Department of Agricultural Sciences

Faculty of Agriculture and Forestry

University of Helsinki

Doctoral Programme in Sustainable Use of Renewable Natural Resources

Root and shoot traits associated with acidity and drought tolerance in *Vicia faba* L. plants

Doctoral thesis

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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Agriculture and Forestry, University of Helsinki, for public examination in Walter Hall, EE-building, Agnes Sjöberginkatu 2 on 25 October 2019, at 12 o´clock noon.

Helsinki 2019

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Cover figure: Schematic drawing of responses and requirements of faba bean under acidic and Al^{3+}-toxicity stresses.

Dedicated to:

My wife Saada and my children Elias, Dina and Lela.

ACKNOWLEDGEMENTS

CONTENTS

ABSTRACT

Background. Abiotic stresses affect the productivity and the evolutionary pathway of adaptation in crops in different agricultural regions. Soil acidity and drought are two major abiotic stresses, when severe, reduce the suitability of fertile lands for crop production, and when moderate, reduce yield and often quality. Faba bean (*Vicia faba* L.) is sensitive to acidity, aluminium toxicity and limitation of soil moisture, and these stresses greatly reduce the yield potential and stability of the crop. The overall objectives of this study were to investigate complementarity in shoot and root morphological and physiological phenotypic markers to acid soil and drought adaptation in pre-flowering faba bean plants, and to identify sources of tolerance for further breeding work. These objectives were tested in light of four hypotheses: acid zone germplasm would have higher acid and Al^{3+} tolerance index than other germplasm (publication I); dry-zone germplasm would have more prolific root systems than wet-zone germplasm (publication II); dry-zone germplasm would maintain its root system growth better in drought than wet-zone germplasm would (publication II); and drought avoidance is based on a combination of leaf gas exchange and exploitation of soil water (publication III).

Materials and Methods. Multiple sets of faba bean accessions were chosen based on their expected exposure to acidity, aluminium, or drought stresses in their regions of provenance, and based on previous research data and reports. Experiments were established in aquaponic, peat and perlite media in controlled/environment growth chambers, greenhouses and a robotic phenotyping facility to evaluate the performance of a range of faba bean accessions in acid, aluminium, and watering treatments. Key root and shoot data were collected and analysed.

Results and Discussion. Acidity and Al^{3+}-toxicity treatments were sufficiently strong to initiate detectable variation in root length, stain score and Al^{3+} tolerance index, SPAD value, stomatal conductance, biomass and leaf area in solution culture, peat, and perlite experiments. Roots behaved differently in response to pH and Al^{3+} treatment differences. Al-tolerant accessions showed contrasting shoot Al content, indicating multiple Al tolerance mechanisms in faba bean. The results of acid tolerance index in aquaponic and perlite media experiments were positively correlated. Trait expression complementarity and variability were observed across the experiments owing to differences in growth media. Accessions differed in root regrowth length in solution culture and in SPAD values and taproot length in perlite medium to changing pH and Al^{3+} concentrations as shown by accession by treatment interactions. Root tolerance index, root regrowth length, and SPAD values were found to be largely informative traits in solution culture, and peat and perlite pot experiments. In aquaponics experiment, 41 µmol/l Al^{3+} was not informative, 82 µmol/l Al^{3+} was informative, 123 µmol/l Al^{3+} was severe. As a result, 82 µmol/l Al^{3+} was used in the next peat and perlite experiments. However, 82 µmol/l Al^{3+} was found to be less informative in peat experiment, hence 123 µmol/l Al^{3+} could be recommended for selection of outstanding accessions in solid media. Overall, accessions responded to acid and Al^{3+} treatments independently. Cultivars Aurora and Messay were found to be Al^{3+} tolerant but acid sensitive; Kassa and GLA 1103 acid tolerant, but Al^{3+}-sensitive; NC 58 and Dosha were tolerant to both Al^{3+} and acidity, while Babylon was sensitive to both. Aquaponic media for mass screening and perlite media for verification experiments were found to be convenient (publication I).

Screening of germplasm for drought was successfully conducted in a perlite-based pot experiment, which allowed quicker screening of a large set of materials and enabled detection of variation in

constitutive traits among accessions. Use of the GROWSCREEN Rhizo phenotyping facility allowed detection of useful differences between treatments and among accessions. In both the screening and phenotyping drought experiments, accessions originating from the drier regions of the world showed drought avoidance behaviour thereby confirming FIGS as a valuable strategy (publication II and III). In germplasm screening, root and shoot dry mass and their fractions, along with SPAD value provided useful information in discriminating accessions with potential drought-avoidance characteristics. In the phenotyping experiment, root traits were strongly and positively correlated with each other and with shoot traits, but these correlations indicated specific plasticity of traits with watering treatments (publication III). In the well watered treatment, total dry mass was correlated with root length traits, whereas in the water-limited treatment, it was correlated with root width and convex hull area. Apparent root length density was positively correlated with second order lateral root length in the well watered treatment and with apparent specific root length in water limited treatment, indicating high surface area to volume ratio to maximize water absorption is a key strategy in droughted condition. In the water-limited treatment, root traits contributing to drought avoidance such as lateral root length and root system depth, convex hull area and root system width, and apparent root length density (publication II and III) were positively associated with shoot traits such as total dry mass, leaf number, and leaf mass fraction reported in publication III. Accession DS70622 exhibited deeper and wider growing roots that filled the root system volume with long and thin laterals. The larger root system combined with moderately high total dry mass and stomatal conductance endorsed this accession as a potential drought-avoiding candidate by effective use of water suitable in transient droughts. Accessions such as DS11320 and ILB938/2 that combined a large and thick root system with low root length density, low specific root length and low stomatal conductance can be recommended as potential sources of drought-avoiding traits by improved water use efficiency suitable in terminal droughts. Future research directions on the development of multiple abiotic stress tolerant cultivars enables climate change resilience in crops. Most acid soils are subject to Al^{3+}-toxicity, and drought can occur on this soils as it occurs in others. Hence, multiple stress tolerance traits, mechanisms and QTLs need to be investigated in faba bean to identify host accessions with multiple tolerance to Al^{3+} and drought stresses for breeding of high yielding materials.

LIST OF ORIGINAL PUBLICATIONS

I. Belachew KY, Stoddard FL. (2017). Screening of faba bean (*Vicia faba* L.) accessions to acidity and aluminium stresses. *PeerJ,* 5:e2963; DOI 10.77171/peerj.2963, 1-19.

II. Belachew KY, Nagel AK, Fiorani F, Stoddard FL. (2018). Diversity in root growth responses to moisture deficit in young faba bean (*Vicia faba* L.) plants. *PeerJ*, DOI 10.7717/peerj.4401, 1-20.

III. Belachew KY, Nagel AK, Hendrik Poorter, Stoddard FL. (2019). Association of shoot and root responses to water deficit in young *Vicia faba* plants. *Front.Plant Sci.* 10:1063, DOI:10.3389/fpls.2019.01063, 1-10.

Statement of contributions in these articles

Author Contributions	I	II	III
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Performed the experiment and organizing data	KYB	KYB	KYB
Analyzed the data	KYB FLS	KYB FLS	KYB HP
Contributed reagents/materials/analysis tools	FLS	FLS KAN	FLS KAN
Wrote the paper	KYB FLS	KYB FLS KAN FF	KYB FLS KAN HP

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ABBREVIATIONS

ABA   abscisic acid

ACCO   ACC-oxidase

APX   ascorbate peroxidase

BC   base cations

CAT   catalase

DAS   days after sowing

DAT   days after the treatment began

EQY   effective quantum yield

FIGS   Focused Identification of Germplasm Strategy

GEP   germin-like protein

GPX   guaiacol peroxidase

H_2O_2 hydrogen peroxide

Ha   hectare

ICARDA   International Center for Agricultural Research in the Dry Areas

ICP-OES   inductively coupled plasma-optical emission spectrometry

LAI   leaf area index

MDA   malondialdehyde (MDA, $CH_2(CHO)_2$),

NADPH   nicotinamide adenine dinucleotide phosphate hydrogen

NCED   9-cis-epoxycarotenoid dioxygenase

O_2^- superoxide

OA   osmotic adjustment

OH^- hydroxyl ions

POD   peroxidase

PSI and PSII photosystem I and photosystem II

RIL   recombinant inbred line

ROS   reactive oxygen species

SLA   specific leaf area

SOD   superoxide dismuatase

SPAC   soil-plant-atmosphere continuum

1 INTRODUCTION

1.1 Faba bean (*Vicia faba* L.): The crop

Faba bean (*Vicia faba* L.) (Figure 1), also known as fava bean, field bean, broad bean, horse bean, bell bean and tick bean (Lim 2012), is an annual herbaceous plant, belonging to the family Fabaceae, subfamily Faboideae, tribe Vicieae and genus *Vicia* L (Muratova 1931). Faba bean is diploid with 12 chromosomes (2n = 12) with large nuclear genome size of 1C DNA = 13330 Mb (Johnston et al. 1999), while all other *Vicia* species contain 14 chromosomes (2n = 14) (Duc et al. 2010).

Figure 1. Faba bean plant, root, stem, leaves, flowers, pods, and seeds.

Faba bean is an erect robust plant growing from 60 to often > 200 cm tall (Lim 2012). The roots possess a taproot system with branching laterals naturally bearing nodules with nitrogen-fixing bacteria *Rhizobium leguminosarium* bv *viciae* and the roots can form endomycorrhizal associations (Duc 1997; Link et al. 2008). The stem with indeterminate growth habit is hollow with or without basal branches arising from the leaf axils, with nodes and internodes bearing leaves, mostly without tendril, and inflorescences with two to twelve flowers in the leaf axil (Ladizinsky 1975; Link et al. 2008). Leaflets are oblong, elliptic or obovate reaching up to 10 cm long and 4 cm wide (Lim 2012). The flowers can be black spotted with veining on a background of white, red, brown or violet. Entirely white flowers are indicators of zero tannin in the seed. Generally pods have short velvet cover, and are short (3-4 ovules per pod) in *minor*, intermediate (4-8 ovules per pod) in *equina*, and

long and hanging (8-12 ovules per pod) in *major* types (Link et al. 2008). With one to two pods per inflorescence each bearing three to four seeds, a plant can bear about twelve pods. The seed can be yellow, black, red, green, beige, brown, or violet in color, and it is gray in zero-tannin genotypes (Duc 1997; Link et al. 2008; Lim 2012).

Flower blooming in faba bean mostly takes place a day after anthesis (Ladizinsky 1975). Owing to their floral biology, three of the faba bean varieties are moderately self/cross-pollinating (Stoddard and Bond 1987) with subspecies *paucijuga* greatly tending to autogamy (Cubero 1973). Natural outcrossing in faba bean is estimated in the range of 2 to 84%, with a mean of 32% and outcrossing is assisted by pollinator insects such as honeybees (*Apis mellifera*), bumblebees (*Bombus* spp.) and other solitary insects (Bond and Poulsen 1983). As Stoddard and Bond (1987) summarized, in the absence of bee-mediated pollination, 36% to 70% seed number reduction can occur in faba bean plants. Geographical location, type of pollinating insects and their activity are sources of variation in outcrossing levels (Duc 1997).

Though faba bean is an old-world legume used since the Late Neolithic and Early Bronze Age (Zohary and Hopf 1973; Zohary 1977; Caracuta et al. 2015, 2016) and is supposed to be the first grain legume consumed by humans (Muratova 1931), its center of origin is still debated (Caracuta et al. 2015; Caracuta et al. 2016). However, the probable origin of faba bean was postulated by Cubero (1973) as Near East, Iraq and Iran, from where secondary origins Ethiopia and Afghanistan were later evolved. Recently, Caracuta et al. (2016) found seed remains of wild *V. faba* that grew 14000 years ago in Mount Carmel, northern Israel, the region where the first documented domestication and faba bean farming was started some 10200 years ago (Caracuta et al. 2015). Today, however, no wild relative of faba bean with 12 chromosomes has been found, and no successful crossing with any other *Vicia* species has been reported (Zeid et al. 2003). In faba bean, seed size is the main indicator of diversity within the species, and *V. faba* L. is divided into two subspecies: *paucijuga* (about < 0.3 g seed size) and *eu-faba* (Adsule and Akpapunam 1996). *Eu-faba* is subdivided into three botanical varieties: var. *minor* Beck (0.4-0.6 g), var. *equina* Pers (< 1.0 g) and var. *major* (1-2.6 g) (Muratova 1931; Cubero 1974; Ladizinsky 1975). *Paucijuga* has been developed in India, *minor* in Ethiopia and Egypt (Muratova 1931), *equina* in the Middle East and North Africa, and *major* in South Mediterranean and China (Cubero 1974).

Currently, faba bean grows world-wide from temperate to tropical, and from humid to semi-arid climatic conditions. According to FAOSTAT (http://www.fao.org/faostat/en/#data/QC), globally, faba bean occupied 2.2 x 10^6 ha and 2.4 x 10^6 ha in 2014 and 2016 with corresponding yield of 4.2 x 10^6 and 4.5 x 10^6 tonnes. The observed increase in yield was because of the increased area of

production. Within the years mentioned, global yield per unit area productivity of faba bean fell 4.5%. Decrease in productivity in Europe alone reached 13.7%, while 19.3% increase in area of production was indicated. China, Ethiopia and Australia, in their order, were the three world leading countries in terms of area coverage and production of faba bean in 2016. In Europe, the United Kingdom is the major producer of faba bean followed by France. Though there has been great fluctuation, the continental average yield in Europe ranged from 2.6 to 3.2 t/ha between 2011 and 2018, and the highest productivity was 6 t/ha from relatively small areas in Ireland and Montenegro between 2010 and 2017 (www.https//ec.europa.eu/eurostat/data/database).

Figure 2. Local food made from faba bean in Ethiopia. (A) faba bean snack: sprouted faba bean boiled with salted water; (B) faba bean sauce: eaten with injera as lunch or dinner; (C) faba bean ful: usually eaten with bread as a breakfast dish.

Faba bean has food and feed values. The *minor* and *equina* varieties, referred to as field beans or horse beans, are mainly used for animal feed, and the large seeded *major* variety commonly called broad beans, are grown for human consumption (Knott 1990; Link et al. 1999; Crépon et al. 2010). Faba bean is an important human food in North Africa, the Middle East and China (Stoddard and Bond 1987; Stoddard et al. 2016). Across accessions, protein content ranges from 27 to 34% of seed dry matter (Duc 1997), and faba bean is considered as a meat substitute (Link et al. 2008). In ancient Rome, it was used for making porridge and a special purée (Renfrew 1973) and it was a preferred dish of the ancient Hebrews (Muratova 1931). Presently, faba bean is used as vegetable in green or dried form, and worldwide, 20% of it has green use (Link et al. 2008). In the Mediterranean region and Mediterranean ethnic markets of North America it is marketed for human consumption (McVicar et al. 2008). In China, extruded starch products (vermicelli) and sauces are made from faba bean (Mathews 2003). Faba bean sprouts seasoned with condiments, or boiled with salted water, or sprouted and roasted are popular snacks in Ethiopia (Figure 2A). Faba bean sauce is consumed as a principal dish with local "*ingera*" [(pancake made from tef (*Eragrostis tef* (Zucc.) Trotter) flour)] during lunch and dinner time (Personal observation) (Figure 2B). In Ethiopia and Sudan, whole or hulled bean sauce is served as a breakfast dish called "*ful*" (Figure 2C), a word probably derived

from the Arabic name "*fûl*" meaning faba bean.

