Screening of faba bean (*Vicia faba* L.) accessions to acidity and aluminium stresses

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Background. Faba bean is an important starch-based protein crop produced worldwide. Soil acidity and aluminium toxicity are major abiotic stresses affecting its production, so in regions where soil acidity is a problem, there is a gap between the potential and actual productivity of the crop. Hence, we set out to evaluate acidity and aluminium tolerance in a range of faba bean germplasm using solution culture and pot experiments.

Methods. A set of 30 accessions was collected from regions where acidity and aluminium are or are not problems. The accessions were grown in solution culture and a subset of 10 was grown first in peat and later in perlite potting media. In solution culture, morphological parameters including taproot length, root regrowth and root tolerance index were measured, and in the pot experiments the key measurements were taproot length, plant biomass, chlorophyll concentration and stomatal conductance.

Result. Responses to acidity and aluminium were apparently independent. Accessions Dosha and NC 58 were tolerant to both stress. Kassa and GLA 1103 were tolerant to acidity showing less than 3% reduction in taproot length. Aurora and Messay were tolerant to aluminium. Babylon was sensitive to both, with up to 40% reduction in taproot length from acidity and no detectable recovery from Al^{3+} challenge.

Discussion. The apparent independence of the responses to acidity and aluminium is in agreement with the previous research findings, suggesting that crop accessions separately adapt to H^+ and Al^{3+} toxicity as a result of the difference in the nature of soil parent materials where the accession originated. Differences in rankings between experiments were minor and attributable to heterogeneity of seed materials and the specific responses of accessions to the rooting media. Use of perlite as a potting medium offers an ideal combination of throughput, inertness of support medium, access to leaves for detection of their stress responses, and harvest of clean roots for evaluation of their growth.

1 **Screening of faba bean (*Vicia faba* L.) accessions to acidity and aluminium stresses**

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9   **Abstract**

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14   germplasm using solution culture and pot experiments.

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16   are not problems. The accessions were grown in solution culture and a subset of 10 was grown

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22   and NC 58 were tolerant to both stress. Kassa and GLA 1103 were tolerant to acidity showing

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25   detectable recovery from Al^{3+} challenge.

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27   agreement with the previous research findings, suggesting that crop accessions separately adapt

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29   the accession originated. Differences in rankings between experiments were minor and

30   attributable to heterogeneity of seed materials and the specific responses of accessions to the

31   rooting media. Use of perlite as a potting medium offers an ideal combination of throughput,

32   inertness of support medium, access to leaves for detection of their stress responses, and harvest

33   of clean roots for evaluation of their growth.

34

Introduction

Growth and yield of faba bean are determined by climatic, edaphic, and management practices that are not independent of each other and interact to affect the chemical characteristics of the soil. Soil acidity has a dramatic impact on most chemical and biological processes of a crop. Faba bean (*Vicia faba* L.) grows best in soils with pH ranging from 6.5 to 9.0 (Jensen, Peoples & Hauggaard-Nelsen, 2010), and is considered to fare poorly at a pH values of 5 or less (French & White, 2005). Nevertheless, some accessions remain productive when soil pH is as low as 4.5 (Singh et al., 2012), with a critical soil pH value of 4.0 below which assimilation of major ions decreases (Schubert, Mengel & Schubert, 1990), and net H^+ release and root growth cease (Yan, Schubert & Mengel, 1992).

Soil acidification is a worldwide problem, and sensitivity to acid soils limits the usage of faba bean in some cropping systems. Besides the simple matter of low pH, soil acidity is associated with high availability of Al^{3+}, which is stressful or toxic to many plants. Acid soil can be managed by the application of lime and the effect of Al^{3+} toxicity is ameliorated by the use of P-containing fertilizers (Liao et al., 2006; Atemkeng et al., 2011). However, these options are not available where farmers are poor or legislation restricts fertilizer usage, they are less effective when cultivars are sensitive (Sun et al., 2008), and acidity in subsoil is harder to address than that in topsoil (Hede, Skovmand & Lopez-Cesati, 2001; Brown et al., 2008; Zheng, 2010). Furthermore, in areas as diverse as sub-Saharan Africa and Western Australia, soil acidity has resulted in changes of cropping sequence. In Ethiopia, faba bean has been abandoned in some agricultural regions where it was previously part of the cropping system and has not been replaced by another food legume (Genanew, Argaw & Adgo, 2012). In areas where soil acidity is severe, cereal rye cropping and livestock grazing may be the only remaining options for farmers (Genanew, Argaw & Adgo, 2012).

Most of the studies on the inheritance of Al^{3+} resistance have been conducted on the major cereal crops (wheat (*Triticum aestivum* L. emend Thell), rice (*Oryza sativa* L.), and maize (*Zea mays* L.)) (Kochian, Pinéros & Hoekenga, 2005), a few legumes such as soybean (*Glycine max* (L.) Merr.) (Liao et al., 2006), pigeon pea (*Cajanus cajan* L.) (Choudhary, Singh & Kumar, 2011), and barrel medic (*Medicago truncatula* L.) (Narasimhamoorthy et al., 2007; Chandran et al., 2008). Studies on this trait in faba bean have been few in number. Nevertheless, wide diversity

65   exists among faba bean landraces for agro-ecological adaptation (French & White, 2005;

66   Wondafrash, 2006) and biotic and abiotic stress resistance (Stoddard et al., 2006; Shifa, Hussien

67   & Sakhuja, 2011; Khazaei et al., 2013). Al^{3+} toxicity tolerance of cv 'Herz Freya' was lower than

68   that of barley and much lower than in yellow lupin and rye (Horst & Göppel, 1986a; Horst &

69   Göppel, 1986b).

70   To identify sources of resistance to acidity and aluminium stresses, an efficient screening method

71   is required. Solution culture, where seedlings are suspended with their roots in an aerated

72   nutrient solution of known Al^{3+} concentration, is suitable for screening of tens or hundreds of

73   accessions, and has proven useful in wheat (Stodart et al., 2007), pigeon pea (Choudhary, Singh

74   & Kumar, 2011; Choudhary & Singh, 2011), barley (*Hordeum vulgare* L.) (Nawrot, Szarejko &

75   Maluszynski, 2001; Echart et al., 2002; Tamas et al., 2006), sorghum (*Sorghum bicolor* L.) (Hill,

76   Ahlrichs & Ejeta, 1989), and barrel medic (Narasimhamoorthy et al., 2007). Selected germplasm

77   can then be tested in soil or potting mix, so differences in growth and yield can be determined.

