of the variability and availability of an efficient screening techniques, but the pace of improvement

depends on easy and reliable phenotyping techniques. The established high throughput hydroponic system facilitated the phenotyping of a large number of accessions in a relatively short time at optimised Al treatment, which successfully differentiated tolerant and sensitive accessions. The mapping populations, segregating populations and new germplasms can be easily screened with this established method for Al toxicity tolerance by considering RRG% measurements and Haematoxylin stains. Identified tolerant accessions, Northfield, AGG70137 and sensitive accessions, Precoz and AGG70530, including known tolerant line, ILL6002 could be used as control lines in each screening to ensure repeatability of screening assays. The identified acidity (Digger, AGG70305 and AGG70085) and Al toxicity tolerant varieties (Northfield, Cassab and PBA Jumbo2) and accessions (AGG70137, AGG70164, AGG70561 and AGG70281) that showed better performance than known tolerant line can be used as tolerant parents in crossing programmes to develop mapping populations. The information regarding the genetic distance between the accessions and Al tolerances in each haplotype will further help to select the parents while considering for the crossing. The landraces with Al tolerant haplotypes, Hap3 and 4 could be worthwhile to use in selection and improvement programmes after assessing their yield potential on the field. The identified signatures of selection can be used as molecular keys to assign lentil germplasm to a specific subpopulation, which would be of great significance for ease of germplasm characterisation in genebanks. The significant markers from this study can be used for MAS breeding after validation in new lentil collections.

5.3.2 Long-term breeding efforts for enhanced performance in Al-toxic soils

The success of breeding programmes for Al tolerance relies on an understanding of physiology, genetics and gene regulatory information of the trait. Lentil crop lacks the information about acid soil Al tolerances as there are only few studies available. Based on earlier lentil report and present study, the presence of variation in Al tolerance among lentil accessions indicated possibility for genetic improvement for Al toxicity tolerance trait.

Traditional breeding approaches involving crossing of contrast accessions for Al tolerance with wide genetic distance and further back crossing techniques could confirm the Al inheritance pattern and tolerance mechanisms in present lentil collection. This also validates the identified novel QTLs in the present work from GWAS study. Molecular and statistical analysis showed the low heritability of Al tolerance trait indicating complex nature and quantitative inheritance, as reported in rice and maize where breeding focused on population improvement (Zeigler et al., 1995). In breeding programmes, allocation of genetic resources in terms of number of progenies need to be maintained in the early stages of evaluation for Al tolerance would benefit from the genetic gain (Anas et al., 2019). The use of this genetic gain information allows the plant breeders to improve the efficiency of selection methods (St. Martin & Futi, 2000; Daetwyler et al., 2015; Engel et al., 2016) hence its worth to consider genetic gain for Al tolerance trait in lentil. The knowledge on the strength of association among important traits is useful tool to improve the quantitative characters. Identification of secondary traits is important in breeding for tolerance to soil acidity and its associated Al toxicity due to their correlations with yield (Ngoune Tandzi et al., 2018). In early lentil study, the significant correlation reported for root regrowth with root and shoot lengths, weights, and pods per plant in hydroponics, and with yield in acid field evaluation (Singh et al., 2012; Singh et al., 2016). In lentil, considering the other agronomic traits with Al tolerance at physiological and molecular level is important and thus QTLs associated with these traits under stress condition could be utilized as indirect molecular predictors of plant performances.

The use of molecular markers in biparental QTL mapping and in natural populations for association mapping has identified the genes/loci for Al tolerances in different crops. The QTL mapping in cereals mainly in wheat generally reported the single dominant gene (Delhaize et al., 1993a), however exceptions in some cultivars were also reported (Tang et al., 2002). Association mapping differs with biparental mapping in which it evaluated

numerous alleles simultaneously in maize and identified the number of genes in different metabolic pathways (Krill et al., 2010). However, lentil lacks such type of studies as there is very limited information on use of molecular markers for Al tolerances. Hence in lentil, the use of inter species along with wild accessions for QTL mapping with denser SNP and different type of molecular markers will help to evaluate inheritance nature, possible tolerance mechanisms and identification of the functional genes for Al tolerance. After QTL are validated, tightly-linked markers can be used to detect, transfer and accumulate desirable genome regions into superior genotypes, a process that is much faster than phenotypic selection, thus aids the MAS in breeding programs. As reported in barley from the tolerance gene *HvMATE*, one gene-specific marker (HvMATE-21indel) was developed, which increased the explained phenotypic variation compared with the other SSR markers and used for selecting the tolerance gene from multiple tolerance sources (Bian et al., 2013). Closer markers or gene-specific markers for identified Al tolerance QTLs will make selection more efficient and consideration of more genes/QTLs that underlay combination of different tolerance mechanisms may achieve better tolerances. Thus, the discovery of more genes/QTLs from different sources in lentil, and their validation through segregation or mutation /transgenic approaches can complement and enhance traditional programmes by gene pyramiding.

Transgenic approach offers unique opportunities for validating gene function in Al tolerance and could also be an alternative technology to increase crop production in acidic soils through development of Al-tolerant cultivars by genetic engineering. Wheat major gene for Al tolerance, malate transporter gene (*ALMT1*) significantly improved Al tolerance in transgenic barley, where transgenic plants showed robust root growth and unaffected root apices under certain Al levels (Delhaize et al., 2004). This type of approach can be utilized in lentils after promising genes are identified for Al tolerance.

In acid soils, Al toxicity affects every aspects of legume nitrogen fixation, including the host plant, the rhizobia and their interaction (Jaiswal et al., 2018). In legumes including lentil, rhizobia response and host plant interaction under Al conditions are still unknown. It important to consider selection for tolerant rhizobia along with tolerant host for successful symbiosis in acidic soils (Artigas Ramírez et al., 2018). Hence pre-breeding screening should include different Al concentrations at different pHs for both host crop and rhizobia. Further breeding programmes, identification of genes and gene expressions studies for Al tolerances should aim to consider symbiotic association of host and rhizobia to increase gain yield of pulses in acid soils (Jaiswal et al., 2018).

5.4 Conclusions

In conclusion, this project combined phenotyping and genotyping of diverse lentil accessions for the Al toxicity tolerance trait. This was enabled by the establishment of a high-throughput hydroponics-based screen, that was validated through soil-based pot trials of accessions identified as having contrasting phenotypes. This approach identified both tolerant accessions and informative SNP markers which are linked to Al toxicity tolerance traits. The high throughput screening method can be used to screen breeding populations or new germplasm collections. The validated Al toxicity tolerant FIGS landraces and identified genetically divergent accessions can be immediately made available to lentil breeding programs. There are other identified tolerant accessions that could be brought into breeding programmes after validation in acid soil conditions. Both mechanistic and genetic analysis identified two different tolerance mechanisms which should be further examined to enable possible combined deployment. Significant marker-trait associations should be followed up further both to enable their deployment in breeding, and to give further insight into the mechanisms used by lentil for tolerance of Al toxicity

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