Another benefit of faba bean is its ecosystem service in sustainable agriculture: biological nitrogen fixation, improving of soil biological, physical and chemical properties, and breaking of crop pest cycles (diseases, insects, and weeds) (Jensen et al. 2010; Rubiales 2010). The use of biofertilizers in crop production decreases the use of inorganic fertilizers (Dashadi et al. 2011), and faba bean is one of the best crops used in rotation with cereals to improve the productivity and meet the nitrogen (N) need of the succeeding crop (Atemkeng et al. 2011; Singh et al. 2012). The crop has the potential to fix free nitrogen at 150 to 300 kg N/ha (Singh et al. 2012), and the N benefit for the following crop reaches 100-200 kg/ha (Jensen et al. 2010). Faba bean has low fertilizer, pesticide, and fungicide requirements thus making it environmentally friendly wherever sustainable agriculture is sought (Duc 1997). Inoculation of faba bean with *Rhizobium leguminosarum* bv *viciae* or co-inoculation with *Azotobacter chroococcum* increased nodulation and total nitrogen content, and improved water and nutrient uptake of the crop under water stress conditions (Dashadi et al. 2011). With appropriate inoculum, this characteristic can be exploited in N-poor agricultural fields. Green manuring of soils at a rate of 9 tonne dry matter of faba bean per ha improved soil organic matter and microbial population, C and N content, soil structure and water holding capacity (Jensen et al. 2010 and references therein).

As a crop, growth and yield of faba bean are determined by climatic, edaphic, and management practices. Faba bean is best adapted to cool and moist agricultural areas (Mathews 2003). Hot, dry spells will result in desiccation injury to the plants and may reduce flowering and seed set (McVicar et al. 2008). Faba beans are especially sensitive to drought during the flowering stage (Duc 1997). In areas where terminal drought is common, early planting of faba bean is the best strategy for drought escape (Duc 1997; Khan et al. 2007; Link et al. 2008). In low-rainfall semi-arid Mediterranean environments, early sowing resulted with greater biomass and yield as compared to the late sown ones (Loss et al.1997). The optimal temperature for faba bean growth is 18-27 °C, it prefers soil types with pH ranging from neutral to alkaline (pH of 6.5 to 9.0) (Jensen et al. 2010), and it is highly sensitive to soil acidity and toxic levels of Al^{3+} concentration (Horst and Göppel 1986a, 1986b).

Under field growth conditions the yield of faba bean can reach as high as 6.15 t/ha (EUROSTAT 2019), but yields are reduced by abiotic stresses such as acid soil and drought stresses. One of the important aspects of soil acidity is the availability of aluminium ion that reduces growth and yield by causing root damage and oxidative stress. Drought, another source of oxidative stress and yield-limiting factor, affects the growth and productivity of faba bean at various stages. Al^{3+}-toxicity and drought interact, and their damage on the crop is synergistic (Yang et al. 2012, 2013).

1.2 Acid soil stress

1.2.1 Acid soil and its implication in crop production

The activity of hydrogen ion (H^+) expressed as pH determines soil acidity (Hede et al. 2001), and when the activity of the H^+ surpass that of the hydroxyl ions (OH^-), the soil is said to be acidic (Abebe 2007). Soil is a complex medium in that soil acidity is related with the amount of H^+ and Al^{3+} ions in exchangeable form (Abebe 2007). Acid soils, with pH < 5.5, are estimated to cover about 30% of the world's total ice-free land, and over 50% of the global potential arable lands are acidic (von Uexküll and Mutert 1995). Combinations of complex processes involving human and natural factors are responsible for the occurrence of acid soils in the world. Natural factors such as soil parent material and processes of weathering, rainfall and temperature conditions, the type of vegetation cover, the uptake of bases by plants and leaching of bases in the soil, and release of carboxylates and protons by plant roots (von Uexküll and Mutert 1995; Hede et al. 2001; Abebe 2007) along with the formation of weak acids such as carbonic acid from decomposition of plant materials all contribute to the soil acidification process (Mukherji and Ghosh 1996). Management practices known to accelerate soil acidification include frequent application of ammonium-based synthetic nitrogen fertilizers, removal of basic cations with plant material from the soil in the form of grain and livestock feed, excessive soil leaching in uncovered crop fields, and acid precipitation from polluted air (von Uexküll and Mutert 1995; Chen 2006; Zheng 2010).

Unless it is coupled with mineral element toxicities and/or deficiencies, plant growth is seldom affected by low pH alone (von Uexküll and Mutert 1995). However, soil pH values as low as 4.0 for faba bean and 3.5 for maize were reported to be critical, below which net H^+ release and root growth were inhibited (Yan et al. 1992). Broadly, the low productivity of acid soils is associated with soil nutrient content and soil physical characteristics. Nutritionally, the combined effects of toxicities arising from Al^{3+}, Mn^{2+} (manganese ion) and Fe^{2+} (iron ion), and deficiencies associated with soil P (phosphorus), Ca (calcium), Mg (magnesium), and K (potassium) are considered as causes of crop failure in acid soils (von Uexküll and Mutert 1995; Vardar and Unal 2007; Dharmendra et al. 2011). Depending on the soil type, physically, acid soils are characterized by low water holding capacity, susceptibility to crusting, erosion and compaction (von Uexküll and Mutert 1995). Moreover, deficiencies of some micronutrients, legume nodulation failures and increased plant disease are other crop production problems associated with acid soils (Dharmendra et al. 2011; Kochian et al. 2004; Vardar and Unal 2007).

In acid soils, crop production is inhibited by Al and Mn toxicities and P deficiency (White and Brown 2010; Zheng 2010), Al toxicity being the primary factor limiting crop production in 67% of global acid soil lands (Eswaran et al. 1997) causing 25–80% yield losses in various crop species (Dharmendra et al. 2011). Aluminium is the third most abundant element next to oxygen and silicon (Ma et al. 2001; Pilon-Smith et al. 2009) constituting about 8% of the earth's crust, and it is a component of primary and secondary minerals (Hede et al. 2001). In neutral or weakly acidic pH, Al exists as a stable complex with oxygen and silicate (Vardar and Unal 2007; Zhang et al. 2009), which is insoluble, biologically inactive deposit and inoffensive to plants (Chen 2006; Brunner and Sperisen 2013). At lower pH, Al is solubilized into an ionic form from the primary and secondary minerals and becomes phytotoxic (Hede et al. 2001). At pH lower than 5.0, $Al(H_2O)_6^{3+}$, which is known as Al^{3+}, is the dominant aluminium species (Vardar and Unal 2007). Naturally, acid soils are high in Al^{3+} concentration and low in base cations (BC) such as Ca^{2+}, Mg^{2+}, and K^+ (Brunner and Sperisen 2013). A ratio of Ca^{2+}/Al^{3+} or BC/Al^{3+} in the soil solution lower than 1 is commonly used as an environmental indicator of possibly adverse effects of Al^{3+} stress. Alternatively, the availability of toxic Al^{3+} in the soils can be indirectly determined from the Al and Ca concentrations in fine roots (Brunner and Sperisen 2013).

A toxic concentration of Al^{3+} in the rhizosphere is associated with harmful changes in root anatomy, morphology, and physiological functions (Liao et al. 2006) and inhibits root growth and function (Pineros and Kochian 2001) by destroying the structure of cells at the root apex (White and Brown 2010; Zheng 2010). Consequently, root tips and lateral roots develop into wrinkled, short, swollen and brittle structures (Vardar and Unal 2007) with limited capacity for nutrient and water absorption (Zheng 2010). Shoot symptoms seen because of Al^{3+} toxicity are the outward expressions of root damage (Vardar and Unal 2007), that may be displayed as reduced shoot photosynthesis and photo-protective systems, reduced water, carbohydrate, and mineral content, organic acid and nitrogen metabolism (Chen 2006). However, plant species exhibit different levels of tolerance to acid soil and Al^{3+}-toxicity.

1.2.2 Mechanisms of Al^{3+} toxicity tolerance

Plant species and genotypes vary in their tolerance and mechanism of tolerance to Al^{3+} toxicity. The existence of these differences indicates that tolerance to aluminium toxicity is genetically controlled (Singh et al. 2011). Genetic variability to Al^{3+}-toxicity tolerance has been indicated in tef (Abate et al. 2013), sorghum (*Sorghum bicolor* L.) (Hill et al. 1989), maize (*Zea mays* L.) (Maron et al. 2010), barley (*Hordeum vulgare* L.) (Cai et al. 2013), wheat (*Triticum eastivum* L. emend Thell) (Stodart et al. 2007), rice (*Oryza sativa* L.) (Nguyen et al. 2002), common bean (*Phaseolus vulgaris* L.) (Eticha

et al. 2010), pigeon pea (*Cajanus cajan* L.) (Choudhary et al. 2011), *Medicago truncatula* Gaertn (Chandran et al. 2008) and other crops. Breeding programs dedicated to the development of Al^{3+} tolerant varieties can benefit from these genetic variabilities to develop novel genotypes with enhanced aluminium tolerance.

Broadly, there are two physiological mechanisms that allow plants to tolerate toxic levels of Al^{3+} in soil solution: excluding Al^{3+} from the root tip (Delhaize et al. 1993; Jorge and Arruda 1997; Hocking et al. 2000; Maron et al. 2010; Castilhos et al. 2011), referred to as external tolerance; and providing Al^{3+} tolerance or detoxification in the plant symplasm, referred to as internal tolerance (Hocking et al. 2000; Hede et al. 2001; Ma et al. 2001; Vardar and Unal 2007; Brunner and Sperisen 2013). Exclusion may involve secretion of organic acids, formation of a rhizosphere pH barrier, selective permeability of the plasma membrane, and Al^{3+} efflux (Arunakumara et al. 2013). However, in Al^{3+} -tolerant plants, aluminium-induced release of organic acids from the roots plays a major role in the tolerance mechanism.

The chief mechanism of Al exclusion involves preventing Al from entering the root symplast by Al-activated release of organic acid anions such as citrate, malate, and oxalate that chelate and detoxify Al^{3+} outside the root tips (Zheng 2010; Brunner and Sperisen 2013). Sensitive plants in this group accumulate Al^{3+} in the epidermal layer and in the cortical layer below the epidermis (Delhaize et al. 1993), and probably this is the Al that is detectable by staining. Plant species and genotypes vary in the type and amount of organic acid they release and the number of genes involving in Al-tolerance, so there is variability in the degree of Al-tolerance (Pellet et al. 1996; Dharmendra et al. 2011). Al^{3+} -activated release of citrate in faba bean (Chen et al. 2012), maize (Jorge and Arruda 1997; Maron et al. 2010) and common bean (Miyasaka et al. 1991), malate in wheat (Delhaize et al. 1993; Pellet et al. 1996) and soybean (*Glycine max*) (Liao et al. 2006) have been reported. In wheat cultivars and lines for example, Al^{3+} -tolerance in cultivar Atlas was found to be multigenic but that in line ET3 was conditioned by the single Alt1 locus, and multigenic tolerance was reported to be 3 times as effective as monogenic tolerance (Pellet et al. 1996). Similarly, Al^{3+} exclusion in oat (*Avena sativa* L.) is controlled by a single gene in which a diverse pattern of Al^{3+} accumulation at root apexes across genotypes was reported (Castilhos et al. 2011). In chickpea (*Cicer arietinum* L.) Al^{3+} tolerance is controlled by a monogenic dominant trait (Singh and Raje 2011).

Internal Al tolerance mechanisms involve chelation of Al entering the root and transportation and sequestration into less sensitive parts of the plant and sub-cellular compartments (Brunner and Sperisen 2013). Organic acid anions and phenolic compounds have been implicated in internal Al^{3+} tolerance (Ma et al. 2001). Plants that accumulate Al in their leaves detoxify it by forming complexes

with organic acids such citrate and oxalate. Presumably, this chelation may reduce the activity of Al^{3+} entering the cytosol and limits its toxicity.

1.2.3 Management of acid soils for crop production

In acid soils, phyto-available P may be naturally low in quantity (Hocking et al. 2000) or the P is bound by clay minerals (Zheng 2010) rendering it unavailable for plant absorption. Additionally, in these soils aluminium is solubilized into Al^{3+} form, which is toxic to many plants (Hede et al. 2001). Consequently, crop production is largely limited in acid soils where low P availability is combined with Al^{3+} toxicity (Hocking et al. 2000). Application of lime to raise soil pH and P fertilizers to increase the phyto-availability of P in the soil are traditional recommendations to improve crop production in acid soils (Figure 3). However, these practices are not always practically and economically successful (Hede et al. 2001), in agricultural regions involving large areas of acid soils and where cropping is predominantly low-input (Hocking et al. 2000). The amelioration of aluminium toxicity through application of lime is expensive, ineffective in the subsoil and in some cases heavy application may have a deleterious effect on the soil structure (Zheng 2010) while application of P fertilizers is not always economical for farmers of developing countries (Atemkeng et al. 2011). In their four-year field study of aluminium chemistry in acid soils with high organic matter content, Brown et al. (2008) found that broadcasting lime raised the soil pH to a depth of 15 cm, but reduction of the activity of Al^{3+} was observed only in the top 5 cm of the soil. Similarly, these authors reported that the application of lime in a band at a soil depth of 5-10 cm did not significantly increase soil pH, so it had no effect on the yield of experimental crops. From the environmental point of view, frequent application of N and P fertilizers in agricultural lands contribute to the pollution of water bodies, resulting in eutrophication (White and Brown 2010). Hence, liming and fertilization practices alone are not sufficient in sustaining agricultural productivity in acid soils unless combined with the development of cultivars able to tolerate Al^{3+} toxicity and access poorly available soil P (Hocking et al. 2000; Hede et al. 2001).
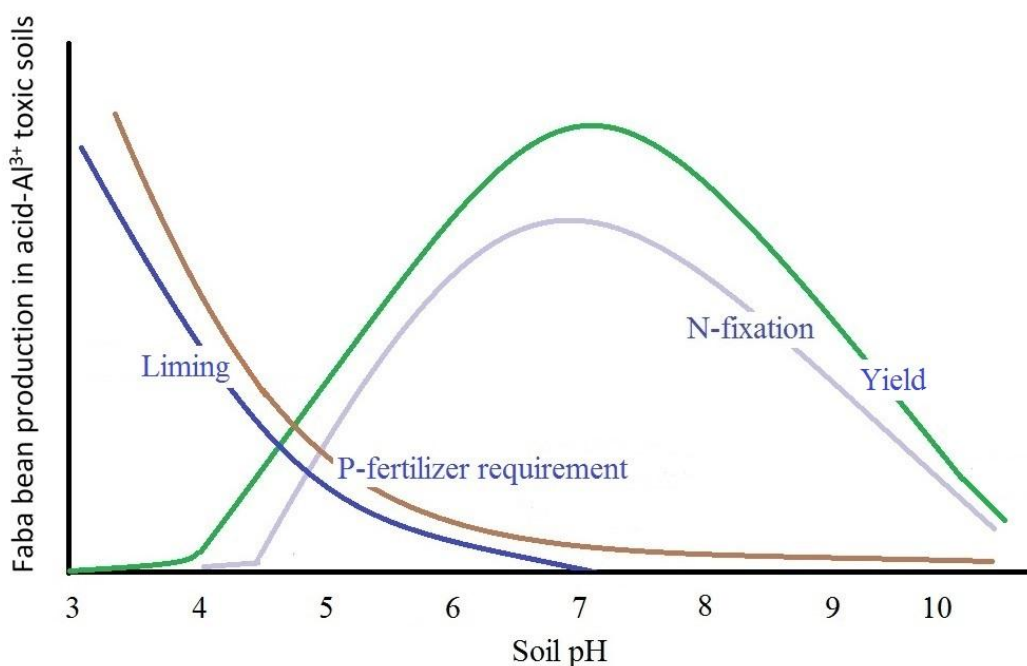
Figure 3. Schematic drawing of responses and requirements of faba bean under acidic and Al^{3+}-toxicity stresses. Faba bean grows best between pH of 6.5 and 9.0. Below 6 soil acidity and below 5 Al^{3+}-toxicity are the major challenges affecting yield and biological N fixation, and P availability and liming requirements.

