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RESEARCH ARTICLE

Aluminum exclusion from root zone and maintenance of nutrient uptake are principal mechanisms of Al tolerance in *Pisum sativum* L.

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Abstract Our study aimed to evaluate intraspecific variability of pea (*Pisum sativum* L.) in Al tolerance and to reveal mechanisms underlying genotypic differences in this trait. At the first stage, 106 pea genotypes were screened for Al tolerance using root re-elongation assay based on staining with eriochrome cyanine R. The root re-elongation zone varied from 0.5 mm to 14 mm and relationships between Al tolerance and provenance or phenotypic traits of genotypes were found. Tolerance index (TI), calculated as a biomass ratio of Al-treated and non-treated contrasting genotypes grown in hydroponics for 10 days, varied from 30% to 92% for roots and from 38% to 90% for shoots. TI did not correlate with root or shoot Al content, but correlated positively with increasing pH and negatively with residual Al concentration in nutrient solution in the end of experiments. Root exudation of organic acid anions (mostly acetate, citrate, lactate, pyroglutamate, pyruvate and succinate) significantly increased in several Al-treated genotypes, but did not correlate with TI. Al-treatment decreased Ca, Co, Cu, K, Mg, Mn, Mo, Ni, S and Zn contents in roots and/or shoots, whereas contents of several elements (P, B, Fe and Mo in roots and B and Fe in shoots) increased, suggesting that Al toxicity induced substantial disturbances in uptake and translocation of nutrients. Nutritional disturbances were more pronounced in Al sensitive genotypes. In conclusion, pea has a high intraspecific variability in Al tolerance and this trait is associated with provenance and phenotypic properties of plants. Transformation of Al to unavailable (insoluble) forms in the root zone and the ability to maintain nutrient uptake are considered to be important mechanisms of Al tolerance in this plant species.

Keywords Aluminium · Biodiversity · Organic acids · Nutrient uptake · Pea · Rhizosphere

Introduction

High soil acidity is a worldwide stress factor limiting productivity of agricultural crops. For the most part, phytotoxicity of acid soils is the result of elevated concentrations of mobile aluminium ions, such as Al^{3+} and hydroxy-Al species (Kochian 1995; Gupta et al. 2013). Plants have developed a number of mechanisms to avoid and/or tolerate Al toxicity: exudation of organic acids (malate, citrate, oxalate) from roots to complex Al in the rhizosphere and prevent its entry into the root; increased rhizosphere pH and secretion of mucilage to immobilize Al outside the root; internal detoxification involving Al chelation and complexation within plant tissues; sequestration of Al in the vacuole, and the actual efflux of accumulated Al from the root apex via transport proteins (Taylor 1991; Ma et al. 2001; Kochian et al. 2004; Delhaize et al. 2012; Liu et al. 2014). Numerous studies performed to investigate the

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mechanisms of Al tolerance in different plant species such as wheat, barley, maize, soybean and *Arabidopsis thaliana*, revealed differences in the significance of a particular mechanism for different plant species (see literature reviewed in the references above). However, little is known about Al tolerance in pea (*Pisum sativum* L.). An Al-tolerant pea cultivar had a higher pH value in the root zone and lower root Al content (Klimashevsky et al. 1972) compared to Al-sensitive cultivar treated with Al. Measuring root growth during a recovery period in Al-free solution distinguished differences in Al toxicity between two pea cultivars (Lazof and Holland 1999). The recovery of pea root elongation depended on the ability to resist Al-induced oxidative stress and the reduction of lignin production (Motoda et al. 2010; Matsumoto and Motoda 2012). Immobilization of Al by pectin located in the pea root border cells was shown to play an important role in protecting the root apex (Yu et al. 2009a).

The important role of calcium (Ca) in alleviating Al toxicity in plants occurs by preventing a decrease in the negative charge on the plasma membrane (PM) caused by Al, decreasing activity of Al^{3+} on PM surface, and maintaining normal hormonal status and Ca^{2+}-related signalling pathways in the presence of toxicant (Rengel 1992; Kinraide et al. 2004; Kobayashi et al. 2013; Pandey et al. 2013). Magnesium (Mg) can also counteract Al phytotoxicity by increasing synthesis and exudation of organic acids, improving carbon partitioning from shoots to roots, regulating cytoplasmic pH and enhancing activity of stress-related enzymes (Rengel and Robinson 1989; Silva et al. 2001; Bose et al. 2011; Pandey et al. 2013). Zhou et al. (2015) showed that the alleviation mechanism of boron (B) could be related to changes in expression of genes involved in Al tolerance. Exudation of phosphate from maize roots might also be involved in immobilization of Al in the rhizosphere (Pellet et al. 1995, 1996; Pineros et al. 2002). In pea, treatment with Ca decreased a negative effect of Al on K uptake by roots (Matsumoto and Yamaya 1986) and treatment with B alleviated Al toxicity in root tips by decreasing Al binding in cell walls and callose formation (Yu et al. 2009b). The role of other nutrient elements in interactions of plants with toxic Al is scarcely understood.