78   Staining of root tips with hematoxylin, measurement of root regrowth after transfer to non-stress

79   conditions, and relative root growth as a measure of root tolerance index after exposing plant

80   roots to toxic levels of Al^{3+} are some of the techniques that are employed (Hede, Skovmand &

81   Lopez-Cesati, 2001) and allow simple ranking of tolerance and sensitivity (Choudhary & Singh,

82   2011). Reliable results are, however, most likely to be obtained by the application of multiple

83   procedures (Narasimhamoorthy et al., 2007).

84   Other morpho-physiological measures may also be informative about aluminium and acidity

85   responses in faba bean. Leaf gas exchange was reduced by 2-3 fold in tomato cultivars as

86   aluminium concentration increased to 50 µmol/l (Simon et al., 1994). Similarly, 0.1 mM

87   aluminium reduced root weight in winter wheat (Szabó-Nagy, 2015) and at pH 4, aluminium

88   concentration of 0.13-0.15 mmol/kg significantly reduced photosynthesis, chlorophyll

89   concentration and transpiration in wheat (Ohki, 1986). Other ionic stresses such as salinity

90   increased chlorophyll a fluorescence level in both mung bean and Brassica seedlings (Misra,

91   Srivastava & Strasser, 2001).

92   For these reasons, we set out to evaluate acidity and aluminium tolerance in a range of faba bean

93   germplasm. Solution culture of seedlings and pot experiments on pre-reproductive plants were

94   both used in order to develop a reliable technique for discriminating sensitive and tolerant

95   germplasm.

96   **Materials and methods**

97   **Plant materials**

98   Thirty faba bean accessions were chosen for this study on the basis of their expected exposure to

99   acidity or aluminium stress in their regions of provenance (Table 1). One accession had to be

100   dropped due to inconsistent germination during the experiment. Twenty Ethiopian accessions

101   were provided from the germplasm collection of the Ethiopian Institute of Agricultural Research,

102   Holeta Agricultural Research Center (HARC) and the remainder were chosen from European and

103   Canadian germplasm used in previous experiments. Acid soils are found in the wet highlands of

104   Ethiopia where faba bean is predominantly grown. Acid soils occupy 41% of the country

105   (Abebe, 2007), and of this area nearly one-third has an aluminium toxicity problem (Schlede,

106   1989). The first solution culture experiments were conducted with seed materials as received, the

107   first pot experiment with material that had been inbred for one generation, and the second

108   experiment with seeds from a single third-generation inbred plant of each accession.

109   **Screening for tolerance to acidity and aluminum in solution culture**

110   **Plant growing conditions**

111   Seeds of uniform size were selected from each of the accessions, washed three times in tap water,

112   disinfected with 1% NaClO (sodium hypochlorite) (w/v) for 5 min and rinsed 3 times with

113   running tap water. The seeds were soaked in tap water for 24 h, transferred to three layers of

114   moist filter paper in 14 cm diameter Petri dishes (14-20 seeds/dish), and incubated for 72 h at 22

115   °C in the dark. The seedlings were then transferred to holes in a lid suspended over a plastic tray

116   (78 cm × 56 cm ×18 cm) containing 0.5 mM $CaSO_4$ solution, and grown in the dark for another 4

117   days in controlled-environment growth chambers at 22/20°C day/night temperatures with 70 %

118   relative air humidity. The solution was continuously aerated with an aquarium pump. Eight

119   seedlings of each of the 29 accessions were used in each tray. Three replicates of five treatments

120   were prepared, and arranged in a split-plot design with replicate as the block, tray (treatment) as

121   the main plot and accession as the subplot. The replicates were separated by 21 days in time

122   rather than physically in space, owing to space restrictions.

123   For the remaining 7 days of the experiment, seedlings were allowed to grow on continuously

124   aerated nutrient solution comprising 4.0 mM $CaCl_2$, 6.5 mM KNO_3, 2.5 mM $MgCl_2$, 0.1 mM

125    $(NH_4)_2SO_4$, and 0.4 mM NH_4NO_3, with fresh nutrient solution culture provided on day 4. The pH

126   was corrected to 7.0 (neutral control) and 4.5 (acid and aluminium treatments) with 10.88 M HCl

127   and 0.5 M Na_2CO_3 solutions. The growing conditions were 16/8 h light/dark regime, 22/20 °C

128   day/night temperatures, 70 % relative air humidity and a photon flux density of 230 $\mu mol\ m^{-2}\ s^{-1}$

129   at the plant canopy. After two days of this 7-day interval, the aluminium treatments were

130   initiated by transferring three sets of seedlings to fresh nutrient solution containing 41, 82, and

131   123 μmol/l aluminum sulfate $[Al_2(SO_4)_3.16H_2O]$. One day later, 4 seedlings from every set of 8

132   were removed for haematoxylin staining (see below) and after a further day, the remaining

133   seedlings were transferred to aluminium-free solutions at pH 4.5 and allowed to recover for 3

134   days, as recommended by Nava et al. (2006), bringing their total growing time to 7 days after

135   their transfer to the nutrient solution culture, the same as for those not exposed to Al^{3+}.

136   At the end of the growth period, the length of the primary root of each seedling was measured

137   with a ruler, showing its tolerance to the stress. On the aluminium-treated seedlings, the length of

138   the primary root from the tip to the point of root thickening (callus), caused by the aluminium

139   treatment, was measured with a ruler and termed as root regrowth length. This measure

140   demonstrates the ability of the plant to recover from the stress.