Yield limitation in acid soils due to toxic mineral elements and mineral deficiencies is generally mitigated by the combined application of agronomic strategies and the development of novel tolerant cultivars (White and Brown 2010). Development of P-efficient cultivars that are able to utilize both soil and applied P in acid soils would be both sustainable and economical (Zheng 2010). However, conventional breeding efforts to identify and develop genotypes with improved P uptake and P use efficiency has been slow unless integrated with molecular approaches (Atemkeng et al. 2011). Incorporating carboxylate-exuding legume species in the cropping system may be a suitable agronomic practice to enhance P uptake in the current and subsequent crops. Hocking et al. (2000) summarized that the exudation of organic acids from plant roots is stimulated by both P deficiency and exposure to Al^{3+}, and that the organic acids are responsible for detoxification of Al^{3+} and solubilization of fixed soil P, indicating the important role of organic acids in enhancing plant nutrient acquisitions in acid soils. Faba bean genotypes known for their P efficiency and malate exudation in acid soils were reported to improve the growth and P uptake of wheat crops grown afterwards (Rose et al. 2010). Similarly, an Al^{3+}-tolerant common bean cultivar exuding citric acid in the rhizosphere was reported to enhance the availability of P from insoluble aluminium phosphate (Miyasaka et al.

1991). For low-input agriculture, breeding of legumes with improved ability to establish symbiotic relationships with arbuscular mycorrhizal fungi may improve P uptake in acid soil (Atemkeng et al. 2011). Hence, integrating the use of Al^{3+}-tolerant and P-efficient cultivars with soil amendment and fertilizer management practices provides the most effective strategy for production of economically important crops in acid soils.

1.2.4 Screening of crops for aluminium tolerance

Several screening methods for detecting Al^{3+}-tolerance in plant species and genotypes have contributed to accelerated breeding efforts to develop acid soil tolerant cultivars. In screening of crop genotypes from a wide germplasm pool, laboratory and greenhouse experiments are commonly employed using nutrient solution culture and solid media such as soil, peat, perlite, and sand (Villagrcia et al. 2001; Wang et al. 2006; Choudhary et al. 2011). Field screening in limed and non-limed plots can be applied to investigate the effect of Al^{3+} on root development or plant growth at different stages of growth (Samac and Tesfaye 2003). However, each method has its own advantages and shortcomings, so relying on a single method may yield misleading results (Samac and Tesfaye 2003; Narasimhamoorthy et al. 2007).

Evaluating shoot and root performance parameters in response to toxic levels of Al^{3+} concentrations provides good separation of tolerant and sensitive genotypes (Wang et al. 2006). Measurements of absolute and relative root length, root re-growth, and staining of roots with hematoxylin (indicator of the Al uptake by sensitive plants) and lumogallion (a fluorescent stain specific for Al) are the commonly used screening methods (Samac and Tesfaye 2003), and the use of Chrome Azurol S in faba bean (Chen et al. 2012) and of eriochrome cyanine R in pea (*Pisum sativum* L.) (Kichigina et al. 2017) were reported as staining procedures as well. Hematoxylin staining and root re-growth tests are not only fast and effective techniques in screening genotypes, but also are advantageous over field screening where large temporal and spatial variation in acidic soils makes it difficult to reliably rank experimental materials (Choudhary and Singh 2011). The hematoxylin staining and root re-growth methods with applications to genotypic selection (Ma et al. 2005; Stodart et al. 2007; Choudhary et al. 2011; Choudhary and Singh 2011), genetic analysis (Echart et al. 2002; Nguyen et al. 2002), and molecular characterization (Chandran et al. 2008; Raman et al. 2005) have been widely used in wheat, pigeonpea, barley, rice, *Medicago truncatula* and other species for Al tolerance. Hematoxylin staining provides a qualitative measure, while root growth measurements are quantitative, so they can be used together to evaluate relative levels of Al tolerance (Stodart et al. 2007). Together with the methods mentioned above, biochemical markers for antioxidant defense system including detection of the activity of enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), germin-

like protein (GEP), and non-enzymatic antioxidants such as ascorbate contents, and malondialdehyde (MDA, $CH_2(CHO)_2$), which is a marker of oxidative lipid injury) and contents of reactive oxygen species such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) in roots and leaves can also be used to discriminate faba bean accessions for Al^{3+}-toxicity tolerance (Zhang et al. 2009; Chen et al. 2012; Nahar et al. 2017).

1.3 Drought stress

Climate change and increase in temperature, land degradation and the recurrence of episodes of drought on one side, and demographic growth and accompanying increase in food, feed, fiber, and biofuel demands on the other side, present major challenges for humanity in the 21st century and beyond. Drought is an episode that limits the growth and development of crops at all stages of their life cycle. In different disciplines, drought is defined in a variety of ways. In agriculture, drought is a gap between the transpiration demand of the crop and available soil moisture (Tuberosa 2012). In rainfed agriculture, crops are exposed to periods of soil and atmospheric water deficit during their life cycle, especially in arid and semi-arid environments. In the semi-arid agricultural zone of tropical Sahel Africa, the length of the growing season was reported to be shorter than it was 50 years beforehand (Hall 2004). In the future, the occurrence and frequency of drought is expected to increase outside the current agricultural regions (Chaves et al. 2002), and under the slow and rapid climate warming setting, an average crop yield decline by 38% and 72% were predicted to happen by the end of the 21st century (Hatfield et al. 2011 and the references in). In faba bean, CO_2 enrichment significantly increased photosynthesis, plant growth, yield and water use efficiency (WUE) under adequate water supply but the effect was negative under drought condition (Wu and Wang 2000), indicating the synergistic effect of drought and elevated atmospheric CO_2 in growth and yield reduction of faba bean that will be even more severe with the increased climate change scenarios in the future.

Food grain and livestock feed production are mainly practiced in rainfed agricultural lands, whereas vegetables and other crops considered high-value are produced in irrigated lands, and this trend is expected to continue for the future (Passioura and Angus 2010). Hence, competition from high value crops will further push grain legumes to marginal lands that will expose them to the risk of drought (Mukeshimana et al. 2014). Under natural conditions, apart from water limitation, crops should cope with various stresses arising from other abiotic and biotic factors, circumstances further complicating their adaptation to moisture stress (Chaves et al. 2002). One of the many solutions suggested to

manage this problem is the development of crop cultivars with minimum yield loss under water-limited conditions.

Depending on the habitats where they originated and evolved, plant species have developed distinctive morphological, physiological and biochemical drought stress tolerance mechanisms that ensure their existence. These modifications are triggered by gene expression changes that provide structural and metabolic abilities for improved function under the stress (Chaves et al. 2002; Farooq et al. 2017). Consequently, crop species and genotypes show great variation in their response to limitation of water, and traits displayed differ with drought type and crop phenology (Passioura 2012). Due to the variable nature of drought, a trait that improves yield in one type of drought may be ineffective in another (Passioura 1983). To exploit sources of genetic variation, many international and national seed banks have been established throughout the world that have accelerated the improvement of agricultural crops in the last century. Sources of genetic variation can also be sought in local landraces and wild relatives. *Vicia faba* ex situ collections in 37 institutions across the world in 2008 identified 38360 known accessions (Duc et al. 2010), the International Center for Agricultural Research in the Dry Areas (ICARDA) with 12015 accessions being the biggest collector in 2009 (croptrust.org). Various reviews and research articles have discussed the different mechanisms of drought tolerance and tried to point to strategies of crop improvement (Levitt 1972; Chaves et al. 2002; Khan et al. 2010; Tuberosa 2012; Stoddard et al. 2016; Farooq et al. 2017; Kabbadj et al. 2017; Negin and Moshelion 2017).

1.3.1 Mechanisms of drought tolerance

Plant responses to drought stress may range from adaptive responses to deadly effects; in the adaptive case, depending on genotype, it may involve a mixture of stress avoidance and tolerance strategies (Chaves et al. 2002). According to Levitt (1972), broadly, drought adaptation mechanisms in crop plants can be categorized into dehydration avoidance and dehydration tolerance. The first involves maintenance of tissue water status through morphological and physiological modifications including vigorous root development, early flowering, osmotic adjustment and accumulation of cuticular waxes. The second involves storage and remobilization of water-soluble carbohydrates and accumulation of molecular protectants that provide a certain level of plant functionality under water limitation.

In this classification, drought escape is grouped with dehydration avoidance mechanisms. Drought escape involves shortening of duration of growth to ensure early flowering, grain development and maturity before the onset of terminal drought (Link et al. 1999; Acosta-Diaz et al. 2009; Farooq et al.

2017). This strategy has been reported to be successful in semi-arid Mediterranean environments in faba bean (Link et al. 2008), in extreme semi-arid Sahelian Africa in cowpea (Hall 2004), and in common bean cultivars (Rosales-Serna et al. 2004; Acosta-Diaz et al. 2009). Drought avoidance (sensu stricto) is a strategy of maintaining high tissue water potential by osmotic adjustment (effective use of water) (Blum 2009; Rosales et al. 2012) and effectively exploring moisture residing in the sub-soil (Matsui and Singh 2003; Songsri et al. 2008; Zhao et al. 2017).

1.3.2 Phenotyping for drought tolerance

In plant pre-breeding, screening and phenotyping of accessions with the objective of quantifying their structure and function in response to environmental changes will have multiple benefits. Firstly, the phenotypic information helps to identify and prioritize traits that can be used as morphological, developmental and physiological selection markers, which can further be aligned with genomic information to identify molecular markers (Passioura 2012). Secondly, the information is useful to identify host genotypes with desirable traits. Hence, by screening and phenotyping of genotypes originating from different growing environments, we can improve our understanding of drought tolerance mechanisms and their significance in crop adaptation.

High throughput screening and phenotyping for drought tolerance is generally hampered by lack of high-precision facilities allowing non-invasive automated data generation (Duan et al. 2018; Atkinson et al. 2019) and the absence of easy and reliable methods (Jeudy et al. 2016). The methods that employ manual measurements of drought response traits are regarded as time-consuming, error-prone, often subjective, and poorly quantified (Duan et al. 2018), so their application is less attractive and ineffective in screening of many plants (Singh et al. 2013). Though various studies indicate the potential of root traits in improving yield under drought, little achievement is recorded in exploiting the existing variation in root traits in crop breeding (Vadez et al. 2008; Zhao et al. 2017). This may be due to difficulties associated with high-throughput root phenotyping (Vadez et al. 2008; Jeudy et al. 2016). However, recently, high-throughput phenotyping facilities and methods have been developed in various institutes.

In the laboratory, noninvasive phenotyping of the root architecture and morphology has been possible through the generation of 2D or 3D images, whereas in field-grown plants usually destructive techniques such as shovelomics (root crown phenotyping) (Atkinson et al. 2019) and trench profiling (side digging and observing the roots) are applied (Wasaya et al. 2018). The quality and extent of data generated largely depend on the type of the growth medium and the technology employed. Conventional root phenotyping techniques employ agar plates (Figure 4A), soil or peat filled

rhizotrons (one sided glass window boxes) (Figure 4B), transparent RhizoTubes (Figure 4C), and pouch systems to generate 2D images non-destructively that will translate to quantitative data using automatic image analysis software (Nagel et al. 2012; Jeudy et al. 2016; Atkinson et al. 2019). Similarly, it is possible to generate 3D images in plants grown in soil-less media such as hydroponic, aeroponic or using any other optically transparent media (Atkinson et al. 2019). On the other hand, nondestructive generation of 3D images in soil media require the application of more advanced technologies that employ one or combination of the three tomographic techniques: X-ray computed tomography (X-ray CT), nuclear magnetic resonance imaging (MRI), and positron emission tomography (PET) (Fiorani and Schurr 2013; Li et al. 2014; Atkinson et al. 2019). However, these tomographic applications are low throughput (Li et al. 2014) and have their own pros and cons. There is also another type of phenotyping system that employs non-image whole plant characterization technique (Figure 4D). This system follows a functional physiological phenotyping approach (Gosa et al. 2018) and allows instantaneous and continuous measurement of physiological functions of each experimental plant in the array within the soil-plant-atmosphere continuum (SPAC) (Negin and Moshelion 2017). In this high-throughput system, each pot is monitored by three probes with an automatic computer-based algorithm that characterizes whole-plant transpiration, biomass gain, stomatal conductance and root flux under three time series treatment conditions: normal, stress, and recovery phases (Halperin et al. 2017).

Figure 4. Examples of phenotyping facilities and methods. (A) agar plate phenotyping and (B) GROWSCREEN-Rhizo phenotyping facility, Jülich Plant Phenotyping Center, Germany; (C) RhizoTubes, being piloted at National Plant Phenotyping Institute (NAPPI), University of Helsinki, Finland; (D) high-throughput functional physiological phenotyping platform, Robert Smith Institute of Plant Sciences and Genetics in Agriculture, Rehovot, Israel.

Growth reduction due to moisture stress on a plant is a result of a complex process involving numerous genes, enzymes, hormones, and metabolites acting either in co-ordination or in parallel or series of actions (Tardieu et al. 2011). The importance of trait-based selection of genotypes for drought tolerance had been reported in various papers, but, due to the large number of suggested traits and the specific nature of traits in crop phenology, prioritization is necessary (Passioura 2012;

Purushothaman et al. 2016; Negin and Moshelion 2017). Traits vary with type and periods of drought (Passioura 2012). Grain yield under drought in chickpea, for example, was associated with crop growth rate, canopy temperature depression, phenology, leaf area index (LAI) at mid-pod filling stage, and shoot biomass at flowering, indicating the importance of breeding for the best combination of traits with the right phenology (Purushothaman et al. 2016). Development of crop cultivars with high drought tolerance or the ability to escape/avoid drought by earliness or by effective use of water for prolonged duration can help maintain both yield and stability under limitation of water (Zhao et al. 2017). In view of this, understanding of the morphological, physiological and biochemical drought response traits and their role in drought adaptation mechanisms has paramount significance.