Pea, along with other legume species, is a relatively sensitive crop compared to cereals (Aniol and Gustafson 1984; Lazof and Holland 1999; Akhter et al. 2009). Toxic Al concentrations inhibited pea root growth and injured root tissues (Lazof and Holland 1999; Motoda et al. 2010), induced oxidative stress (Yamamoto et al. 2001; Panda and Matsumoto 2010; Matsumoto and Motoda 2012) and inhibited nutrient uptake (Matsumoto and Yamaya 1986).

Significant intraspecific variability in Al tolerance was reported for various crops. In wheat cultivars, Al tolerance positively correlated with malate efflux from root apices (Ryan et al. 1995), Ca influx and transport from root to shoot (Huang et al. 1995) and shoot Ca content (Foy 1996), but negatively correlated with root Al content (Foy 1996; Khabaz-Saberi and Rengel 2010). A lower root Al content (Stass et al. 2006) and higher root citrate and phosphate exudation (Pellet et al. 1995) was observed in an Al-tolerant maize cultivar, compared to Al-sensitive one. Comparing 25 sorghum genotypes demonstrated the important role of maintaining nutrient homeostasis to alleviate Al stress (Bernal and Clark 1997). Root retention of Al, preventing its transport to the shoot, was proposed as a mechanism of Al tolerance comparing 9 rice cultivars (Jan and Pettersson 1989). Al-tolerant soybean genotypes possessed higher P efficiency (Liao et al. 2006; Liang et al. 2013), responded more actively to toxic Al by citrate, malate and oxalate exudation (Silva et al. 2001; Liao et al. 2006), and responded to Mg or Ca treatments by alleviating Al toxicity (Silva et al. 2001). However, intraspecific variability in Al tolerance of pea was not studied, except our recent report describing significant genotypic variation in root re-elongation of 19 pea genotypes following Al exposure (Vishnyakova et al. 2015).

Our study aimed to investigate intraspecific variability of pea in Al tolerance, to select contrasting genotypes and use them for revealing the mechanisms underlying genotypic differences in this trait. The selected genotypes is a promising model for the in-depth study of pea tolerance to acidic soils and for breeding highly productive Al-resistant cultivars.

Materials and methods

Plant material

Seeds of pea (*Pisum sativum* L.) were obtained from the Pea World Collection (PWC) of the N.I. Vavilov Research Institute of Plant Genetic Resources (VIR, Saint-Petersburg). Selection of genotypes was carried out to provide maximum coverage of the species genetic biodiversity. The following phenotypic and economic traits of pea genotypes were taken into account: geographical origin, purpose of use, morphotype, individual seed biomass, seed yield, seed color, seed surface and period of maturity (see Table 1 in Online Resource 1 for details). The catalogue collection numbers of the studied varieties are given in the text, Online Resource 1 and figures throughout this paper.

Primary screening for Al tolerance using root re-elongation assay

The recently proposed root re-elongation assay to rapidly assess Al tolerance in pea (Vishnyakova et al. 2015) was

used to screen 106 genotypes. For this purpose, seeds (25 seeds per genotype) were germinated in the dark for 3 days at 21 °C in special containers filled with nutrient solution (μM): KH_2PO_4, 400; KNO_3, 1200; $MgSO_4$, 250; $CaCl_2$, 60; pH 4.5. Well germinated seeds (from 10 to 20 depending on genotype) were selected, washed with water, placed in fresh nutrient solution supplemented with 110 μM $AlCl_3 \times 6H_2O$ and incubated for 24 h at 21 °C in growth chamber (7000 lx, 12 h photoperiod with minima/maxima temperatures of 19/21 °C respectively). Then seedlings were transferred to fresh nutrient solution without Al and incubated for 2 days as described above. The roots were removed from solution and stained with 0.1% eriochrome cyanine R for 10 min. Staining of roots with eriochrome cyanine R was repeatedly used for rapid assessment of Al tolerance in different plant species using root re-elongation assay. Recently we adapted this method to estimate root re-elongation of pea treated with Al and have already published these results (Vishnyakova et al. 2015). Eriochrome cyanine R is a dye that forms color complexes with cations such as Al and Fe. So, some staining probably could be observed on roots of plants grown in Al-free solution in the presence of Fe as a micronutrient. However, intensity of such staining is almost invisible and dramatically low as compared with Al-treated roots. Actually, if the root tip is significantly damaged by Al, it contains Al in tissues, stained purple and does not re-elongate. If the damage is less, the root re-elongates, some staining is present on damaged zone, but the re-elongating root zone is not stained because there is no Al in tissues developed after transferring the plants to Al-free solution. Root damage by Al was coloured purple and the root re-elongation zone was measured as the length of uncoloured zone from the root tip named as increment of root (IR). Genotypes having contrasting response to Al toxicity were repeatedly assessed as described above.