141   The protocol for staining and scoring aluminium response followed Polle, Konzak & Kittrick

142   (1978) and was based on the affinity of haematoxylin for chelated aluminium. The sets of 4

143   seedlings were transferred to distilled water with occasional shaking for 60 min to remove

144   aluminium from the root surface. The root tips were immersed in the hematoxylin stain (2 g/l

145   hematoxylin and 0.2 g/l potassium iodate (KIO_3) in distilled water) for 30 min and rinsed in three

146   changes of distilled water for an hour. Photographs of root tips were taken using a stereo

147   microscope fitted with an AxioCam ERc 5s imaging device and saved for later scoring of each

148   root tip.

149   Means of the 4 seedlings per replicate were used for statistical analysis.

150   **Testing of acidity and aluminium response in pot experiments**

151   According to the results of solution culture, accessions representing different combination of

152   Al^{3+}- and acidity response (Table 2) were selected for further evaluation. The experiment was

153   conducted twice, first on peat-based medium and again on perlite.

Peat growing medium

155   The peat medium was prepared with 9 parts peat (FPM 420 F6, Kekkilä Oy, Vantaa, Finland) to

156   1 part sand (v:v). The pots were 7.5 l in size and were sunk into sand to prevent overheating of

157   the roots by direct sunlight. The average nutrient composition of the peat in g/m^3 basis included

158   nitrogen 2000, phosphorus 500, potassium 2000, and limestone (Ca and Mg) 1.5. The natural pH

159   of the medium was 4.5 and in one-third of the pots was adjusted to pH 7.0 by liming. Half of the

160   pots at pH 4.5 were supplied with 3 l of 82 μmol/l $Al_2(SO_4)_3.16H_2O$ solution at sowing, and all

161   other pots were given 3 l of tap water bringing the medium to field capacity. Thus, there were

162   three treatments, pH 7.0, pH 4.5, and pH 4.5 with Al^{3+}.

163   Plants were grown in the open air for 58 days from 28 May to 24 July 2015 at the University of

164   Helsinki. Before sowing, seeds were inoculated with *Rhizobium leguminosarum* biovar. *viciae*

165   (faba bean strain, Elomestari Oy, Tornio, Finland). Four seeds of each accession were sown

166   directly in individual pots at a depth of 2 cm and a week after emergence they were thinned to 3

167   plants per pot. The experiment was terminated when plants started flowering.

Perlite growing medium

169   Nutrient solution culture was prepared using a complete fertilizer (Superex Peat, Kekkilä Oy,

170   Vantaa, Finland) containing 6.8 mmol/l NO_3^-, 0.9 mmol/l NH_4^+, 0.29 mmol/l urea, 1.1 mmol/l

171    $H_2PO_4^-$, 6.7 mmol/l K^+, 1 mmol/l Mg^{2+}, 27.8 μmol/l $H_2BO_3^-$, 3.1 μmol/l Cu^{2+}, 48.3 μmol/l Fe^{2+},

172   18.2 μmol/l Mn^{2+}, 0.52 μmol/l MoO_4^{-2}, 4.6 μmole/l Zn^{2+}, and 1 mmol/l SO_4^{-2}. To provide

173   calcium, 2 mmol/l $CaCl_2$ was added. According to the pH requirement of the nutrient solutions

174   for each treatment, the pH of the solution was adjusted using HCl and Na_2CO_3.

175   Plants were grown in an open-sided greenhouse cage for 42 days from 18 May to 30 June 2016

176   at the University of Helsinki. For each plant, two 3-l pots were stacked on top of each other in

177   order to provide depth for rooting. Each pot was 20 cm deep and 15 cm in diameter, with 4

178   drainage holes that were 2 cm in diameter in the bottom pots and 3 cm in the top pots, so the

179   impairment of root growth was minimal. The bottom pots were filled with 2 l of perlite and 1 l of

180   fine sand on top of the perlite to hold the top pot firmly, and the top pots were filled with 3 l of

181   perlite. Two seeds per top pot were planted and each pot was irrigated with tap water to field

182   capacity. Plants were thinned to one per top pot after five days. For 10 days after sowing, pots

183   were watered with 200 ml of tap water every other day. Thereafter, each pot received 200 ml of

184   nutrient solution of the corresponding treatment every other day for 32 days. Treatments were

185   implemented by fertigation with neutral pH (7.0) nutrient medium, acid medium at pH 4.5, and

186   pH 4.5 + 82 μmol/l $Al_2(SO_4)_3.16H_2O$. The experiment was terminated when plants started

187   flowering, even though this was fewer days than in the peat experiment.

188   **Experimental design**

189   The peat and perlite experiments were a split-plot design, with 4 replicate blocks, 3 treatments

190   (neutral, acid and aluminium) as the main plots and accessions as subplot.

191

192   **Data collected**

193   **Peat experiment**

194   The rate of leaf photosynthesis and stomatal conductance were measured using a LI-6400

195   Portable Photosynthesis System (LI-COR, Lincoln, Nebraska, USA) at 1400 μmole/m^2/s

196   radiation level and adjusted cooling temperature of 25 °C, on 6 cm^2 leaf area in bright sunlight

197   from 11:00 a.m. to 2:00 p.m. One leaf per pot was used, chosen from the middle of the shoot

198   having been fully exposed to direct sunlight at 25, 35, and 45 days after sowing. Leaf chlorophyll

199   concentration was measured using an optical chlorophyll meter, SPAD-502 (Minolta Camera Co,

200   Ltd, Japan), on the same days. Two leaves per plant were measured and the average of the two

201   was recorded. Data from 35 days after sowing are presented here.

202   At the end of the experimental period, 52 days after sowing, the leaves of each plant in the pot (3

203   plants per replication) were severed from the stem and the total leaf area was measured using a

204   LI-COR Model LI-3000A Portable Area Meter (LI-COR, U.S.A.) and the average values were

205   calculated for each replicate. Plant roots were carefully removed from both the pot and the peat

206   and scored for nodule quantity and quality. Nodule quantity was measured by two people

207   scoring, zero for absent, 1 for a few nodules, 2 moderate quantity, and 3 for prolific nodule

208   production. Similarly, nodule quality was also scored by two people as follows. "0" was given

209 for absent, "1" was given when >50% of the nodules were white in color, "3" was given when
210 >50% of the nodules were pink in color, "2" was given when each white and pink nodules were
211 nearly equal by numerous. In both cases, the average of the two scores were taken as a value of
212 each unit of plant per replication. Finally, the root and shoot dry weight were taken after drying
213 the biomass in oven at 76 °C for 48 hours. Samples of shoot and potting medium were taken for
214 aluminium analysis.