1.3.2.1 Morphological markers

Plants growing under different levels of soil moisture undergo variable morphological changes in shoot and root biomass partitioning, root and shoot architecture, flowering and grain development patterns. This variability has been studied in different grain and forage legumes. In response to drought treatments, common bean cultivars showed significant differences in shoot biomass gain, pod and seed weight, and relative water content (Rosales-Serna et al. 2004). In many plants, drought limits production of new leaves and accelerates the death of the existing ones, ultimately leading to reduced total leaf area (Wilkinson and Davies 2010). Decrease in leaf production in alfalfa in response to moderate drought was suggested as means to maintain relative water content (Erice et al. 2010). Similarly, large variation in shoot biomass productivity, specific leaf area (SLA) and LAI were observed among genotypes of chickpea in response to limitation of water (Purushothaman et al. 2016). In soybean, water stress during the pod filling stage caused early leaf death and termination of pod filling, resulting in considerable yield loss (Constable and Hearn 1978).

In order to address the optimum grain yield, optimizing the amount of biomass at anthesis is important. Insufficient vegetative growth means that there is less biomass to partition into yield, whereas excessive vegetative growth can lead to exhaustion of soil water and premature termination of grain filling (Passioura and Angus 2010). In their study on drought tolerance response of 40 lentil (*Lens culinaris* Medik) landrace collections, Sarker et al. (2005) found that stem length, taproot length and lateral root number were highly correlated with yield, with stem length alone contributing 85% of the variance observed in seed yield per plant. Faba bean genotypes tested in two contrasting water regimes showed significant variation in shoot, root and nodule dry mass (Kabbadj et al. 2017), and drought stress resulted in significant reduction in plant height and yield per plant reaching 22-29 cm and 6.5 g, and acceleration of 8-11 days to maturity (Link et al. 1999). Reduction of plant height due

to drought was positively correlated with greater yield and it was reported as a useful trait for drought adaptation in faba bean (Link et al. 1999).

The timing of flowering is one of the important traits in drought-affected environments. Optimal flowering time ensures a good balance between water used before and after flowering. Crops that flower after the optimal time are exposed to increased risk of moisture stress during grain filling (Passioura and Angus 2010). The progressive decline of soil moisture that starts prior to flowering may be the reason for the occurrence of terminal drought in legumes (Farooq et al. 2017). Thus, breeding of cultivars that flower close to the optimal time in a given environment will have a positive outcome (Passioura and Angus 2010). However, variability of rainfall makes the practicality of this strategy difficult. Significant reduction in number of days to maturity observed in tolerant common bean cultivars was associated with high grain filling rate, and maturity acceleration was reported to mitigate the effect of terminal drought (Rosales-Serna et al. 2004). Differences in yield and number of days to maturity were reported in cowpea cultivars under severe drought conditions (Hall 2004). A drought-sensitive common bean cultivar showed about 42% pod biomass reduction under severe drought (25% field capacity) while the corresponding decrease in a tolerant cultivar was only 17% (Rosales et al. 2012).

Adaptation to a specific agro-climatic zone is also one of the sources of morphological variation among crop genotypes in response to drought stress. Link et al. (1999) reported that among faba bean genotypes collected from geographically distant sites, North African and Latin American genotypes were more adapted to drought stress than European genotypes. Similarly, Zhao et al. (2017) demonstrated wide variation in root architectural and morphological traits of European faba bean cultivars that were associated with adaptation to specific agro-climatic zones. Northern cultivars showed greater reduction in total dry mass than southern cultivars under water stress condition. In southern cultivars, the individual size of lateral roots and their proportion in the whole root system were important traits contributing to water uptake in deeper soil layers (Zhao et al. 2017). In drought-tolerant chickpea genotypes, high root length density was reported to confer grain yield advantages during terminal drought (Purushothaman et al. 2017). Greater root length and thinner whole root system especially that of laterals in common bean (Abenavoli et al. 2016) along with deeper root growth and early stomatal closure in chickpea (Soltani and Sinclair 2012) were regarded as key target traits in genetic improvement of these crops for drought prone environments.

1.3.2.2 Physiological markers

One of the early symptoms of crops in drought stress is closure of stomata and subsequent reduction of carbon assimilation and rate of photosynthesis, which may be associated with reduced relative water content and leaf water potential (Rosales et al. 2012). Faba bean accessions from drought-prone environments showed higher stomatal density and reduced gas exchange while maintaining warmer leaves and higher leaf relative water content under drought stress than those from non-drought prone regions (Khazaei et al. 2013b). In their study involving nine faba bean inbred lines at the vegetative stage of growth, Khan et al. (2007) reported that moisture stress reduced water usage and shoot dry mass, and drought-tolerant genotypes such as ILB938/2 showed lower stomatal conductance and warmer leaves, while sensitive genotypes like Aurora/1 showed higher stomatal conductance and cooler leaves. Under severe drought (25% field capacity), a sensitive common bean cultivar showed 63% and 70% decrease in transpiration and stomatal conductance, respectively, resulting in a 61% reduction in net photosynthesis (Rosales et al. 2012). In common bean genotypes, there was a strong positive correlation between stomatal conductance and photosynthesis under well watered and water stress conditions, and stomatal conductance in the non-stressed treatment was 10-fold higher than stressed treatment (Mukeshimana et al. 2014). Khan et al. (2007) concluded that stomatal conductance, leaf temperature and $\Delta^{13}C$ were suitable physiological markers of drought tolerance at the pre-flowering stage in faba bean.

Drought during flowering damages seed set through pollen sterility or abortion of embryos and can later impede filling (Passioura and Angus 2010). In some sensitive common bean genotypes, drought stress during flower initiation caused closure of stomata and reduction in assimilation rate beginning from the second day of drought treatment while leaf water potential was unaffected (Acosta-Diaz et al. 2009); presumably this is a risk aversion behaviour induced by enhanced ABA concentration due to drought. Yield reduction during the reproductive and grain filling stage was summarized to be in the range of 26% in mashbean (*Vigna mungo* L.) to 65% in faba bean (Farooq et al. 2017). Kabbadi et al. (2017) reported that under drought stress, C and N content of shoots and roots in faba bean were highly reduced in sensitive genotypes. However, in a tolerant genotype, with greater correlation with growth performance, N content in shoots and roots was increased by 17% and 38%, respectively, and leaf relative water content, chlorophyll content, and efficiency of PSII were less affected under water stress. In drought treatment, a strong positive correlation between chlorophyll density and transpiration efficiency in peanut (Arunyanark et al. 2008), and a negative correlation between carbon isotope discrimination and transpiration efficiency in faba bean (Khan et al. 2007) have been reported. In lentil, chlorophyll content was positively correlated with root dry mass and root length, and

chlorophyll content was recommended as a useful trait to discriminate lentil genotypes for drought tolerance (Kumar et al. 2012). In their study of the effect of drought on two common bean cultivars (sensitive and tolerant to drought), Rosales et al. (2012) found that the drought-tolerant cultivar maintained seed production in terminal drought, due to early response, and fine-tuning of stomatal conductance, CO_2 diffusion and fixation, and by increased water use and avoidance of reactive oxygen species (ROS).

Accumulation of assimilate reserves in stems and roots before the occurrence of drought and remobilization of the reserves during the reproductive period is a coping strategy maintained by crop plants to avoid reproductive failure (Blum 2015). In sorghum and soybean genotypes, water stress during grain filling stage resulted with 17-25% stem dry mass reduction, which may be associated the relocation of stored assimilates from the stem to the grain (Constable and Hearn 1978). This indicates that storage of reserves before flowering is an important factor in maintaining yield and survival of plants during periods of drought (Chaves et al. 2002). Moreover, staying green and delaying senescence and maintaining CO_2 assimilation under drought stress was discussed by Farooq et al. (2017) as a suitable drought-adaptive strategy. In their experiment to determine shoot traits related with drought tolerance in common bean genotypes, Mukeshimana et al. (2014) found that those with a slow rate of wilting under water stress maintained green stems, showed smaller reductions in biomass and pod number, and recovered more rapidly.

1.3.2.3 Biochemical markers

Most legume crop species have evolved to undergo osmotic adjustment (OA) and osmoprotection under drought stress (Rosales et al. 2012; Kabbadj et al. 2017) by active accumulation of solutes when the leaf water potential is low (Blum 2015). By retaining leaf turgor pressure, OA enables the absorption of more water from the soil (Blum 2015) and enhances efficient use of water by the crop under drought condition (Blum 2009). The solutes are either N-containing compounds such as proline or other amino acids and polyamines or hydroxyl compounds such as sucrose, mannitol (sugar alcohol), and oligosaccharides (Farooq et al. 2017). In common bean, higher accumulation of proline was associated with maintenance of leaf water content and relative water content (Rosales et al. 2012). Proline and sugar provide maintenance of membrane integrity, while mannitol helps scavenging hydroxyl radicals and stabilizes macromolecular structures (Farooq et al. 2017). Under water stress, proline and glycine betaine accumulated in roots, shoots and nodules of faba bean genotypes, the first reaching 100-1000-fold increase in roots and nodules compared to the control (Kabbadj et al. 2017). Especially higher levels of glycine betaine accumulation were observed in nodules (Kabbadj et al.

2017), suggesting that this compound may also be involved in providing osmotic stress tolerance to faba bean nodule rhizobia.

Under drought stress, the accumulation of abscisic acid (ABA) in desiccating roots and its effect on closure of stomata leads to reduction of intercellular CO_2 and photosynthesis (Chaves et al. 2002), a characteristic feature that may leading to greater water use efficiency but not necessarily to greater yield (Blum 2009). Further, reduction of intercellular CO_2 leads to transfer of electrons to oxygen at photosystem I (PSI) leading to the generation of ROS such as O_2^-, H_2O_2, and the OH radical that may result in photo-oxidation (Rosales et al. 2012). At higher concentration above the level of plant defense, ROS cause oxidative damage to lipids, proteins, nucleic acids and DNA, and cause changes in the behaviour of biomolecules and result in cell death (Farooq et al. 2017 and references in).

In response to this, plants have evolved to generate antioxidant defence through synthesis of antioxidants such as ascorbate, carotenoids and phenolics, and trigger antioxidant enzymes such as SOD, CAT and reductase that scavenge ROS (Kabbadj et al. 2017). Higher accumulation of proline and mobilization of antioxidant enzyme activities such as ascorbate peroxidase (APX) and CAT in drought-stressed faba bean genotypes in response to higher peroxidation levels of H_2O_2 in roots, shoots and nodules were reported as plant adaptation strategies during water stress (Kabbadj et al. 2017). Rosales et al. (2012) reported that severely droughted sensitive common bean cultivars showed 46% and 55% increased levels of MDA and H_2O_2, respectively, as compared to the control treatment. In this study, higher antioxidant enzyme activities of SOD, CAT, APX and a higher level of reduced ascorbic acid concentration were correlated with higher peroxidation levels observed in the sensitive cultivar. However, the accumulation of phenolic compounds and flavonoids was high in both tolerant and sensitive cultivars under severe drought.

Phytohormones play a regulatory role to endogenous and exogenous stimuli and may act either collectively or independently in response to drought. Among plant hormones, some consider ABA as a drought tolerance hormone whereas others consider it as a drought survival hormone. Under drought, the majority of ABA is produced in three organs, namely: the root, the leaf, and the seed (or fruit) (Blum 2015). Drying soils and increase in air vapor pressure deficit induce the synthesis of ABA in roots and shoots, respectively, and ABA induces the closure of stomata by interfering with the uptake of potassium by leaf guard cells (Wilkinson and Davies 2010), resulting in increased leaf turgor and reduction of transpiration (Blum 2015). Apart from its role in closure of stomata, ABA increases root hydraulic conductivity and facilitates water uptake and transport in the plant (Tardieu and Davies 1993), and has a role in robust root development (Wani et al. 2016). So, root-based ABA contributes to both enhanced water uptake and reduced water loss through stomata. However, this

twofold gain by the plant is transitory. Following the attainment of higher leaf water potential due to the effect of ABA, plants become unable to absorb extra water from the soil owing to reduced water potential gradient between the roots and the soil (Blum 2015), ultimately the plant suffers from reduced carbon assimilation rate and inability to absorb the available moisture in the soil, leading it to an unproductive quiescent state. On the other hand, the effect of ABA on yield can be beneficial or harmful depending on the growth stage of the plant when drought stress occurs. During early stage drought, ABA sensitive plants may benefit from enhanced ABA accumulation as it checks both growth and soil moisture use, hence conserving moisture for terminal growth stage (Blum 2015). Under drought stress, in maize yield and ABA accumulation were negatively correlated, and similarly drought-tolerant wheat accumulated lower ABA and showed cooler mid-day canopy temperature (Blum 2015 and references in).

The expression of ABA and its effect on the plant during drought stress can be affected by the signaling of other hormones. Soil drying increases the synthesis and transport of ethylene in shoots and reduces the transport of cytokinins, the stomatal opening hormone, from roots to shoots (Wilkinson and Davies 2010). The stay-green trait is controlled by cytokinin (Blum 2015). Ethylene seems to possess a dual role in promoting and inhibiting the opening of stomata (Kazan 2015). For example, under ozone pollution, ethylene antagonizes stomatal closure responses to ABA (Wilkinson and Davies 2010), but on the other hand it promotes closure of stomata by promoting nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) mediated production of ROS in guard cells (Kazan 2015). However, the effect of ethylene on stomatal mechanics is not well understood. Generally, under water stress the concentrations of ABA and ethylene tends to increase while those of auxin, gibberellins and cytokinin decrease in most plants.

In conclusion, a large root system (in depth, width, and volume) for effective soil moisture extraction, a higher level of OA, higher levels of stomatal conductance (and sink accumulation), cooler leaf/canopy temperature, and lower levels of ABA concentration are all associated with higher drought tolerance and greater yield. This ideotype follows the anisohydric (water spending) model described in Blum (2015), a crop type which can be most effective in moderately drought-prone environments. On the other hand, higher levels of ABA accumulation, lower levels of stomatal conductance (and low sink accumulation and growth rate during drought stress) and the corresponding higher leaf temperature, are associated with drought surviving crop cultivars usually exhibiting lower yield. According to Blum (2015), this model fits with isohydric ideotype (water saving), and such crop cultivars are well suited to harsh dryland agricultural areas such as

Mediterranean climates known with early season rainfall and terminal drought especially when they incorporate heat tolerance mechanisms to avoid the heat built up during stomatal closure.