Plant growth conditions in hydroponic culture

Seeds were surface sterilized and scarified by treatment with 98% H_2SO_4 for 30 min, rinsed with tap water and germinated on filter paper in Petri dishes for 3 days at 25 °C in the dark. Seedlings were transferred to plastic pots (one pot with 5 uniform seedlings per genotype and treatment) containing 1 l of sterile nutrient solution (μM): KH_2PO_4, 400; KNO_3, 1200; $Ca(NO_3)_2$, 60; $MgSO_4$, 250; KCl, 250; $CaCl_2$, 60; Fe-tartrate, 12; H_3BO_3, 2; $MnSO_4$, 1; $ZnSO_4$, 3; NaCl, 6; Na_2MoO_4, 0.06; $CoCl_2$, 0,06; $CuCl_2$, 0.06; $NiCl_2$, 0,06. The nutrient solution was acidified up to pH 4.7 via addition of 1 M HCl and supplemented or not with 80 μM $AlCl_3 \times 6H_2O$. Such Al concentration was chosen because preliminary experiments showed that during the experiment, a complete growth inhibition of Al sensitive genotypes treated with 110 μM Al occurred. Plants were cultivated for 10 days in a growth chamber (ADAPTIS-A1000, Conviron, UK) with 7000 lx, 12 h photoperiod and minima/maxima temperatures of 18/23 °C respectively. The nutrient solution was changed and where necessary the supplement was added on the 5th day after planting. Transpiration losses were compensated daily via addition of sterile deionized water to maintain constant solution volume. Root and shoot fresh weight (FW) of individual plants and pH of nutrient solution were determined. Roots were washed for 1 min in deionized water to remove unbound Al and nutrients from root surface. Aliquots of nutrient solution were centrifuged at 7000 rpm for 10 min, acidified with HNO_3 up to final concentration of 0.5% to prevent microbial activity and stored for elemental analysis. The plants were dried at room temperature and stored for elemental analysis. Experiments for each pea genotype were repeated three times with 5 plants each.

Root exudation of organic acid anions

On harvest day, the nutrient solution of each pot (containing root exudates accumulated for 5 days) was centrifuged (Model 5804R, Eppendorf, USA) for 10 min at 4500 g, vacuum filtered through 0.45 μm filters (Waters, USA) and concentrated at 45 °C using a rotary vacuum evaporator (Heidolph Hei-VAP Precision, Heidolph Instruments GmbH & Co, Germany). The concentrates were passed through a column of ion exchange resin DOWEX 50Wx8, evaporated to dryness, dissolved in 0.5 ml of deionized water and filtered through 0.22 μm centrifuge tube filters (Corning Costar Spin-X, Corning Inc, USA) for subsequent chromatographic analysis using the UPLC system Waters ACQUITY H-Class (Waters, USA) as previously described (Kuzmicheva et al. 2014). Organic acids were separated on column ACQUITY CSH C18 (Waters, USA) and determined using UV detector at 210 nm. Standards comprised organic acids from analytical grade reagents of Sigma-Aldrich (USA).

Elemental analysis

The dried roots and shoots were ground and digested in a mixture (1:1) of concentrated HNO_3 and 38% H_2O_2 at 70 °C using DigiBlock (LabTech, Italy). Contents of elements in digested plant samples (Al, B, Ca, Co, Cu, Fe, K, Mg, Mn, Mo, Ni, P, S and Zn) and concentration of Al in the acidified nutrient solutions were determined using an inductively coupled plasma emission spectrometer ICPE-9000 (Shimadzu, Japan) following manufacturer instructions.

Statistical analysis

Statistical analysis of the data was performed using the software STATISTICA version 10 (StatSoft Inc., USA) and an ANOVA software (Vorobyev et al. 2013). Fisher's LSD test (ANOVA), correlation analysis, and cluster analysis (standardized values, Ward's method for linkage rules, 1-Pearson-r distance measure) were applied.

Results

Screening of 106 pea genotypes showed that after treatment with Al the IR varied significantly from 0.5 to 1.5 mm for the most Al-sensitive genotypes to 13–14 mm for the most Al-tolerant genotypes (Fig. 1). The distribution of genotypes on the basis of this trait was normal: MV = 7.6; SD = 3.6; CV = 47 ± 3; As = −0.03 ± 0.23; Ex = −0.79 ± 0.46.

Four clusters were recognized on approximately 40% dissimilarity level when pea genotypes were clustered based on phenotypic and economic traits listed in Materials and methods section (Fig. 2). The most Al-sensitive genotypes tended to group together in cluster A, whereas most of the Al-tolerant genotypes distributed among clusters B, C and D. Indeed, mean IR value of genotypes combined in cluster A significantly differed from other clusters (Fig. 3). The IR value correlated with geographic origin (R = 0.21, $P = 0.027$, n = 106), purpose of use (R = 0.39, $P < 0.001$, n = 106) and individual seed biomass (r = −0.53, $P < 0.001$, n = 106) values. Analysis of these results showed that the genotypes combined in cluster A mainly originated from South America, Australia or Africa, were related to forage peas and characterized by small individual seed biomass and seed yield (Fig. 3).

Based on the screening results, the most Al-sensitive (8473, 2759, 1903, 3654, 3283, 1443, 5158, 6778) and Al-tolerant genotypes (7376, 9053, 1776, 8633, 8264, 9038, 0836, 8762, 8353, 7899, 4376, 9504, 9507, 7307) were used for further experiments. Treatment with 80 μM $AlCl_3$ for 10 days significantly decreased root biomass of all genotypes except 6778, 7307, 0836 and 8353 (Fig. 4a), whereas shoot biomass decreased in all genotypes except 6778, 7307 and 8357 (Fig. 4b). TI varied from 30% (genotype 3283) to 92% (genotype 8353) for root biomass and from 38% (genotype 2759) to 90% (genotypes 6778 and 8353) for shoot biomass (Fig. 4c). The IR value positively correlated with root TI (r = +0.68, $P < 0.0001$, n = 22), but not with shoot TI (r = +0.32, $P = 0.15$, n = 22), whereas root and shoot TIs were closely correlated (r = +0.73, $P < 0.0001$, n = 22).