215 **Perlite experiment**

216 On day 41, stomatal conductance was measured using a Leaf Porometer (Decagon Devices, Inc,
217 Pullman WA, U.S.A.) once per plant. On day 42, canopy temperature was measured using a
218 FLUKE Model 574 Precision Infrared Thermometer (Fluke Corporation, Everett, WA, U.S.A.)
219 and leaf chlorophyll concentration was measured using the SPAD-502 as described above. Roots
220 were carefully removed from the perlite and taproot length was measured with a ruler. The root
221 and shoot dry weight were determined as described above. Root to shoot dry weight ratio was
222 calculated by dividing the root weight by the corresponding shoot weight.

223

224 **Aluminium concentration analysis**

225 Samples of shoot and growing media were milled and representative 250 mg sub-samples were
226 digested with acid in a microwave oven (Mars Express, CEM, USA) conforming to EPA 3052 (9
227 ml 70% HNO_3 and 1 ml 30% H_2O_2 at 1600 W and 175°C; EPA, 1996). The concentration of Al
228 was measured with inductively coupled plasma- optical emission spectrometry (ICP-OES),
229 Thermo Scientific iCAP 6000 Series (Thermo Fisher Scientific, Waltham, MA USA). The limit
230 for detection of Al by the ICP was 0.032 mg/l.

231 **Data analysis**

232 Analysis of variance was conducted using SPSS version 22.0 (IBM Inc., Chicago, IL, USA)
233 software package. Accession and culture media were treated as fixed effects and replicate as a
234 random effect in the split-plot design. Treatment means were separated by LSD (5%).

235 **Results**

236 **Solution culture experiment**

237 Taproot length at pH 7.0 ranged from 18 cm in Gora to 27 cm in Obse and EK02016-1, and at
238 pH 4.5 from 14.8 cm in Tesfa to 22.9 cm in Dosha (both $P < 0.05$; Figure 1). Accessions Kassa,
239 NC58 and GLA1103, all showed less than 3% reduction in root length in acid condition, whereas
240 8 accessions showed significant reductions, with the greatest in Tesfa, Divine and EH06006-6
241 which showed 30-40% setback.

242 Two of the three aluminium treatments were relatively uninformative. The difference in mean
243 root lengths between the 0 and 41 µmol/l aluminium treatments was not statistically significant
244 (1.2 cm longer with aluminium than without). The 123 µmol/l treatment did not result in a
245 significant difference in root length from the 82 µmol/l treatment, and very few of the roots
246 regrew, so it appeared to be too strong a dose. Hence, only the 82 µmol/l treatment provided
247 informative results and we present only those here.

248 Roots grown in aluminium-free solutions did not stain with haematoxylin (Figure 2A). The
249 minimum haematoxylin stain score in the 82 µmol/l treatment was found in Dosha, followed by
250 Gebelcho, while the maximum was in Babylon, followed by Degaga ($P < 0.05$; Table 1).
251 Babylon showed no apparent root regrowth following transfer to aluminium-free solution,
252 whereas Hachalu showed the most vigorous regrowth ($P < 0.05$). Accessions EH06006-1,
253 followed by Kontu, Messay, and Aurora, all produced longer roots in the 82 µmol/l aluminium
254 treatment than in the acid treatments (Figure 1), although these differences were not significant.
255 Aluminium treatment caused significant reductions in root growth in Gora and Walki.

256 Summing the ranks of hematoxylin staining, root regrowth, and root length setback by
257 aluminium (Table 1) indicated that Gebelcho, Aurora and Messay were the three most tolerant
258 accessions, whereas Kassa, Babylon and Divine were the most susceptible.

259 Comparison of these results showed that acidity response and aluminium responses have some
260 independence. On this basis, 10 accessions were chosen for further experiments (Table 2).

261 **Experiments in peat and perlite potting media**

262 In the peat experiment, leaf area was significantly different in all 3 treatments, neutral >
263 aluminium > acid, but leaf temperature did not show significant effects of treatment. Rate of
264 photosynthesis increased in the order neutral < acid < aluminium, and shoot dry matter was
265 greatest in the aluminium treatment. Nodule quantity, nodule quality and root dry matter were all

266   highest in the neutral treatment with no significant difference between acid and aluminium

267   treatments (Table 3). Similarly, shoot dry weight and root to shoot dry weight ratio were highest

268   in neutral treatment. Chlorophyll concentration and stomatal conductance were significantly

269   lower in the neutral treatment than in the other two, which did not significantly differ (Table 4).

270   In the perlite experiment, many results were similar. Chlorophyll concentration was again

271   significantly lower in the neutral than in the other two treatments (Table 4). Babylon and GLA

272   1103 showed high leaf chlorophyll concentration in both acid and aluminium treatments. On the

273   other hand, Kassa in the acid, and NC 58, Gebelcho and Messay in the aluminium treatment

274   showed low chlorophyll concentrations (Figure 3A). There was no significant difference in

275   stomatal conductance or leaf temperature between treatments. Differences in root and shoot dry

276   matter were also not significant. Root length, however, was significantly greater in the neutral

277   treatment than the other two. In Aurora, Babylon, EH 06006-6, Messay and Tesfa, the setback

278   due to acidity was greater than the LSD, but in the other 5 accessions the effect of acidity was

279   very small (Figure 3B). The effect of aluminium on reducing root length was significant in Kassa,

280   whereas Gebelcho, Dosha and Aurora showed slightly greater taproot length under aluminium

281   stress.

282   Chlorophyll concentration in the two pot experiments showed similar effects of accessions

283   except that Aurora was an outlier (Table 4). Without Aurora, the correlation coefficient of the

284   other 9 accessions was 0.853 (P < 0.01). Stomatal conductance values in the two experiments

285   were also highly correlated, again with the exception of Aurora, and with the same r value. Root

286   and shoot dry weights, however, were not significantly correlated between the two experiments.

287   Aurora produced the largest shoot and second largest roots in peat, whereas EH 06006-6 did so

288   in perlite.