1.4 FIGS as a mining tool of adaptive traits

The Focused Identification of Germplasm Strategy (FIGS) is an approach to mine adaptive traits in a wide germplasm collection potentially suitable for a certain agricultural environment (Street et al. 2016). The main principle in FIGS is that the influence of environment in natural selection and distribution of accessions in agro-climatic space so strong. This strategy is applied to filter the traits of interest in a given genetic resource collection (Khazaei et al. 2013a). In FIGS, habitat characterization and development of priori information based on trait-environment relationship is needed so that best-bet accessions are defined (Upadhyaya et al. 2011; Bari et al. 2012) and when this is not the case, FIGS has been shown to be a valuable tool in predicting and mining trait information lacking due to incomplete documentation or characterization as reported in Nordic barley landraces (Endresen 2010). The strategy has been tested in finding agriculturally beneficial traits for drought adaptation in faba bean (Khazaei et al. 2013a), phenology driven yield improvement in chickpea (Berger et al. 2004), stem rust tolerance (Bari et al. 2012) and Russian wheat aphid (Bouhssini et al. 2011) in wheat.

1.5 Interaction of aluminium and drought stresses

Drought and Al^{3+}-toxicity are the two major abiotic stresses contributing to the greatest share of global yield loss in most agricultural regions. More importantly, drought and Al^{3+} toxicity are known to interact. The combined effect of drought and Al^{3+}-toxicity stresses have been studied in several crops including soybean (Goldman et al. 1989; Nian et al. 2004; Joris et al. 2013), common bean (Yang et al. 2012), and barley and wheat (Tang et al. 2002; Karmanenko et al. 2011).

Al^{3+} stress inhibits root growth, and its effect on shoot is secondary as it is induced by the damage it causes on roots. On the other hand, drought stress inhibits shoot growth but promotes root growth to reach the available water in acidic sub-soil (Yang et al. 2013). When drought occurs in acid aluminium toxic soils, the effect on both root and shoot growth will be profound. Combined Al^{3+}-drought stresses in common bean resulted in severe reduction of root elongation by 45-68%; partly due to the direct effect of Al^{3+} injury on root tips and partly due to the effect of Al^{3+} on the reversion of drought-enhanced expression of 9-cis-epoxycarotenoid dioxygenase (NCED), an enzyme that regulate the biosynthesis of abscisic acids in roots (Yang et al. 2012). Al^{3+}-tolerant wheat uses more water and yields higher than Al^{3+} sensitive ones in soils with sub-soil acidity (Tang et al. 2002). In soybean, combined treatment of Al^{3+} (100 μM $AlCl_3$) and PEG-6000 resulted in higher shoot and

root growth reduction than either of the treatments alone (Nian et al. 2004). Soybean plants grown under drought and Al^{3+} stress did not recover from desiccation within the recovery period (Goldman et al. 1989), indicating, unless that Al tolerance is integrated, drought tolerance cannot be fully utilized in crops growing in aluminium-toxic acid soils because of a poorly developed root system.

Both Al^{3+}-toxicity and drought are oxidative stresses resulting in the production of ROS. The mechanisms of tolerance for the oxidative damage is controlled by enzymatic and non-enzymatic antioxidant defense systems and/or through biochemical detoxification and cell protection systems. In a drought and Al^{3+}-tolerant rice cultivar, root to shoot ratio, relative water content and chlorophyll content were affected by neither Al^{3+} nor drought nor the combined treatments, and tolerance to these stresses was associated with increased activity of SOD, guaiacol peroxidase (GPX), APX and CAT (Pandey et al. 2014). In mung bean (*Vigna radiata* L.), Al^{3+} stress induced reduction of leaf relative water content by 16-23% and was accompanied by a 50-82% increase in the concentration of the osmoprotectant molecule proline in 48-72 h (Nahar et al. 2017). Drought suppressed the Al-induced expression of *MATE* gene involving in the biosynthesis of citrate, but Al-enhanced expression of *ACCO* and reversion of *NCED* expression were responsible for inhibition of root elongation under combined Al-drought stress condition (Yang et al. 2012).

Hence**,** screening and phenotyping of a wide range of accessions for novel traits of drought, acidity and aluminium toxicity tolerance, and incorporating traits of interest into modern cultivars is a key strategy to improve faba bean production and extending its production environments where these stresses are prevalent.

2 OBJECTIVES OF THIS STUDY

The overall objectives of this study were to investigate complementarity in shoot and root morphological and physiological phenotypic markers to acid soil and drought adaptation in pre-flowering faba bean plants, and to identify sources of tolerance for further breeding work.

The specific objectives were:

to identify faba bean accessions that perform relatively well in acid soils, investigate the underlying responses to acid soil and Al^{3+}-toxicity, and to develop a reliable technique for discriminating sensitive and tolerant germplasm (Publication I);

to identify faba bean accessions that perform relatively well in drought stress, and investigate the underlying root responses to drought stress. The first hypothesis, tested with a germplasm survey, was that dry-zone germplasm would have more prolific root systems than wet-zone germplasm. The second hypothesis, tested with the phenotyping robot, was that dry-zone germplasm would maintain its root system growth better in drought than wet-zone germplasm would (Publication II); and

to investigate shoot response traits to drought avoidance under contrasting soil moisture conditions and evaluate the complementarity of shoot and root traits. The hypothesis was that drought avoidance is based on a combination of leaf gas exchange and exploitation of soil water, so evaluation of both roots and shoots was needed (Publication III).

3 MATERIALS AND METHODS

To achieve the objectives mentioned in Section 2, faba bean accessions for acid soil study were chosen based on their expected exposure to acidity or aluminium stress in their regions of provenance; and for drought tolerance study, the original set of 201 wet-adapted and 201 dry-adapted accessions studied by Khazaei et al. (2013a) was reduced to 88 based on differences in canopy temperature depression measured in the glasshouse (Khazaei et al. 2013a), country of origin and availability of seeds. Ten other accessions (7 from Ethiopia and 3 from Europe) were selected based on previous research findings and observations. An outline of experimental works of this study is presented in Table 1, and measurements and instruments applied in each experiment indicated in Tables 2 and 3. Designs and statistical tools and plant growth management conditions applied in experiments are summarized below in sub-sections.

Table 1. Summary of methods applied in the original publications (I-III) of this study.

Method	Publication
Germplasm screening for acidity and Al^{3+}-toxicity tolerance in nutrient solution culture	I
Testing acidity and Al^{3+}-toxicity responses in peat growing medium	I
Testing acidity and Al^{3+}-toxicity response in perlite growing medium	I
Growth medium and shoot Al concentration analysis using ICP-OES*	I
Multivariate statistics	I, II, III
Germplasm screening for drought adaptation	II, III
Root and shoot phenotyping of selected accessions in automated phenotyping platform GROWSCREEN-Rhizo	II, III
Quantification of root images using PaintRHIZO and WinRHIZO	II

* Inductively coupled plasma-optical emission spectrometry.

3.1 Tests for acidity and Al^{3+}-toxicity tolerances

3.1.1 Germplasm screening in solution culture

The experiment was conducted in growth chambers at the University of Helsinki, Finland in spring 2015. In total, 29 faba bean accessions were chosen (listed in Table 1 of publication I) based on their expected exposure to acidity or aluminium stress in their regions of provenance. Twenty accessions were delivered by Ethiopian Institute of Agricultural Research, Holeta Agricultural Research Center (HARC) and the others were chosen from European and Canadian germplasm used in previous experiments. These accessions were tested for their response to acidity and Al^{3+} toxicity in 5 treatments (at pH 7.0 (control)), pH 4.5 (treatment for acid or control for Al^{3+}), and pH 4.5 acid + 41, 82, 123 µmol/l $Al_2(SO_4)_3 \cdot 16H_2O$) in 3 replications arranged in a split-plot design (replication as blocks, tray as main plot, and accessions as sub-plots). The three levels of Al^{3+} concentrations were

adopted from Choudhary and Singh (2011). The replicates were separated by 21 days in time rather than physically in space, owing to space restrictions. Data on the root system were taken on the eight and eleventh days after the seedlings were transferred into the solution culture as described in 3.3.1. A.

3.1.2 Testing in pot experiments: peat and perlite media

A subset of 10 accessions representing different combination of Al^{3+} and acidity response in solution culture (listed in Table 2 of publication I) were selected for further evaluation. The experiment was conducted twice at the University of Helsinki, first on peat-based medium in open air (in summer 2015) and again on perlite medium in an open-sided greenhouse cage (in summer 2016) until the beginning of flowering. The peat and perlite experiments were conducted in a split-plot design, with four replicate blocks, three treatments (neutral, acid and 82 µmol/l $Al_2(SO_4)_3.16H_2O$) as the main plots and accessions as subplot. In these pot experiments, the 41 and 123 µmol/l aluminium treatments were abandoned as they were found to be relatively uninformative in the solution culture experiment.

3.2 Tests for drought adaptation

3.2.1 Germplasm screening

A set of 89 accessions (listed in Table 1 of publication II) from wet and dry growing regions of the world was defined according to the Focused Identification of Germplasm Strategy (Khazaei et al. 2013a) and screened in spring 2016 at University of Helsinki, Department of Agricultural Sciences greenhouse facility in a perlite-sand medium under well watered condition arranged in a randomized complete block design (RCBD) with 4 replications. The experiment was designed to maximize expression of potential root mass by providing plentiful moisture and nutrients. At BBCH stage 39 (Meier 2001), when there were approximately 9 visibly extended internodes, 30-34 days after sowing (DAS), measurements indicated in Table 3 were taken.

3.2.2 Root and shoot phenotyping

Eight accessions were chosen (listed in Table 4 of publication II) from the germplasm survey according to morphological and physiological data. In order to get more information on root systems, phenotyping of these selected accessions under contrasting soil moisture level was required. Consequently, access to specialized equipment was needed, and access was given to the root phenotyping facilities at the Jülich Plant Phenotyping Center (JPPC), Forschungszentrum Jülich GmbH, Germany. The hypotheses were that accessions with a strong root system would be more capable of withstanding the stress of water deficit than those with small or weak root systems that rapidly run out of access to water (Publication II), and drought avoidance is based on a combination

of leaf gas exchange and exploitation of soil water (Publication III). Thus, the eight accessions were subjected to drought adaptation test using the automated root and shoot phenotyping platform GROWSCREEN-Rhizo using rhizotrons with a size of 90 x 70 x 5 cm (Nagel et al. 2012) at JPPC in winter 2017. The experiment was arranged in a split-plot design, with 4 replicate blocks, 2 treatments (well watered and water limited) as the main plots and 8 accessions as subplots. Shoot and root data indicated in Table 3 were taken during and at the end of the experimental period.

3.3 Measurements

Table 2. Measurements, instruments and methods used during experiments (publication I).

Measurement	Acidity and Al^{3+} tolerance*			
	Germplasm survey	Peat assay	Perlite assay	Instruments / methods / reference
Taproot length	Yes		Yes	Ruler
Root regrowth length	Yes			According to Nava et al. 2006
Hematoxylin stain score	Yes			According to Polle et al. 1978
Hematoxylin stain root imaging	Yes			Stereo microscope fitted with an AxioCam ERc 5s imaging device
Acid root tolerance index	No			Ratio of taproot lengths grown at pH 4.5 to pH 7.0 treatment
Aluminium root tolerance index	Yes			Ratio of taproot lengths grown at pH 4.5 with & without Al treatment
Leaf rate of photosynthesis		Yes		LI-6400 Portable Photosynthesis System (LI-COR, Lincoln, NE, USA)
Stomatal conductance		Yes	Yes	LI-6400 Portable Photosynthesis System (LI-COR, Lincoln, NE, USA) and Leaf Porometer (Decagon Devices, Inc., Pullman, WA, USA)
Canopy temperature			Yes	FLUKE Model 574 Precision Infrared Thermometer (Fluke Corporation, Everett, WA, USA)
Leaf chlorophyll concentration		Yes	Yes	SPAD-502 (Minolta Camera Co, Ltd., Tokyo, Japan)
Total leaf area		Yes		LI-COR Model LI-3000A Portable Area Meter (LI-COR, USA)
Root nodule quality and quantity		Yes		Scoring based on color (white vs pink) and presence and absence of nodule
Root and shoot dry mass		Yes	Yes	Weighing using sensitive balance to the nearest 0.01g
Potting medium and shoot Al content analysis		Yes		ICP-OES (according to EPA 1996)

*3 treatment conditions: neutral (pH 7.0), acid (pH 4.5), acid + aluminium (pH 4.5 + 41, 82, 123 μmol/l $Al_2(SO_4)_3 \cdot 16H_2O$).

Table 3. Measurements, instruments and methods used during experiments (publication II & III).

Measurement	Survey	Phenotyping for drought		Instruments / methods / reference	Publication
		Root	Shoot		
Seed weight	Yes			Weighing 10 dried seeds per accession	II
Stomatal conductance	Yes		Yes	Leaf Porometer (Decagon Devices, Inc., Pullman, WA, USA)	II, III
Leaf surface temperature	Yes		Yes	FLUKE Model 574 Precision Infrared Thermometer (Fluke Corporation, Everett, WA, USA)	II, III
Leaf chlorophyll concentration	Yes		Yes	SPAD-502 (Minolta Camera Co, Ltd., Tokyo, Japan)	II, III
Maximum quantum yield of photosystem II (Fv/Fm)			Yes	Photosynthesis Yield Analyzer (MINI-PAM 3, Heinz Walz GmBH, 91090 Effeltrich, Germany)	III
Effective quantum yield (EQY)			Yes	Photosynthesis Yield Analyser (MINI-PAM 3, Heinz Walz GmBH, 91090 Effeltrich, Germany)	III
Leaf count			Yes	The first three leaf counts were taken 2, 9, 16 and during plant harvest at 19 DAT	III
Total dry mass			Yes	Weighing to the nearest 0.01g	III
Leaf and stem dry mass			Yes		III
Leaf and stem dry matter content (%)			Yes	Calculated as leaf & stem dry mass (g) divided by leaf & stem fresh mass (g), respectively, multiplied by 100	III
Root and shoot dry mass	Yes		Yes	Weighing using sensitive balance to the nearest 0.01g	II, III
Root to shoot dry mass ratio	Yes		Yes	Root dry mass divided by shoot dry mass	II, III
Root mass fraction	Yes		Yes	Root dry mass divided by whole plant dry mass	II, III
Shoot mass fraction			Yes	Soot dry mass divided by whole plant dry mass	III
Apparent specific root length (m/g)			Yes	Taken as total root length (whole root system length visible at the transparent plate of the rhizotrons) (m) divided by whole root system dry mass (g)	III
Apparent root length density (cm/cm^3)			Yes	Calculated from total root length (cm) divided by volume (cm^3) of peat enclosed in the convex hull area marked in the rhizotrons multiplied by the breadth of the box (5 cm)	III
Taproot length		Yes		Root images obtained with GROWSCREEN-Rhizo were analyzed using PaintRHIZO software package (according to Nagel et al. 2009 & 2012)	II
First order & 2nd order lateral root lengths		Yes			II
Total root length		Yes			II
Root system depth		Yes			II
Root system width		Yes			II
Convex hull area		Yes			II
Sample plant actual whole root system length		Yes		Root images obtained using manual root scanner EPSON A3 Transparency Unit (Model EU-88, Japan) were analyzed using WinRHIZO software (according to Mühlich et al. 2008)	II

3.3.1 Exposing of plants to acidity and Al^{3+}-toxicity treatments (publication I)

A. Treatment management: solution culture screening experiment

Seeds of uniform size were selected, washed, surface sterilized, soaked in water for 24 h and transferred to Petri dishes with moist filter paper layers for 72 h at 22 °C in the dark for germination. Seedlings were allowed to grow in plastic trays (30 accessions each with 8 units of seedlings) covered with a lid with holes through which roots were suspended inside aerated 0.5 mM $CaSO_4$ solution for 96 h in a climate-controlled growth chamber (Figure 5). Then plants were grown in aerated nutrient solution adjusted to pH of 7.0 (control), pH 4.5 (for acid and Al^{3+} treatments) for 3 days. On the fourth day, fresh nutrient solution culture was provided for each treatment with corresponding pH adjustment and the 3 aluminium treatments were initiated and were grown for 24 h in Al containing nutrient solution. From the 8 seedlings of each accession, 4 were taken for hematoxylin staining and the remaining 4 were left for 3 days in the tray to recover in Al-free nutrient solution adjusted to pH 4.5. The length of the tip to the point of callus formation was presented as root regrowth length. Fresh nutrient solutions were also provided for the control and acid-grown plants at the same time to maintain the uniformity of experimental conditions.