Eleven genotypes (Al-sensitive 3654, 8473, 2759, 1903; Al-tolerant 8762, 9507, 8633, 6778, 7307, 0836, 8353) were selected for more detailed study. Amongst these, significant genotypic differences in Al content were found in root (Fig. 5a) and/or in shoot (Fig. 5b). These Al contents were not correlated with IR or TI values, however minimal root Al contents were observed in two Al-sensitive genotypes 2759 and 2759. Shoot Al content was similar in all genotypes, except that 3654 (Al-sensitive) and 6778 (Al-tolerant) had 6 and 3 times higher Al-contents as compared with other genotypes (Fig. 5b). At the end of experiment, Al concentration in nutrient solution significantly differed depending on pea genotype (Fig. 5c) and negatively correlated with IR (r = −0.77, $P = 0.006$, n = 11), root TI (r = −0.84, $P = 0.001$, n = 11) and shoot TI (r = −0.67, $P = 0.025$, n = 11). Moreover, final pH of nutrient solution negatively correlated with final solution Al concentration (r = −0.69, $P = 0.020$, n = 11), but positively correlated with IR (r = +0.63, $P = 0.038$, n = 11), root TI (r = +0.75, $P = 0.007$, n = 11) and

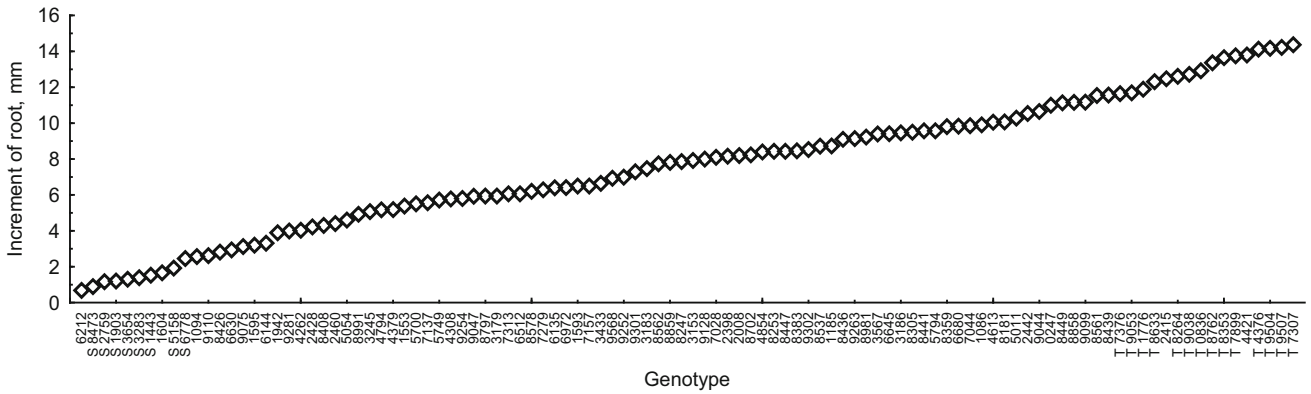


Fig. 1 Root re-elongation (increment of root) of 106 pea genotypes. Seedlings were treated with 110 μM $AlCl_3$ for 1 day, transferred to fresh nutrient solution without aluminum and incubated for 2 days. Standard errors are less then symbol size (n varied from 10 to 30 depending on genotype). The most Al-tolerant or the most Al-sensitive genotypes chosen for further experiments are marked by T or S, respectively

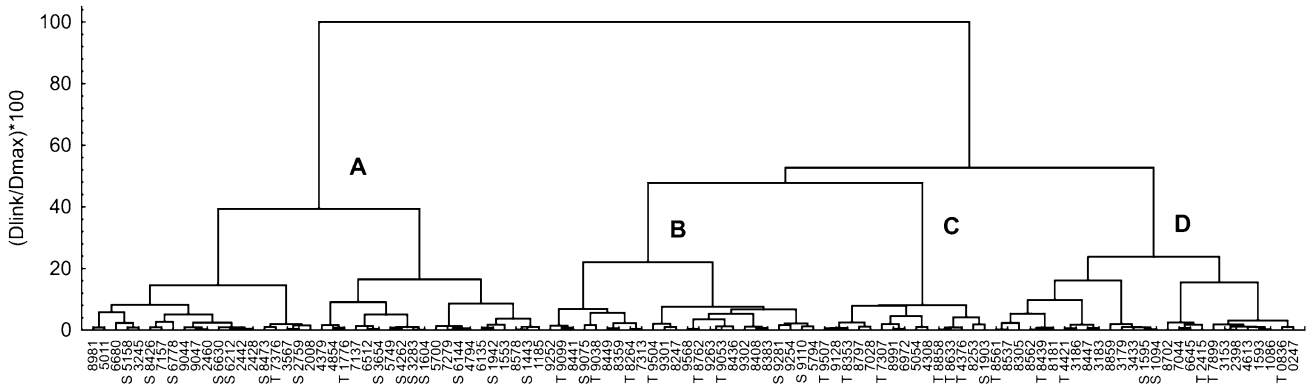


Fig. 2 Dendrogram showing relationships among the studied pea varieties based on cluster analysis of the data for phenotypic and economic traits (see "Materials and methods" section for details). Ward's method for linkage rules, 1-Pearson-r distance measure. Capital letters indicate numbers of clusters. The 20 most Al-tolerant or 20 most Al-sensitive genotypes (accordingly to the data shown in Fig. 1) are marked by T or S, respectively. The properties used for cluster analysis are presented in supplemental Table 1