289   **Shoot and growing medium Al concentration**

290   There was no significant difference between treatment means in shoot aluminium accumulation

291   (Table 3). However, there was a highly significant difference among accessions, with Babylon

292   followed by Aurora showing low shoot Al concentration. Aluminium concentration in peat

293   growing medium was far higher in aluminium-treated medium at 1107 mg/kg than the other two

294   (150 mg/kg neutral, 124 mg/kg acid).

295 **Discussion**

296 Great variation among accessions was observed in acidity tolerance ranging from below 3% to

297 40% setback in taproot growth. Similarly, accessions showed variable responses to aluminium

298 treatments ranging from no apparent taproot regrowth to prolific root elongation during recovery

299 after being exposed to aluminium treatment. Generally, accessions showed independent tolerance

300 to acidity and aluminium treatments (Table 2). Accessions tending to tolerate both acidity and

301 aluminium were found to be moderately tolerant. The independence of these responses to acidity

302 and aluminium is in agreement with Kidd & Proctor (2001), who showed that crop accessions

303 separately adapt to H^+ and Al^{3+} toxicity as a result of the difference in the nature of soil parent

304 materials (organic vs mineral) where the accession originated.

305 In general, the results obtained in solution culture experiment were confirmed in the two pot

306 experiments. Babylon, however, performed unexpectedly well in the peat experiment in

307 comparison with the other experiments, and results of leaf traits of Aurora were outliers in the

308 regression of the peat and perlite experiments. Part of this difference may be attributed to

309 heterogeneity of the samples, as the experiments were started with material as received, and part

310 to specific responses of accessions to the rooting media. The heterogeneity was also shown in the

311 highly variable responses to hematoxylin staining within some accessions. Nevertheless, results

312 of the perlite experiment, conducted using third-generation inbred seed materials, showed great

313 similarity with those of the solution culture experiment (compare Figures 1 and 3B). Inbreeding

314 of accessions to produce lines that are as homozygous as possible is well known to be necessary

315 in cross-pollinated crops like faba bean.

316 The combination of reduced leaf size and increased chlorophyll concentration was indicative of

317 stress due to either acidity or aluminium. Multiplying these two values together (e.g., from the

318 treatment means in Tables 3 and 4) showed that even though the chlorophyll concentration was

319 higher in the stress treatments, chlorophyll content per plant was lower. By these measures,

320 certain accessions showed little stress from one or the other treatment, particularly in the perlite

321 experiment, as confirmed by their root lengths and stomatal conductance values. Those

322 accessions reported in solution culture experiment as tolerant in either of the stresses, such as

323 Kassa in the acid treatment, showed lower chlorophyll concentrations in the tolerated stress

324 treatment, whereas those accessions reported as sensitive to acid and/or aluminum stress, such as

325   Babylon, showed higher concentrations (Figure 3A). Similarly, GLA 1103 (aluminium sensitive)

326   and NC 58 and Messay (aluminium tolerant) showed the highest and the lowest photosynthesis

327   rates in aluminium treatment (Table 3), respectively. In certain accessions of wheat, a relatively

328   low concentration (16 µmol/l) of Al increased chlorophyll a and b concentrations (Alamgir &

329   Akhter, 2010), but 130 µmol/l decreased leaf chlorophyll concentration (Ohki, 1986).

330   Shoot Al concentration analysis indicated conflicting results among Al-tolerant accessions. The

331   shoot Al concentrations of Aurora, Gebelcho and Dosha was low, whereas those of NC 58 and

332   Messay were relatively high (Table 3), and the accession by treatment interaction was not

333   statistically significant. Faba bean can exclude aluminium by chelation with exuded citrate (Chen

334   et al., 2012), and this would be seen as stronger hematoxylin in susceptible accessions, as shown

335   for wheat (Polle, Konzak & Kittrick 1978). Its mechanism of Al tolerance may also include

336   sequestration, as found in other species (Osaki, Watanabe & Tadano, 1997).

337   In terms of taproot growth along with root and shoot dry weights, accessions responded in

338   several ways to the acidity and aluminum treatments. There was significant variation among

339   treatments in taproot length (neutral > acid >aluminium) but not in root dry weight, showing that

340   roots were shorter and thicker in stressed conditions that otherwise. Taproot length of tolerant

341   accessions including GLA 1103, NC 58 and Kassa was not affected by acidity, whereas that of

342   Aurora, Tesfa and Babylon was severely affected. Similarly, Kassa, Babylon, Tesfa and EH

343   06006-6 were greatly affected by the chosen concentration of aluminium, but the root lengths of

344   Gebelcho, Dosha and Aurora were slightly longer under this stress (Figure 3B). While this

345   increase was not statistically significant, it was consistent with proposed root growth

346   enhancement properties of aluminium under acidic conditions (Kinraide, 1993; Osaki, Watanabe

347   & Tadano, 1997). In Al-stimulated plants, such as, *Acacia mangium* Willd. and some lines of

348   *Oryza sativa* L. (Osaki, Watanabe & Tadano, 1997), available Al^{3+} at 44 µmol/l concentration

349   applied as $Al_2(SO_4)_3$ was reported to increase the activity of roots for P uptake and initiation of

350   many laterals.

351   The rhizotoxicities of Al^{3+} and H^+, may partially alleviate each other, due to the competition of

352   the positively charged cations for binding sites of the negatively charged epidermal layers of the

353   roots (Kinraide & Parker 1987; Kinraide et al. 1992). At low soil pH, Al^{3+} has a stronger effect

354   in amelioration of toxicity that is caused by cations such as H^+, Na^+, K^+, Ca^{2+}, and Mg^{2+} (Fawzy,

355 Overstreet & Jacobson, 1954; Kinraide & Parker, 1987; Kinraide, Ryan & Kochian, 1992; Yan,

356 Schubert & Mengel, 1992; Kinraide, 1993; Kinraide, 2003). This reciprocal alleviation is

357 probably the reason for the observed slight increase in taproot length and the development of

358 smooth root surface (without callus marking) in some accessions treated with 41 μmol/l Al^{3+} in

359 the first experiment.