Figure 5. Faba bean accessions receiving three levels (41, 82, and 123 μM) aluminium treatments in one of the full sets of replicates.

B. Treatment management: peat and perlite media pot experiments

Peat assay. The peat medium was prepared in 9:1 peat:sand (v/v) ratio and poured into 120 pots each with 7.5 l capacity. The pots were sunk into sand-beds prepared in open air. The treatments included in this experiment were pH 7.0 (control), pH 4.5 (acid), and pH 4.5 + 82 μmol/l $Al_2(SO_4)_3.16H_2O$. Upon delivery, the pH of the peat was 4.5, so one-third of the pots were brought to pH 7.0 by liming, one-third were used for acid treatment as they were, and the remaining one-third were supplied with

3 l of 82 µmol/l $Al_2(SO_4)_3.16H_2O$ solution at sowing while the rest of the pots each supplied with 3 l of tap water to bring them to field capacity. Seeds were inoculated with *Rhizobium leguminosarum* biovar. *viciae*, 4 seeds/pot were sown at 2 cm depth and a week after emergence they were thinned to 3 plants per pot. Data were taken 25, 35, 45 and 58 days after sowing (DAS). Then, at day 58, the experiment was terminated when plants started flowering.

Figure 6. Acidity and Al^{3+} toxicity tests in perlite medium.

Perlite assay. Plants were grown on two 3-l pots that were stacked on top of each other to provide adequate depth for root growth. Adequate drainage holes were provided for each pot. The bottom pot was filled with 2 l of perlite and on top of the perlite 1 l of fine sand was put to ensure firm hold for the top pot (Figure 6). The top pot was filled with 3 l of perlite alone. Two seeds per pot were sown and each pot was supplied with tap water to field capacity. After 5 days, each pot was thinned to 1 plant and for 10 days after sowing, pots received 200 ml of tap water every other day. Then, for the next 32 days, each pot received 200 ml of nutrient solution prepared using 1 g/l complete fertilizer (Superex Peat; Kekkilä Oy, Vantaa, Finland) supplied with 2 mmol/l $CaCl_2$ on every other day with nutrient solution adjusted to corresponding treatment conditions: pH 7.0 (control), pH 4.5 (acid), and pH 4.5 + 82 µmol/l $Al_2(SO_4)_3.16H_2O$. Data were taken 41 and 42 days after sowing. Then, at day 42, the experiment was terminated when plants started flowering.

3.3.2 Plant and treatment management in drought adaptation experiments (publication II and III)

A. Plant growth management: germplasm survey experiment

In this experiment, the bottoms of 3 l capacity pots were lined with a membrane then filled with 0.2 l of sand at the bottom, 2.6 l of perlite at the middle, and 0.2 l of sand on the top (Figure 7). Two seeds per pot were sown and thinned to one plant after 5 days. Fertilizer nutrient solution prepared from 1 g/l of Superex Peat (Kekkilä Oy, Vantaa, Finland) supplemented with 2 mmol/l $CaCl_2$ was automatically applied at 200 ml per pot every other day from sowing to the end of the experimental period, 34 DAS.

Figure 7. Germplasm survey for drought adaptation. Four replicates were sown at seven-day intervals.

B. Plant growth management: root and shoot phenotyping experiments

This experiment was conducted during the vegetative stage of plant growth, covering 28 days from seed soaking to destructive harvesting of experimental plants. Pregerminated seedlings of each accession showing uniform root growth were transplanted into the rhizotrons. The growth medium used was GRAB-ERDE, a dark peat-based substrate (Plantaflor Humus Verkaufs-GmbH, Germany). The initial moisture content of the peat-soil was 66.3%. The well watered treatment rhizotrons were filled with the peat without drying and part of the peat was air dried to 40% moisture content and filled into the rhizotrons for the water-limited treatment.

Figure 8. Phenotyping of 8 accessions in contrasting watering treatments using GROWSCREEN-Rhizo automated phenotyping system, Jülich Plant Phenotyping Center, Germany.

The boxes were then fixed in the robotic system in the greenhouse and tilted at 43° from vertical (Figure 8). During the establishment phase, each seedling in the well watered treatment received 200 ml water in the automatic irrigation system and those in water-limited treatment received 50 ml of water to their roots manually. Thereafter, the well watered plants were given 100 ml of water every 12 h until the end of the treatment period. In the water-limited treatment, plants received the second 50 ml of water 4 days after transplanting with no watering afterwards. Data (Table 3) were taken five days a week, from these, data taken at 5, 12, and 19 days after treatment (DAT) started were presented in Publication II, and shoot and root morphological and physiological data that were recorded 15 to 19 DAT were presented in Publication III.

3.3.3 Statistical analysis

Analysis of variance was conducted using SPSS version 22.0 or 24.0 (IBM Inc., Chicago, IL, USA) software package, and treatment means were separated by either LSD or Duncan's Alpha (5%) (Publication I, II, and III). Accession and culture media were treated as fixed effects and replicate as a random effect in the split-plot design and repeated measure analysis was conducted for SPAD value (Publication I). Root images were analyzed using PaintRHIZO and WinRHIZO software packages following the methods developed by Mühlich et al. (2008) and Nagel et al. (2009, 2012). Frequency distributions were used to check for the presence of outliers. Independent-samples t-test was conducted to test difference between the group means of the dry-adapted and wet-adapted sets in the

germplasm survey. Two-way ANOVA was used to test the main effect of treatment, the main effect of accession, and the treatment by accession interaction effect on multiple sampling dates (Publication II). Genotypic means (across treatments) and phenotypic means (treatment means) were both tested. Two-tailed Pearson correlations were calculated and principal component analyses between roots and shoot measurement data were performed. Hierarchical cluster analysis of root and shoot data was used to construct a dendrogram using average linkage between groups. Correlation network of traits was conducted using R software package (Publication III).

4 RESULTS AND DISCUSSION

4.1 Responses to acidity and Al^{3+}-toxicity

4.1.1 Germplasm screening to acidity and Al^{3+}-toxicity in solution culture

Root responses to aluminium depended on the concentration of Al^{3+} ion in the nutrient solution. At the lower concentration of Al^{3+} (41 µmol/l Al^{3+}), larger values of root regrowth length and Al^{3+} tolerance index were observed, whereas the stain score was high at higher Al^{3+} concentration (123 µmol/l Al^{3+}), indicating increased sensitivity of roots to higher concentration Al^{3+}. Root regrowth length at 41 µmol/l Al^{3+} was 2.6- and 17-fold longer than those at 82- and 123 µmol/l Al^{3+}, respectively (Table 4). The root regrowth length at 82 µmol/l Al^{3+} was 6.6-fold longer than at 123 µmol/l Al^{3+} (Table 4), and stain score at 123 µmol/l Al^{3+} was about 2-fold higher than the score observed at the minimum concentration. In both cases, it appeared that root resiliency and the extent of chelation of Al^{3+} depended on the Al^{3+} concentration in the growth medium. The root tolerance index was significantly higher at 41 µmol/l Al^{3+} than the rest and was even larger than acidity tolerance index, indicating an enhancement of root growth in the low concentration of Al^{3+} beyond than in acidic medium alone. In similar research, root growth in faba bean was inhibited at 50 and 100 µmol/l Al^{3+}, but not at 10 µmol/l Al^{3+} (Zhang et al. 2009). The Al^{3+} tolerance index fell by 67% to 87% in a tolerant faba bean cultivar as Al^{3+} concentration increased from 50 up to 400 µm (Chen et al. 2012), and the difference between the tolerant and sensitive cultivars increased as treatment time increased from 2 h to 24 h (Chen et al. 2013).

Lower Al^{3+} concentration (41 µmol/l) tended to enhance root elongation whereas higher Al^{3+} concentration (82 and 123 µmol/l) and acid (pH 4.5) media reduced it. In a similar study, aluminium tolerant and sensitive cultivars of faba bean did not show significant differences in Al^{3+} stain score at lower concentration of Al^{3+} (50 µM), but at concentrations of 100 µM and above, the difference was significant (Chen et al. 2012). Furthermore, roots were largest in the neutral followed by the 41 µmol/l Al^{3+} treatment, and there was no difference in root length between 82- and 123 µmol/l Al^{3+} treatments. In pea, Al^{3+} stress (500 µM) reduced root growth by 31 and 35% after 48 and 72 h exposure (Nahar et al. 2017).

Table 4. Accessions and treatment means of root regrowth length at three levels of Al^{3+} treatments in solution culture medium, n=4.

Accessions	Root regrowth length (cm) following Al^{3+} treatments of		
	41 μmol/l Al^{3+}	82 μmol/l Al^{3+}	123 μmol/l Al^{3+}
Alexia	2.05	0.15	0.00
Aurora	1.72	0.82	0.33
Babylon	0.03	0.00	0.00
Bulga 70	2.24	0.28	0.33
CS 20 DK	1.34	0.18	0.07
Degaga	1.16	0.35	0.00
Divine	1.47	0.41	0.04
Dosha	2.80	1.28	0.10
EH 06006-6	1.52	0.32	0.08
EK 02016-1	2.43	0.77	0.00
Fatima	2.64	1.25	0.15
Gebelcho	1.55	0.87	0.05
GLA 1103	2.87	0.46	0.07
Gora	1.25	0.42	0.07
Hachalu	1.74	1.51	0.09
Holetta-2	1.82	0.56	0.08
Kassa	1.91	0.16	0.11
Kontu	0.92	0.33	0.00
KUSE	1.03	0.57	0.00
Mélodie	1.79	1.01	0.18
Messay	0.91	0.79	0.14
Moti	2.73	1.02	0.09
NC 58	0.68	0.94	0.03
OBSE	0.60	0.58	0.07
SSNS-1	2.60	0.35	0.17
Tesfa	1.79	0.67	0.20
Tumsa	3.01	1.29	0.19
Walki	1.62	0.61	0.13
Wayu	0.52	0.51	0.04
SE		0.24	
LSD (5%)		0.67	
Treatment mean	1.63	0.63	0.10
SE		0.04	
LSD (5%)		0.12	
P-value			
Treatment		**	
Accession		***	
Treatment x Accession		***	

, * are significant at 0.01 and 0.001, respectively, SE is standard error, LSD is least significant difference.

Aluminium, and other elements such as sodium, cobalt, selenium, and silicon are not required by all plants but can enhance growth in certain taxa (Pilon-Smits et al. 2009), and under certain environmental conditions aluminium can improve plant growth (White and Brown 2010). A low

concentration of Al^{3+} under acidic condition enhanced root growth in wheat seedlings (Kinraide 1993). Root length in tea [*Camellia sinensis* L. (Kuntze)] was enhanced by 2.5-fold at 400 µmol Al^{3+} at pH 4.2 treatment as compared to the root length observed in the control (without Al^{3+}), presumably due to the accompanying increase in Al^{3+}-induced activities of antioxidant enzymes (Ghanati et al. 2005). Similarly, in *Quercus serrata* Murray, increase in root biomass was reported at 2500 µmol Al^{3+} at pH 3.5 (Broadley et al. 2012 and references in).
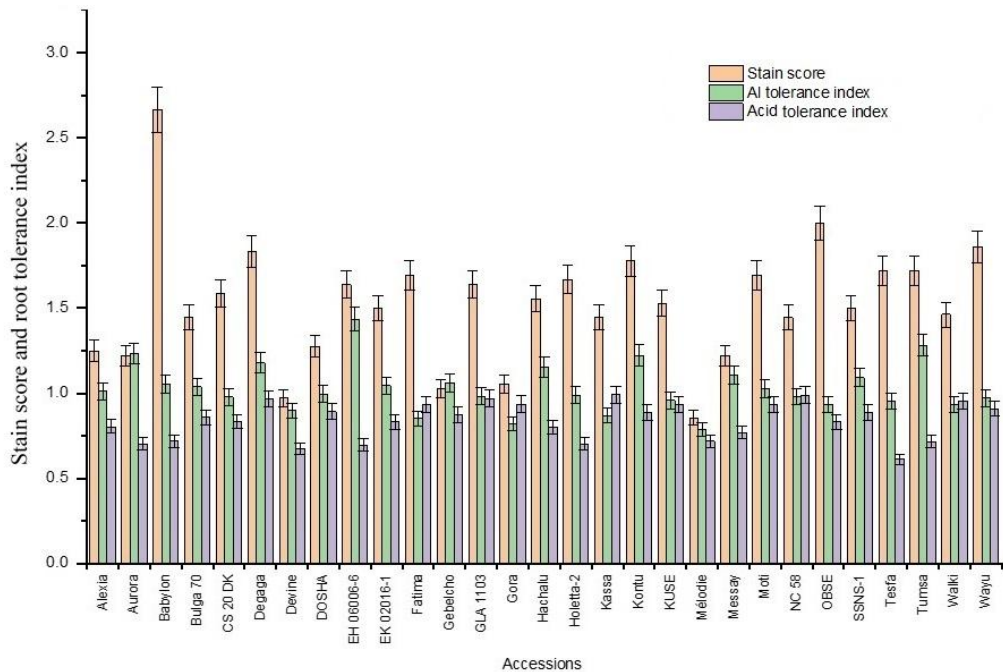
Figure 9. Stain score, acid and aluminium tolerance indices of 29 faba bean accessions grown in acid and Al^{3+} solution culture treatments. Mean stain score and Al^{3+} tolerance index (mean values of across the three Al^{3+} treatments), mean acid tolerance index. Stain score: 0, <25% stain; 1, 25%<x≤50% stain; 2, 50%<x≤75% stain; 3, >75% stain. Stain score: $\bar{x}$ = 1.02, 1.68, and 1.87 at 41, 82, 123 µmol/l Al^{3+}, respectively. Al^{3+} tolerance index: $\bar{x}$ = 1.12, 0.99, 0.98 at 41, 82, 123 µmol/l Al^{3+}, respectively. Mean acid root tolerance index was 0.84.