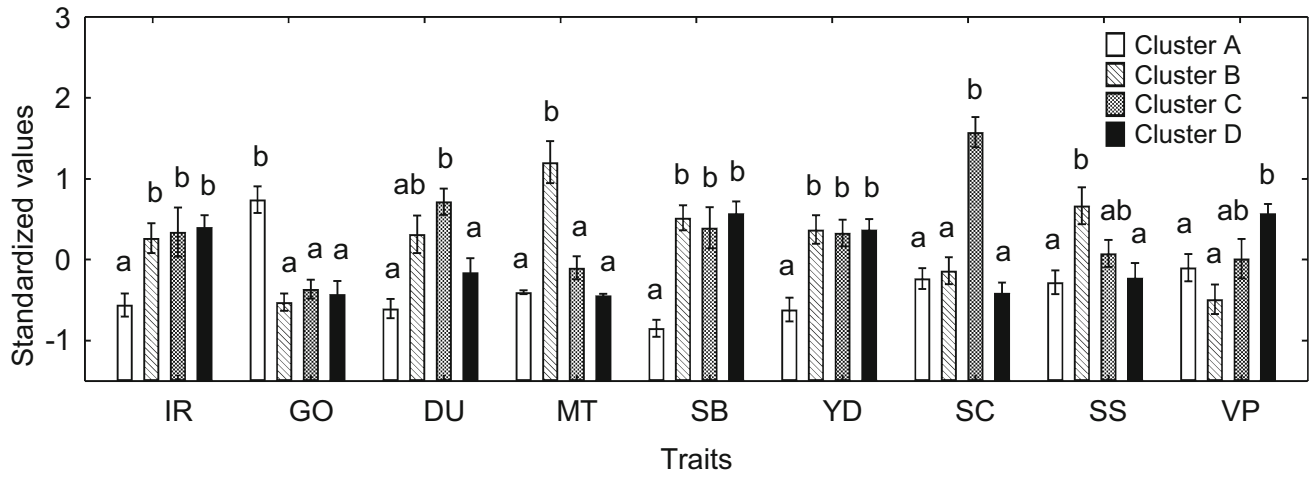


Fig. 3 Means for the clusters combining pea genotypes according to the dendrogram shown in Fig. 2. Traits: increment of root (IR), geographic origin (GO), direction of use (DU), morphotype (MT), individual seed biomass (SB), seed yield (YD), seed color (SC), seed surface (SF), vegetation period (VP). Note that IR was not included into the cluster analysis. Vertical bars indicate standard errors (n = 40, 24, 15 and 27 for clusters 1, 2, 3 and 4, respectively). Different lowercase letters indicate significant differences between clusters for each trait (LSD test, $P \leq 0.05$)

shoot TI ($r = -0.68$, $P = 0.022$, $n = 11$). Significant correlation between final pH of nutrient solution and IR ($r = +0.45$, $P = 0.035$, $n = 22$) or root TI ($r = +0.52$, $P = 0.013$, $n = 22$) was also observed for 22 pea genotypes presented in Fig. 4. Slight turbidity of the Al treated solutions was observed for all pea genotypes, suggesting precipitation of Al.

The major organic acid anions exuded by pea roots and detected in the nutrient solutions at the end of experiments were acetate, citrate, lactate, pyroglutamate, pyruvate and succinate (Fig. 6). In the absence of Al, maximum exudation of organic acids was observed for Al-tolerant genotypes such as 6778 (acetate, citrate and succinate), 0836 (acetate, citrate and pyruvate) and 8353 (acetate, lactate and pyroglutamate). However, in the presence of Al, maximum exudation was observed in Al-sensitive genotypes 8473 (acetate and pyruvate), 2759 (citrate, pyroglutamate and pyruvate) and 1903 (acetate and succinate), as well as in Al-tolerant genotypes 8353 (lactate) and 6778 (pyruvate and succinate). Malate was detected in solutions of Al-untreated 2759 (67 ± 16 μg g^{-1} DW) and 0836 (380 ± 139 μg g^{-1} DW) genotypes only. In general, when summing all organic acids, Al treatment increased exudation of these compounds, particularly in Al-sensitive genotypes 3654, 2759 and 1903, as well as in Al-tolerant genotypes 6778 and 7307 (Fig. 6). However, in some cases Al-treatment decreased organic acid exudation, namely lactate (genotypes 3654, 2759, 8633 and 8353) and pyroglutamate (3654, 1903, 8762, 9507, 8633 and 8353). No correlations were found between organic acid exudation and TI or Al content in plants, except that root Al