360 There was an important effect of pH but not of aluminium on nodule quantity and quality. Low

361 pH reduced nodule mass by 48% and nodule quality by 29%. Similarly, acidity significantly

362 decreased nodule numbers (as much as 20-33%) and activity in red clover inoculated by two

363 different bacterial strains (Shirokikh, Shirokikh & Ustyuzhanin, 2005). Decreased plant

364 dependence on fixed nitrogen was reported in subterranean clover (*Trifolium subterraneum* L.)

365 growing in low soil pH and high soil aluminium (Unkovich, Sanford & Pate, 1996). Correa,

366 Aranda & Barneix (2001) reported that alfalfa (*Medicago sativa* L.) and *Lotus glaber* Mill.

367 varied in their nodulation and nitrogen fixation ability at pH 4.0 due to variation in host and/or

368 strain tolerance to low pH. In this study, only one inoculum was used, so we were unable to test

369 the effect of its genotype, but it is known that rhizobium symbionts of faba bean differ in their

370 sensitivity to low pH, and their sensitivity to Al concentration depends on the other ions in the

371 solution (Kinraide & Sweeney, 2003; Bayoumi Hamuda et al., 2009).

372 **Conclusion**

373 Responses to acidity and aluminium toxicity were independent. A concentration of 82 μmol/l

374 aluminum sulfate [$Al_2(SO_4)_3.16H_2O$] was suitable for discriminating the sensitivity of faba bean

375 accessions, whereas 41 μmol/l had too little effect on root growth and 123 μmol/l was too much.

376 Taproot length, root length setback by treatment and root regrowth procedures were found to be

377 sound methods for preliminary screening of accessions for acidity and aluminium tolerance. At a

378 later stage, taproot length, root and shoot biomass coupled with chlorophyll concentration and

379 stomatal conductance provided reliable discrimination between accessions. Results, generally

380 agreed between experiments, with some differences attributable to uniformity of seed and to

381 specific responses of rooting medium.

382 High throughput screening for acidity and Al tolerance at an early stage of seedling growth was

383 possible in the solution culture growth medium. Nevertheless, for further screening of faba bean

384 accessions for an extended growth period, perlite was found to be an appropriate medium owing

385 to its inert nature, free drainage allowing changes of treatment medium, provision of support for

386 plants allowing monitoring of leaf and shoot responses to the acidity and aluminium stresses, and

387 easy extraction of entire root systems for evaluation without encumbering medium.

388 Kassa, GLA 1103, Aurora, Messay, NC 58 and Babylon were selected for future breeding,

389 genetics and physiological studies on Al tolerance in faba bean.

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535   Table 1. The 29 accessions of faba bean, their country of origin and source, and their response to
536   82 µmol/l Al^{3+} in solution culture, n = 3.

| | Origin | | Aluminium responses | | | | | |
| | | | Haematoxylin stain | | Root regrowth | | Al root tolerance index (82 µmol/l Al^{3+} / 0 µmol/l Al^{3+}) | | |
Accession	Country	Source	Score	Rank	Length (cm)	Rank	Value	Rank	Rank sum
Alexia	Austria	Gleisdorf	1.5	14	0.2	2	1.04	21	37
Aurora	Sweden	Svalöf Weibull	1.3	20	0.8	20	1.14	25	65
Babylon	Netherlands	Nickerson Limagrain	2.8	1	0.0	1	0.98	15	17
Bulga 70	Ethiopia	HARC	1.8	9	0.3	5	0.97	13	27
CS 20 DK	Ethiopia	HARC	1.9	7	0.2	4	1.03	19	30
Degaga	Ethiopia	HARC	2.4	2	0.4	8	1.14	26	36
Divine	France	INRA	1.7	12	0.4	9	0.82	4	25
Dosha	Ethiopia	HARC	0.8	24	1.3	26	0.95	12	62
EH 06006-6	Ethiopia	HARC	1.7	10	0.3	6	1.47	29	45
EK 02016-1	Ethiopia	HARC	1.6	13	0.8	18	1.04	20	51
Fatima	Canada	Univ. Saskatchewan	2.1	4	1.3	25	0.89	9	38
Gebelcho	Ethiopia	HARC	1.0	23	0.9	21	1.07	22	66
GLA 1103	Austria	Gleisdorf	1.8	8	0.5	11	0.99	16	35
Gora	Ethiopia	HARC	1.3	19	0.4	10	0.59	1	30
Hachalu	Ethiopia	HARC	1.4	17	1.5	28	1.03	18	63
Holetta-2	Ethiopia	HARC	1.3	21	0.6	13	0.93	10	44
Kassa	Ethiopia	HARC	2.0	5	0.2	3	0.83	5	13
Kontu	Finland	Boreal	2.3	3	0.3	7	1.23	28	38
KUSE	Ethiopia	HARC	1.5	15	0.6	14	0.88	7	36
Melodie	France	INRA	1.4	16	1.0	23	0.79	3	42
Messay	Ethiopia	HARC	1.4	18	0.8	19	1.19	27	64
Moti	Ethiopia	HARC	1.8	9	1.0	24	1.02	17	50
NC 58	Ethiopia	HARC	1.1	22	0.9	22	0.94	11	55
OBSE	Ethiopia	HARC	2.0	5	0.6	15	0.87	6	26
SSNS-1	Canada	Univ. Saskatchewan	2.0	5	0.4	8	1.08	23	36
Tesfa	Ethiopia	HARC	1.7	11	0.7	17	0.89	8	36
Tumsa	Ethiopia	HARC	2.0	5	1.3	27	1.11	24	56
Walki	Ethiopia	HARC	1.5	14	0.6	16	0.77	2	32
Wayu	Ethiopia	HARC	1.9	6	0.5	12	0.97	14	32
SE			0.29		0.23				
LSD (5%)			0.82		0.66				

537

538   Table 2. Summary of acid and aluminium responses of the 10 faba bean accessions chosen from
539   the solution culture experiment for further investigation.

		Aluminium response		
		Tolerant	Intermediate	Sensitive
Acidity response	Tolerant	NC 58, Dosha		Kassa, GLA 1103
	Intermediate	Gebelcho		
	Sensitive	Aurora, Messay	Tesfa, EH 06006-6	Babylon

540

541   Table 3. Accessions and treatment means of leaf and nodule characters measured in either peat or
542   perlite potting media, n = 4.