Root regrowth length after Al^{3+} treatments varied among accessions. At 41 and 82 µmol/l Al^{3+} Tumsa, Dosha, Fatima, and Moti, and at 123 µmol/l Al^{3+} Bulga 70 and Aurora showed high root recovery rates. At the opposite end, Babylon consistently showed little or no recovery at all treatment levels. In the step from 41 µmol/l Al^{3+} to 82 µmol/l Al^{3+} Alexia, Kassa, Bulga 70, GLA1103 and SSNS-1 showed the highest reductions in root regrowth length (Table 4). Similarly, among 32 pigeon pea genotypes, root regrowth decreased significantly with an increase in Al^{3+} concentration from 10 to 50 µM, and genotypic variation ranged from no regrowth in sensitive ones to 1.5 cm in tolerant

genotypes at 50 µM Al^{3+} (Choudhary et al. 2011). Large Al^{3+} tolerance indexes were observed in EH06006-6 and Tumsa followed by Aurora, and values were low in Mélodie and Gora followed by Kassa (Figure 9). Root tolerance index ranging from 0.47 to 1.14 was recorded in 32 *Medicago truncatula* genotypes in response to 25 µM Al^{3+} treatment (Narasimhamoorthy et al. 2007), indicating wide variability. On the other hand, Kassa and NC 58 showed high values of acid tolerance index and Tesfa showed the opposite (Figure 9). This indicated that the responses of accessions to acidity and Al^{3+}-toxicity were independent. In maize cultivar BR 201 F, root elongation was not affected by acid treatment (pH 4.3), but exposure of roots to 20 and 50 µM Al^{3+} caused inhibition of root elongation, whereas other cultivars showing sensitivity to acidic media showed root elongation under the same Al^{3+} treatment, presumably due to amelioration of H^+ toxicity by Al^{3+} (Llugany et al. 1995). This might result partly from differences in soil chemical composition in regions where the accessions evolved and adaptation to distinct response mechanisms to H^+ and Al^{3+} ions in acidic solution as discussed in Shavrukov and Hirai (2016). In pea, variability in Al^{3+} tolerance among genotypes was suggested to relate to place of origin (Kichigina et al. 2017). Generally, Aurora from Sweden combined low stain score, high Al^{3+} tolerance index, and greater root regrowth length (indicators of Al^{3+}-toxicity tolerance) (Figure 10A), whereas OBSE from Ethiopia, combined high stain score, low Al^{3+} tolerance index and lower root regrowth (indicators of Al^{3+} susceptibility) (Figure 10B).
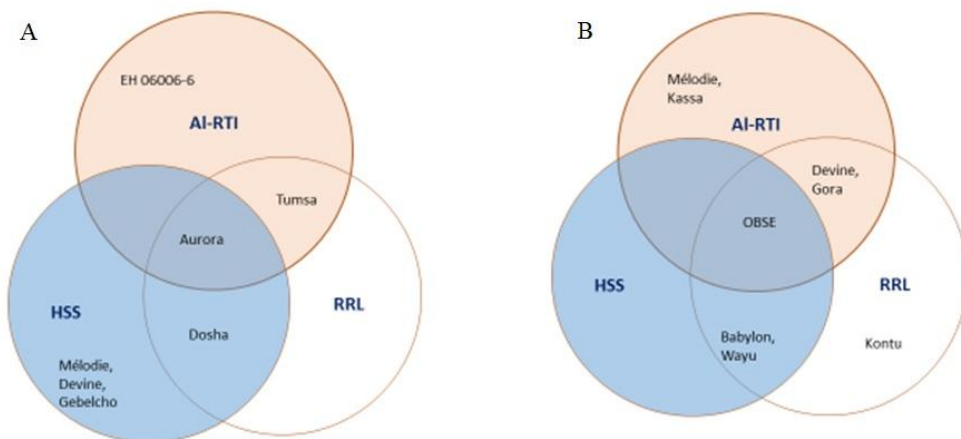
Figure 10. Distribution of tolerant and sensitive accessions against Al^{3+} tolerance indicator variables. (A) Accessions combining Al^{3+}-tolerance traits: lower hematoxylin stain score (HSS), larger Al root tolerance index (Al-RTI), and larger root regrowth length (RRL). (B) Accessions combining Al^{3+}-sensitivity traits: higher HSS, lower Al-RTI and lower RRL.

Aluminium-tolerant accessions showed contrasting shoot Al contents. Shoots of Aurora, Dosha and Gebelcho contained relatively little Al whereas other tolerant accessions including NC 58 and Messay accumulated more, indicating multiple Al tolerance mechanisms in faba bean that may involve

chelation and/or sequestration. Compound Al^{3+} tolerance mechanisms have been reported in some legumes. In white lupin (*Lupinus albus* L.) organic acids such as citrate and malate as well as phenolic compound genistein were involved in Al^{3+}-toxicity tolerance through external and intercellular detoxification (Valentinuzzi et al. 2016). Activation of catalase enzymatic defense system and citrate exudation were reported as mechanisms of Al tolerance in chickpea (Sharma et al. 2016). Similarly, root zone detoxification of Al^{3+} and maintaining the ability to take up nutrients from the soil under the stress were reported as Al tolerance mechanisms in pea (Kichigina et al. 2017).

4.1.2 Responses to acidity and Al^{3+} toxicity in pot experiments

In the peat experiment, SPAD value was significantly different (P<0.01) between treatments at 25 and 35 DAS, stomatal conductance (P<0.05) at 35 DAS, leaf area and total dry mass (P<0.001) and root and shoot mass fraction (P<0.01) at 58 DAS (Table 5). Total dry mass followed the sequence neutral > Al^{3+} > acid, whereas root mass fraction followed acid = Al^{3+} > neutral. In pigeon pea in sand medium, root and shoot dry mass were reduced in response to 50 μM Al^{3+} treatment and the effect was more pronounced in roots than in shoots (Choudhary et al. 2011). Taproot length in the perlite experiment was in the order neutral > acid = Al^{3+}. This is in agreement with the findings reported in soybean that root and shoot length and dry mass were reduced at 150 μmol/l Al^{3+} at pH of 4.0, but the calculated root mass fraction was larger than in the acid treatment alone (Shamsi et al. 2008). This may indicate that decreased root length and increased thickness are coordinated responses to toxic levels of Al^{3+} by faba bean and other legumes. SPAD values in both peat and perlite experiments and stomatal conductance in peat were greater in Al^{3+} and acid treatments than in the neutral, but leaf area was in the order neutral > Al^{3+} > acid treatments, and mean SPAD values decreased through time in the peat experiment. A similar increase in leaf chlorophyll concentration in stressed plants was reported in wheat in response to salinity stress (Shah et al. 2017). In an aluminium-tolerant sorghum cultivar, stomatal conductance progressively increased with time in Al^{3+}-treated plants (Peixoto et al. 2002), which may be due to increased respiration triggered by the extra energy demand of the plant in ameliorating the effect of the stress or the plant was physiologically adapted to the stress.

Table 5. Accessions and treatment means of SPAD value, stomatal conductance, leaf area, total dry mass, and root and shoot mass fractions in peat potting medium, n=4.

Accession	SPAD value			Stomatal conductance (mol H₂O/m²/s)	Leaf area (cm²)	Total dry mass (g)	Root mass fraction	Shoot mass fraction
	25 DAS	35 DAS	45 DAS	35 DAS	58 DAS			
Aurora	40.4	38.7	38.0	0.442	831	11.49	0.28	0.72
Babylon	45.4	40.8	38.4	0.495	722	10.31	0.34	0.66
Dosha	40.9	33.3	31.7	0.393	640	9.47	0.24	0.76
EH 06006-6	42.2	36.2	34.5	0.366	551	8.44	0.23	0.77
Gebelcho	40.6	34.6	32.6	0.489	617	8.90	0.24	0.76
GLA 1103	44.6	39.7	37.7	0.623	606	8.51	0.29	0.71
Kassa	37.6	34.8	29.9	0.503	533	8.06	0.22	0.78
Messay	38.3	34.3	31.7	0.473	547	8.27	0.23	0.77
NC 58	39.9	34.7	32.3	0.405	569	9.11	0.23	0.77
Tesfa	40.7	35.4	32.8	0.576	475	6.72	0.25	0.75
SE	0.7	0.6	1.0	0.037	32	0.39	0.01	0.01
LSD (5%)	2.0	1.7	2.7	0.104	89	1.11	0.03	0.03
Treatment								
Neutral	38.5	34.5	32.9	0.354	780	10.98	0.22	0.78
Acid (pH 4.5)	41.9	37.4	34.9	0.513	491	7.43	0.27	0.73
Acid + 82 µmol/l Al³⁺	42.8	36.8	34.1	0.564	556	8.38	0.27	0.73
SE	0.4	0.3	0.5	0.020	17	0.22	0.01	0.01
LSD (5%)	1.1	0.9	1.5	0.057	49	0.61	0.02	0.02
P-value								
Treatment	**	**	ns	*	***	***	**	**
Accession	***	***	***	***	***	***	***	***
Accession x treatment	ns	ns	ns	ns	ns	ns	ns	ns
Date x accession		***						

*, **, *** are significant at 0.05, 0.01 and 0.001, respectively, ns is not significant, SE is standard error, LSD is least significant difference.

In the peat experiment, accession differences were also observed in SPAD values, stomatal conductance, leaf area, total dry mass, root and shoot mass fractions (Table 5). SPAD values were high in Babylon and GLA 1103 at 25 DAS and decreased with time (Table 5), whereas GLA 1103 and NC 58 showed the highest and lowest values of stomatal conductance, respectively at 35 DAS. Interaction of date by accession for SPAD value was significant (P < 0.001) and the reduction of SPAD value through time was high in Dosha and Gebelcho and low in Aurora. Aurora had the largest leaf area at 58 DAS followed by Babylon, while Tesfa and Kassa had the lowest areas (Table 5). In similar studies variability in shoot and root biomass reduction among pea genotypes (Kichigina et al. 2017) and reduction of leaf area and total dry mass in tomato cultivars were reported (Simon, Smalley et al. 1994). In contrast, reduction of stomatal conductance (Simon, Kieger et al. 1994) in tomato cultivars, and reduction of chlorophyll content in wheat (Ohki 1986) due to Al³⁺ treatment have been reported. In the perlite experiment, there were no differences among accessions in SPAD values and

taproot length, but there were accession by treatment (P<0.01) interactions, indicating phenotypic trait plasticity in response to treatments. Acid and Al^{3+} tolerance indices were significantly different among accessions (P<0.01) and treatment by accession interactions were also significant (P<0.001). The acid tolerance index was high in Kassa, GLA 1103 and NC 58, and low in Aurora, Babylon, Tesfa, and Messay. The Al^{3+} tolerance index was high in Gebelcho, Dosha, Aurora and Messay, and low in Kassa, Babylon and Tesfa. Similarly, variability of genotypes in Al^{3+} tolerance index was reported in pea (Kichigina et al. 2017).

In conclusion, acidity and Al^{3+}-toxicity treatments were sufficiently strong to initiate detectable variation in root length, stain score, Al^{3+} tolerance index, SPAD value, stomatal conductance, biomass and leaf area in solution culture, peat, and perlite experiments. Roots behaved differently in response to pH treatment and pH + Al^{3+} concentration differences. Accessions responded to acid and Al^{3+} treatments independently. Al^{3+}-tolerant accessions showed contrasting shoot Al content, indicating the presence of multiple Al tolerance mechanisms in faba bean. Trait expression complementarity and variability were observed across the experiments owing to differences in growth media. The values of the acid tolerance index in the solution culture and perlite media experiments were positively correlated with each other (R=0.97, n=10, P<0.01), but the results of Al^{3+} tolerance index were not significantly correlated. The effect of Al^{3+} was small (P>0.05) (Table 5) in the peat experiment, even though its concentration was the same as in the aquaponic experiment, indicating that a higher concentration may be necessary in solid potting medium. As a result of this, Babylon responded well in peat medium while the opposite was true in aquaponics and perlite media. This is in agreement with soybean genotypes that responded differently to toxic levels of Al^{3+} due to differences in screening media (Villagarcia et al. 2001) in which 2-fold Al^{3+} concentration was needed in hydroponic medium to produce the same effect in plants tested in sand medium.

Accessions adjusted their root regrowth length in solution culture, and SPAD values and taproot length in perlite medium to pH and Al^{3+} concentrations as demonstrated by accession by treatment interactions. Root tolerance index and root regrowth length were found to be informative traits in solution culture, and SPAD values in peat and perlite pot experiments. The rate of photosynthesis in peat, and stomatal conductance, leaf temperature and biomass weight in perlite were relatively uninformative about responses to acidity and Al^{3+}-toxicity. In germplasm screening, 41 μmol/l Al^{3+} was not informative, 82 μmol/l Al^{3+} was moderate, 123 μmol/l Al^{3+} was severe, hence, 82 μmol/l Al^{3+} for mass screening in aquaponic and 123 μmol/l Al^{3+} for selection of outstanding accessions for higher Al^{3+} tolerance is recommended. Aquaponic media for mass screening and perlite media for verification experiments were found to be convenient.

4.2 Responses to drought adaptation

4.2.1 Germplasm screening to drought adaptation

In the screening experiment conducted in well watered condition, significant differences among accessions and between wet and dry sets were observed in constitutive potentially drought adaptive traits, namely chlorophyll concentration along with shoot and root dry mass. Accessions originating from dry regions of the world had significantly higher chlorophyll concentration, shoot and root dry mass, hence verifying the hypothesis that accessions from the dry set would produce larger root system than from the wet set. In similar research, faba bean accessions adapted to dry southern European environments had larger root system than those adapted to wet northern European environments (Zhao et al. 2017). Accessions DS70622 and DS74573 had largest root and shoot dry mass coupled with intermediate stomatal conductance, and accession DS11320 coupled high stomatal conductance with low leaf surface temperature, large root dry mass and above average shoot dry mass (publication II). Drought responsive or acquired (drought adaptive traits expressed in response to water deficit) traits such as leaf surface temperature and stomatal conductance differed significantly among accessions but not between the two sets, indicating response similarity between the two sets in the absence of the stress. In a different study, faba bean accessions from dry environments showed higher stomatal density and reduced gas exchange under drought stress than those from wet environments (Khazaei et al. 2013b). Based on the criteria mentioned in Table 4 of publication II, eight accessions were selected and investigated further under well watered and water-limited treatments with the aim of assessing root and shoot drought responsive and constitutive traits and testing whether accessions from the dry set differ from the wet set (publications II and III).