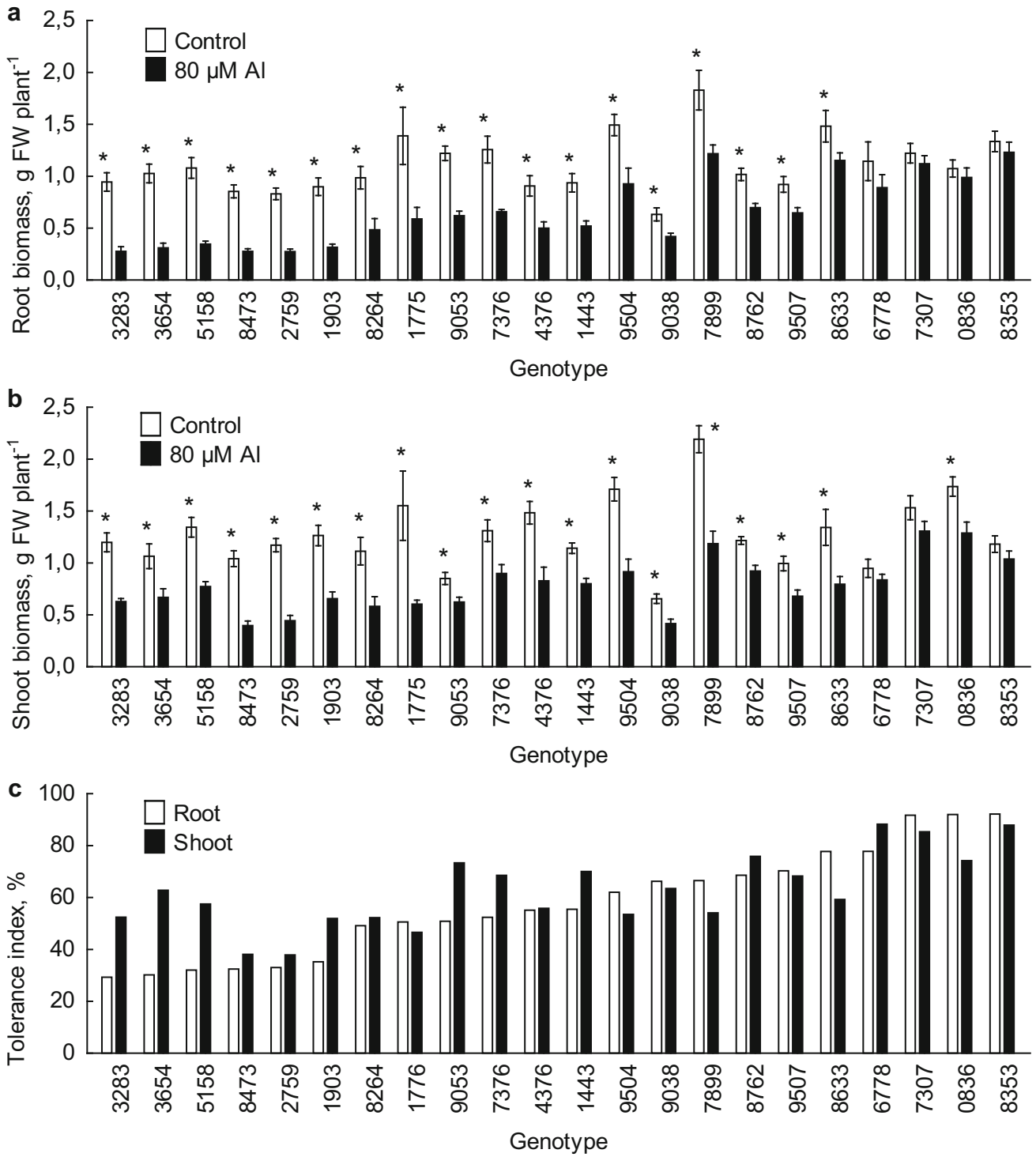


Fig. 4 Effect of aluminium on biomass of root (**a**) and shoot (**b**) and tolerance index (**c**) of the selected pea genotypes. Vertical bars indicate standard errors (n = 15). Asterisks indicate significant differences between untreated (control) and Al-treated (80 μM Al) plants for each genotype (LSD test, $P \leq 0.05$). Genotypes are shown in order of increasing root tolerance index from left to right

content negatively correlated with pyroglutamate ($r = -0.72$, $P = 0.013$, $n = 11$) or the sum of all organic acids ($r = -0.65$, $P = 0.032$, $n = 11$) exuded by Al-treated plants.

Treatment with Al significantly decreased contents of macronutrients K, Mg and S in roots of all genotypes, except for Al-tolerant genotypes 7307 and 0836 (Table 2 in Online Resource 1). Root Ca content also decreased in Al-