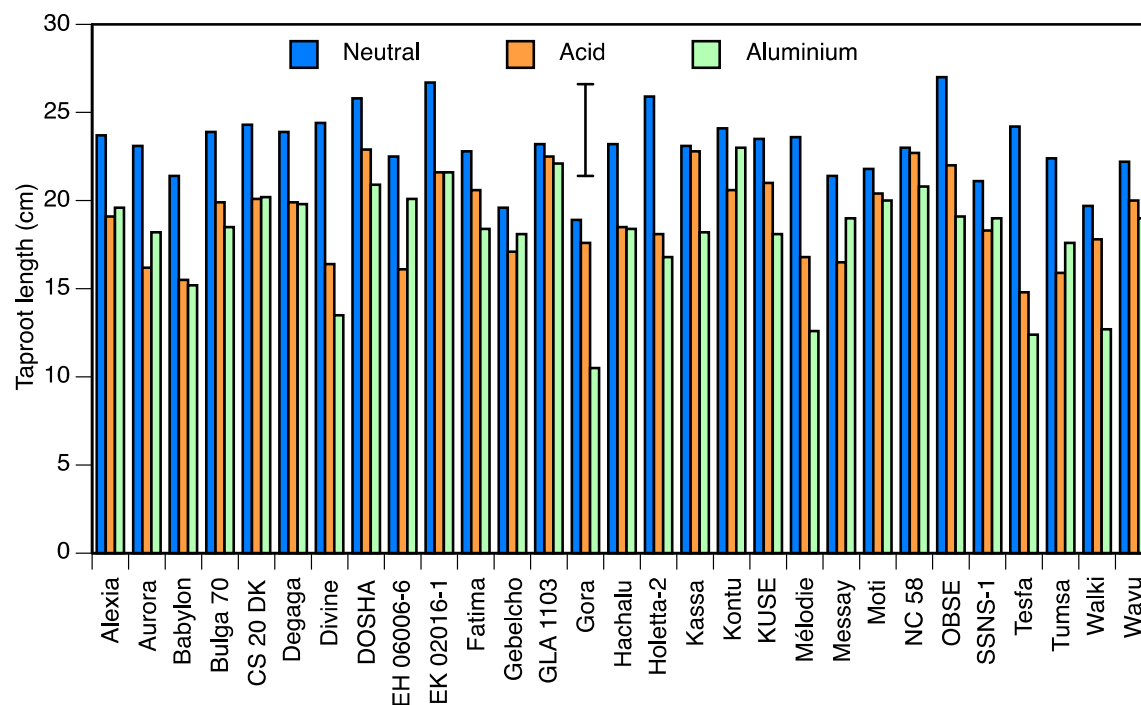
	Peat experiment					Perlite experiment
Accession	Leaf area (cm^2)	Photosynthesis (μmole/m^2/s)	Nodule quantity	Nodule quality	Shoot Al (mg/kg)	Canopy temperature (°C)
Aurora	831	16.4	2.33	2.50	43.1	22.5
Babylon	722	18.6	1.75	2.42	44.1	22.8
Dosha	640	14.8	1.08	1.58	45.2	23.2
EH 06006-6	551	15.1	0.83	1.08	68.6	23.7
Gebelcho	617	16.9	0.83	0.92	42.9	23.0
GLA 1103	606	20.1	2.08	2.58	49.3	22.6
Kassa	533	16.5	1.08	1.33	50.8	22.9
Messay	547	17.2	1.67	2.33	59.5	22.7
NC 58	569	14.7	1.00	1.17	79.1	22.8
Tesfa	475	18.3	0.92	1.33	72.8	22.2
SE	32	0.72	0.27	0.32	8.2	0.24
LSD (5%)	89	2.02	0.75	0.90	23.1	0.66
Treatment						
Neutral	780	15.6	1.90	2.10	44.7	22.7
Acid	491	16.7	0.98	1.50	57.4	22.8
Aluminium	556	18.3	1.20	1.58	64.6	23.0
SE	17	0.39	0.15	0.18	4.49	0.13
LSD (5%)	49	1.11	0.41	0.49	12.7	0.36
P-value						
Treatment	***	ns	*	ns	ns	ns
Accession	***	***	***	***	*	**
Treatment x Accession	ns	ns	ns	ns	ns	ns

543   *, **, *** p< 0.05, 0.01, 0.001, respectively, ns (not significant) p>0.05, SE is standard error

544   Table 4. Accession and treatment means of leaf, root and shoot traits measured in both peat and
545   perlite potting media, n = 4.

	Chlorophyll concentration (SPAD unit)		Stomatal conductance (mol $H_2O/m^2/s$)		Root dry weight (g)		Shoot dry weight (g)		Root to shoot dry weight ratio	
Accession	Peat	Perlite	Peat	Perlite	Peat	Perlite	Peat	Perlite	Peat	Perlite
Aurora	38.7	35.2	0.44	0.52	3.16	1.52	8.32	2.89	0.40	0.56
Babylon	40.8	39.2	0.50	0.29	3.48	1.45	6.83	3.55	0.52	0.42
Dosha	33.3	35.2	0.39	0.31	2.27	1.76	7.20	4.45	0.33	0.40
EH 06006-6	36.2	38.4	0.37	0.20	1.89	2.12	6.55	5.08	0.30	0.42
Gebelcho	34.6	35.8	0.49	0.31	2.08	1.87	6.82	4.93	0.32	0.39
GLA 1103	39.7	40.2	0.62	0.47	2.40	1.44	6.11	2.89	0.43	0.53
Kassa	34.8	34.2	0.50	0.37	1.75	1.57	6.31	3.85	0.28	0.41
Messay	34.3	36.1	0.47	0.29	1.88	1.52	6.40	4.42	0.30	0.34
NC 58	34.7	36.1	0.41	0.28	2.03	1.52	7.08	4.40	0.30	0.35
Tesfa	35.4	34.8	0.58	0.36	1.61	1.66	5.11	4.82	0.34	0.35
SE	0.61	0.79	0.04	0.03	0.13	0.11	0.32	0.25	0.02	0.03
LSD (5%)	1.72	2.21	0.10	0.08	0.37	0.30	0.89	0.70	0.06	0.07
Treatment										
Neutral	34.5	35.1	0.35	0.37	2.43	1.77	5.38	4.34	0.28	0.42
Acid	37.4	36.7	0.51	0.33	2.05	1.59	6.10	3.94	0.39	0.42
Aluminium	36.8	37.7	0.56	0.32	2.28	1.57	8.54	4.10	0.39	0.41
SE	0.33	0.43	0.02	0.02	0.07	0.06	0.17	0.14	0.01	0.014
LSD (5%)	0.94	1.21	0.06	0.05	0.20	0.16	0.49	0.39	0.03	0.04
P-value										
Treatment	**	**	*	ns	ns	ns	***	ns	*	ns
Accession	**	***	***	***	***	***	***	***	***	***
Treatment x Accession	ns	**	ns	ns	ns	ns	ns	ns	ns	ns