4.2.2 Phenotyping for drought adaptation

Drought affected total root length (taproot + laterals + second order laterals), root system depth, width and convex hull area at 5, 12, and 19 DAT, in which larger values were recorded in the well watered treatment. Treatment differences in total root length and root system width increased from 2 times at 5 DAT to 3.7 times at 19 DAT, indicating the increased severity of drought with time. Plants grown in the well watered treatment had 3 times more convex hull area than plants grown in the water-limited treatment (publication II). At the end of the treatment period, drought treatment significantly lowered the biomass weight and stomatal conductance, and increased leaf and stem dry matter content and SPAD value (publication III). Accession DS70622 showed high stomatal conductance in the well watered treatment and intermediate in the water-limited treatment. In both treatments, DS11320 and ILB938/2 showed intermediate and low stomatal conductance, respectively, and Mélodie/2 showed high stomatal conductance in the water-limited treatment (publication III). In common bean, drought

stress resulted in reductions of rooting depth by 14%, total root length by 35 %, root biomass by 29% and root volume by 14% (Sofi et al. 2018), and significant decreases in stomatal conductance and chlorophyll content were reported in common bean genotypes due to drought (Soureshjani et al. 2019). Stomatal behaviour that decreases transpiration rate in a drought-tolerant wheat cultivar was reported to improve integral water use efficiency and photosynthetic rate in the drought condition (Li et al. 2017). However, higher leaf stomatal conductance in common bean was positively correlated with larger grain yield under drought stress (Polania et al. 2016), a response that may be associated with effective use of water during drought.

Accessions adjusted their taproot and second order lateral root lengths, biomass weight and apparent root length density differently in response to the watering treatments, as shown by the significant accession by treatment interactions. Lateral root length contributed to two-third of the total root length. Accessions from the dry set such as DS70622 and DS11320 with larger root and shoot dry mass in the screening experiment also showed deep root systems across the three time points and larger biomass at the end of the treatment period. In comparison, DS70622 exhibited the longest lateral roots and greatest total root length in both treatments, and maximum convex hull area in water-limited treatment, and the smallest taproot and lateral root length differences between the two treatments. Accessions from the wet set, Mélodie/2 and WS99501, showed the opposite in the water-limited condition (publication II). A drought-tolerant common bean cultivar, Topaz, was reported to possess high values of root to shoot ratio, total root length and root volume under drought stress (Sofi et al. 2018), and drought tolerance in recombinant inbred lines (RILs) of common bean was positively correlated with root depth and root and shoot system vigour (Polania et al. 2017). Total dry mass in faba bean accessions from southern Europe (Mediterranean climate) was less affected by drought than those from north Europe (Sweden and Estonia) (Zhao et al. 2018).

Root growth in depth and width was more or less balanced in the two drought avoiding accessions, DS70622 and DS11320. In both treatments, DS70622 and DS11320 showed linearly parallel increase in convex hull area across time points, and as a group, the two accessions clearly diverged from the benchmark accession ILB938/2 (Figure 11 A & B). However, DS70622 and DS11320 largely differed one from the other by the production of lateral roots filling the convex hull area (Figure 11 C & D), and the differences were significantly large in the water-limited treatment where DS70622 developed the longest lateral roots. This morphological dimorphism in response to treatments is a potential trait for drought avoidance. This is in agreement with the concept that root system architecture is extremely responsive to drought stress (Ye et al. 2018). Similarly, the length of second order lateral roots shown by DS70622 were twice and 38 times that of DS11320 in well watered and

water-limited treatments, respectively (publication II). This may be the reason for the high apparent root length density and apparent specific root length shown by DS70622 in both treatments reaching 2-fold and 3-fold larger than that of DS11320. On the other hand, DS11320 showed 1.2-fold and 1.5-fold greater root mass fraction than DS70622, indicating a feature of coarser root development (publication III).
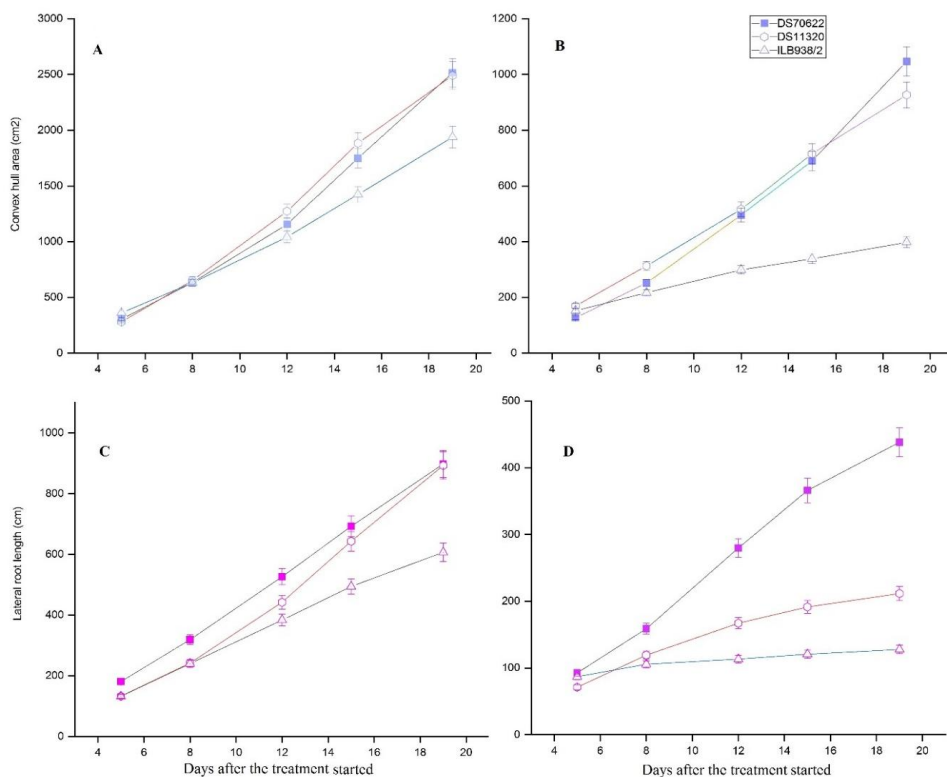

Figure 11.Convex hull area and lateral root length of DS70622, DS11320, and ILB938/2. (A) Convex hull area in well watered treatment; (B) Convex hull area in water-limited treatment; (C) Lateral root length in well watered treatment; (D) Lateral root length in water-limited treatment.

Root length plasticity under contrasting watering regime was reported to be a promising trait for peanut (*Arachis hypogaea* L.) drought tolerance (Thangthong et al. 2018). In drought-tolerant peanut genotypes, root length density in contrasting watering regimes was more variable in the upper soil layer (0-40 cm) than the lower layer (40-100 cm), indicating the greater association of root length density variability with depth of soil under moisture stress in that drought avoiding genotypes had greater root length density at deeper soil layer in response to drought (Songsri et al. 2008). Similarly, under water stress condition, root dry mass and root length density of drought tolerant cowpea cultivar tended to distribute downwards (Matsui and Singh 2003).

4.2.3 Correlation of traits for drought avoidance

In both the screening and phenotyping experiments, accessions originating from the drier regions of the world showed drought avoidance behaviour, thereby confirming FIGS as a valuable strategy. In germplasm screening, root and shoot dry mass and their fractions along with SPAD value provided useful information in discriminating accessions with potential drought avoidance characteristics. In the phenotyping experiment, root traits were strongly and positively correlated with each other and with shoot traits, but these correlations indicated specific plasticity of traits with watering treatments (Figure 3 in publication III). In the well watered treatment, total dry mass was correlated with root length traits, whereas in the water-limited one, it was correlated with root width and convex hull area. Similarly, apparent root length density was positively correlated with second order lateral root length in well watered treatment, and with apparent specific root length in the water-limited treatment, indicating that a high surface area to volume ratio to maximize water absorption is a key strategy in drought. In the water-limited treatment, root traits such as lateral root length and root system depth, convex hull area and root system width, and apparent root length density contributing for drought avoidance (publication II and III) were positively associated with shoot traits such as total dry mass, leaf number, and leaf mass fraction reported in publication III. This is in agreement with Blum (2015) that water scavenging plants maintain relatively larger biomass under drought condition. In faba bean, maintaining larger shoot mass under limitation of water at the flowering stage was reported as an indicator of drought tolerance (Khan et al. 2007).

4.3 Breeding for root traits

Root traits have a potential to improve yield under various abiotic stress conditions, yet little has been done to exploit the information on root traits in breeding of high yielding cultivars (Zhao et al. 2017). Evaluation of accession responses to acidity and Al^{3+}-toxicity in solution culture and in solid media, peat or perlite had benefits special to each media. In solution culture, the roots were visible from the top and side of the box and changing the entire growth medium at a given time was more easily conducted. The possible buffering effect to acidity that may arise from certain solid media such as vermiculite (Indrasumunar and Gresshoff 2013) is not a concern in solution culture. It was also possible to harvest the entire roots without any damage, and marking the damaged part and recovery scars from the roots after the Al^{3+} treatment were easily conducted. Root tips remain intact or unbroken so that they can be used for staining procedure. However, the lack of natural soil resistance to root growth, the artificial supply of oxygen and the direct supply of nutrient solution to the roots may alter their behaviour from what is natural in field grown plants. Hence, verification of results in solid media, perlite or peat was important.

Handling of roots in perlite medium was easier than in peat medium. In perlite, plants were continuously provided with solution culture containing the appropriate treatment of pH and Al^{3+} until the end of the experimental period. Harvesting and separation of roots from the perlite was convenient, so intact roots provided taproot length without difficulty. In contrast, extraction of roots from peat led to considerable breakage and loss of roots. The long fibers in the peat were tightly held in the growing roots, so making it difficult to harvest the roots in their entirety.

In contrast, when the peat was pounded and sieved (thus breaking or removing the long fibers in it), separation of roots from the medium was not difficult in the GROWSCREEN Rhizo-box drought experiment. The GROWSCREEN Rhizo phenotyping platform provided high throughput two dimensional images non-invasively. Acquisition of image data on a daily basis offered an opportunity to monitor root growth rates and architecture as a whole as well as the different classes of roots. However, extraction of roots from the medium showed that 68-75% of the root system was invisible for imaging, so the system is not comprehensive, a typical feature and limitation of rhizotrons as discussed in Atkinson et al. (2019). Furthermore, the system is uniquely designed for acquisition of morphological and architectural root traits (Nagel et al. 2012), so it lacked checking mechanisms for components of the plant-water relationship. Naturally, accessions differ in vigour, so they exhibit corresponding differences in their water requirement for growth and development in the real situation (Negin and Moshelion 2017).

Hematoxylin staining has been shown to be a useful indicator of Al^{3+} tolerance for species known to tolerate Al^{3+}-toxicity through chelation when genetically homogenous seeds are available. Various staining procedures have been applied to study Al^{3+} tolerance in soybean, faba bean and pea (Akhter et al. 2009; Chen et al. 2012; Kichigina et al. 2017) that confirmed the success of the procedure. The test of Al^{3+}-toxicity tolerance was made using seeds as received, so the roots showed wide variation between individuals within an accession. This affected the interpretation of data in the course of data analysis. Hence, root regrowth length as indicator of resiliency and recovery from the damage of Al^{3+} and root tolerance index as indicator of root length setback were used as primary parameters to evaluate the treatment effect on different accessions. These two root growth parameters were found to be highly informative in the heterogeneous faba bean accessions.

Roots in GROWSCREEN Rhizo-boxes showed to be highly variable in response to drought treatments and the morphological dimorphism observed in accession to well watered and water-limited treatments were indicators of water scavenging behaviours of accessions such as DS70622. This behaviour was further supported by the positive correlation observed among stomatal conductance, apparent specific root length and Fv/Fm in the well watered treatment, indicating that

the maximum surface area to volume ratio of roots meets the transpiration demand of the plant during the course of photosynthesis. Plants with higher values of specific root length (long but thin roots), root length density and root system depth were reported to maintain yield under drought (Comas et al. 2013). However, methods for screening large numbers of accessions for water-scavenging ability have yet to be developed.

4.4 Conclusions and recommendations

Managing of acidic media and application of aluminium toxicity was demonstrated for a large set of material in a seedling-based aquaponic technique, where root tolerance index and root regrowth provided detection of differences among treatments and accessions. Further verification experiments were conducted in two pot-based media, each of which had its pros and cons. The peat-based medium was useful to evaluate response differences between treatments and accessions using SPAD value, stomatal conductance, shoot and root dry mass. However, owing to its operative simplicity in handling and separation of roots as well as because of its inert nature, the perlite medium was more convenient than the peat. Based on the overall results: Aurora and Messay were found to be Al^{3+} tolerant but acid sensitive; Kassa and GLA 1103 acid tolerant, but Al^{3+}-sensitive; NC 58 and Dosha were tolerant to both Al^{3+} and acidity, while Babylon was sensitive to both.

Screening of germplasms for traits potentially associated with drought was successfully conducted in the perlite-based pot experiment, which allowed quicker screening of a large set of materials and enabled detection of constitutive traits indicating variation among accessions. The GROWSCREEN Rhizo phenotyping facility allowed detection of variation between treatments and among accessions. The dark peat-based medium in GROWSCREEN Rhizo provided contrasting background for capturing root images nondestructively. Accessions showed wide plasticity for watering treatments, and those from the drier regions revealed superior drought avoidance characteristics in both the screening and phenotyping experiments. DS70622 exhibited deep- and wide-growing roots that explored the root volume with long and thin laterals. A larger root system combined with moderately high total dry mass and stomatal conductance endorsed this accession as a potential drought avoiding candidate by effective use of water. Accessions such as DS11320 and ILB938/2 that combined large and thick root system but with low root length density, specific root length, and low stomatal conductance can be recommended as potential sources of drought avoiding traits by improved water use efficiency.

4.5 Future directions

Oxidative stresses such as soil acidity, drought, waterlogging, heat and salinity are major constraints in crop production. Among these, acidity and drought are two of the most important stresses affecting the yield and stability of faba bean.

Mechanisms of Al^{3+}-toxicity tolerance, biochemical markers for antioxidant and enzymatic defence system and quantitative trait loci (QTL) and underlying genes associated with these traits need to be investigated.

Effective use of water under drought condition is a function of osmotic adjustment, stomatal gas exchange and water scavenging behaviours of plants. These physiological and morphological behaviours need to be further investigated in faba bean to identify host accessions for breeding of high yielding materials under drought stress. Together with this, methods of detection to water scavenging potential in a more rapid and easier way is a research area for investigation.

Most acid soils are subject to Al^{3+} toxicity, and drought and Al^{3+} toxicity interact. Al^{3+} stress inhibits root growth, so they are less likely to find soil moisture, and its effect on shoot is secondary as it is induced by the damage it causes on roots. On the other hand, the effect of drought is more pronounced on shoot growth than on root growth. When drought occurs in acid Al^{3+}-toxic soils, the combined effect on both root and shoot growth has been shown to be synergistic in other crop species. Thus, drought tolerance cannot be fully utilized in crops growing in Al^{3+}-toxic acid soils unless Al^{3+} tolerance is also present. Hence, finding or developing for faba bean accessions with combined tolerances to Al^{3+} and drought stress and integrating traits of tolerance in modern cultivars will have significant effect on the productivity and production area expansion of the crop in the future.

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