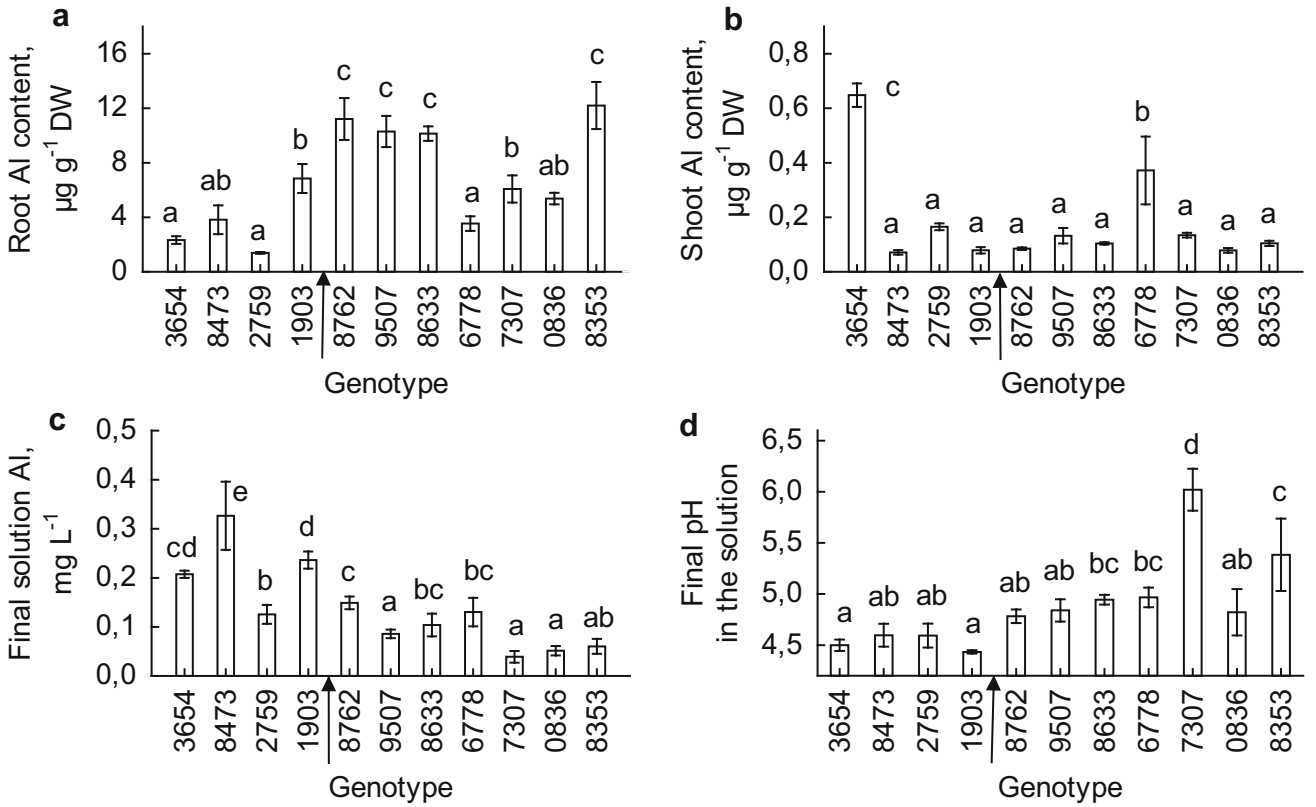


Fig. 5 Aluminium content in root (**a**) and shoot (**b**) of the selected pea genotypes and final solution Al concentration (**c**) and pH (**d**) in the end of experiments with Al-treated plants. Vertical bars indicate standard errors (for Al in plants n = 5; for solution Al and pH n = 3). Different lowercase letters indicate significant differences between means (LSD test, $P \leq 0.05$). Genotypes are shown in order of increasing root tolerance index from left to right and the arrow differentiates Al sensitive from Al resistant genotypes