546   *, **, *** p< 0.05, 0.01, 0.001, respectively, ns (not significant) p>0.05, SE is standard error

547

548   Figure 1. Taproot lengths in solution culture.
549   Taproot length of seedlings of 29 accessions of faba bean grown in solution culture for 10 days
550   in neutral (pH 7.0) or acid (pH 4.5) nutrient solution, or 8 days in the acid solution followed by 2
551   days in aluminium-containing acid (pH 4.5, 82μmol/l Al^{3+}) solutions. Error bar shows LSD (5%).
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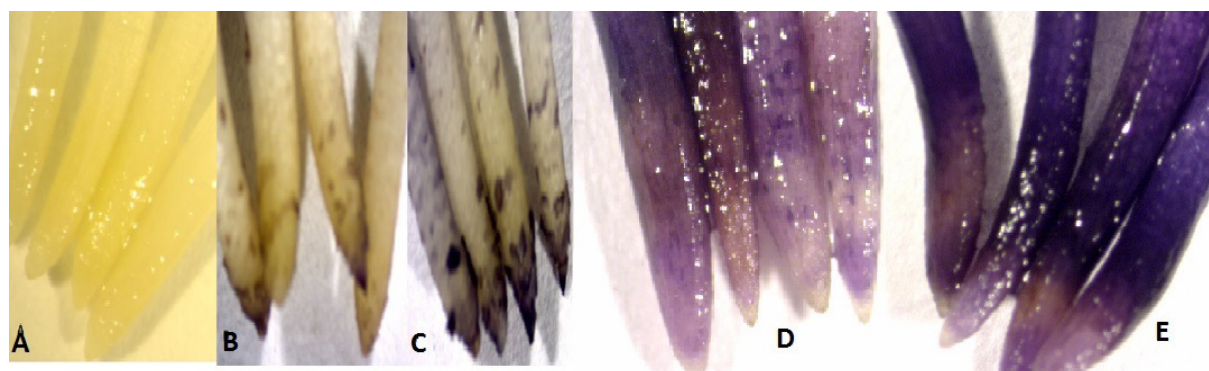
555   Figure 2. Haematoxylin staining of root tips of faba bean.
556   **A**, acid treatment without aluminium; **B-E**, after 82 μmol/l Al^{3+} treatment; B, accession Dosha,
557   score 0, $\leqslant$ 25% stain; **C**, accession Aurora, score 1, 25% <x$\leqslant$50% stain; **D**, accession Wayu,
558   score 2, 50%<x$\leqslant$75% stain; **E**, accession Babylon, score 3, >75% stain.

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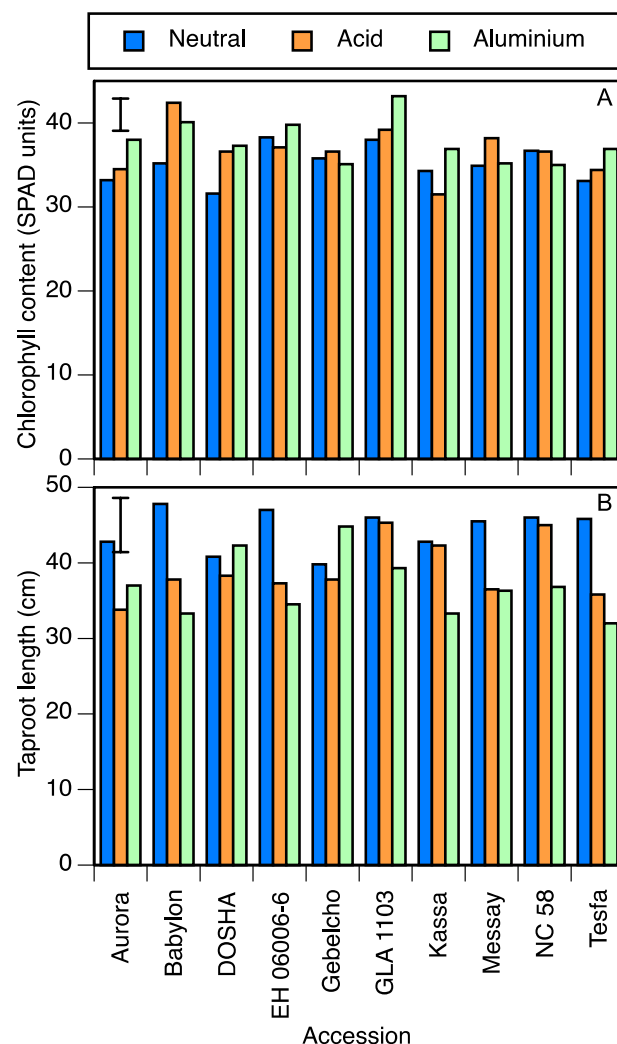
Figure 3. Chlorophyll concentration and taproot length.

A, Chlorophyll concentration (SPAD units) and **B**, taproot length (cm) in 10 accessions of faba
bean grown in perlite and fertigated with neutral (pH 7), acid (pH 4.5) or aluminium containing
acid (pH 4.5, 82 μmol/l Al^{3+}) solutions. Error bar shows LSD (5%).