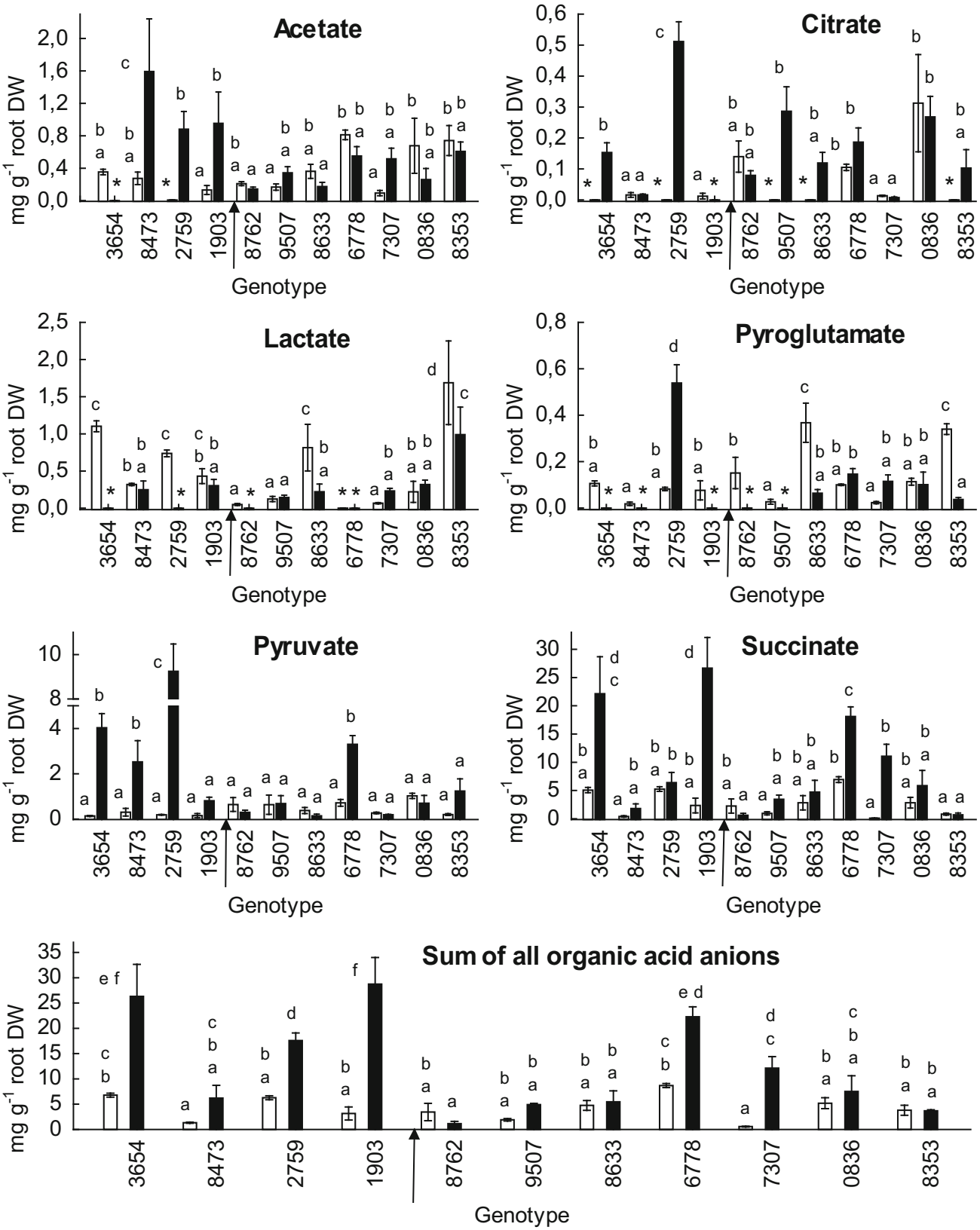
treated plants, except for Al-tolerant genotypes 7307, 0836 and 8353. In contrast, root P content was increased in all genotypes, except for Al-tolerant genotype 8633. Significant decrease in shoot K, Ca, Mg and S contents was found in all genotypes, except for Al-tolerant genotype 7307, which had increased content of these elements, and genotype 0836, which had increased S content (Table 3 in Online Resource 1). Shoot P content was decreased in genotypes 8473, 8762, 8633 and 6778.

Al treatment generated large variation in plant micronutrient uptake, depending on element, genotype and plant organ (root or shoot). Several Al-treated genotypes, particularly those which were Al-sensitive, had increased content of B, Fe and Mn in roots (Table 4 in Online Resource 1) and B and Fe in shoots (Table 5 in Online Resource 1). Molybdenum content of Al-sensitive genotypes increased in roots, but was not affected or even decreased in shoots, mostly of Al-tolerant genotypes. Majority of the genotypes, except for Al-tolerant 7307 and 0836, had decreased content of Co, Mn, Ni and Zn in both root (Table 4 in Online Resource 1) and shoot (Table 5 in Online Resource 1).

Two Al-tolerant genotypes 7307 and 0836 significantly differed from other genotypes by maintaining nutrient uptake in the presence of toxic Al (Tables 4 and 5 in Online Resource 1). When root TI was plotted against the effects of Al on root nutrient (B, Ca, Co, Fe, K, Mg, Mn and Mo) concentrations, the more tolerant genotypes consistently showed smaller percent changes in root element composition, independently whether the element content was decreased or increased by Al treatment (Fig. 7). Similar relationships were found between root TI and shoot Ca, Co, K, Mg, Mn, Ni and Zn contents, as well as between shoot TI and shoot Ca, K and Mn contents (data not shown).

Discussion

Significant variation among 106 pea genotypes was found in response to toxic Al estimated by the root re-elongation assay and by the root and shoot growth responses to Al treatment. This is in agreement with substantial intraspecific variability in this trait previously found in


Acetate
Citrate
Lactate
Pyroglutamate
Pyruvate
Succinate
Sum of all organic acid anions
mg g-1 root DW
Genotype
3654
8473
2759
1903
8762
9507
8633
6778
7307
0836
8353

◀**Fig. 6** Exudation of organic acid anions by roots of the selected pea genotypes. The plants were incubated in nutrient solution for 5 days. White and black columns indicate untreated control and Al-treated plants, respectively. Vertical bars indicate standard errors (n = 3). Asterisks show not detected. Different lowercase letters indicate significant differences between means (LSD test, $P \leq 0.05$). Genotypes are shown in order of increasing root tolerance index from left to right and the arrow differentiates Al sensitive from Al resistant genotypes

other legumes such as cowpea (Horst 1987; Kolawole et al. 2000; Jemo et al. 2007), clover (Baligar et al. 1987) and soybean (Liao et al. 2006; Villagarcia et al. 2001).

For the first time, we detected relationships between Al tolerance and provenance or phenotypic traits of pea varieties such as geographic origin, purpose of use, individual seed biomass and seed yield. The only previous report described correlation between Al tolerance of sorghum varieties with their agronomic traits like biomass, height and days to flowering (Anas and Yoshida 2004). Previously, we also found that tolerance to Cd negatively correlated with seed number, individual seed weight and seed N content of 99 pea genotypes (Belimov et al. 2015b). Interestingly, Al-tolerant or Cd-tolerant pea genotypes were less productive (that is characteristic of forage peas) and probably less domesticated. Although better understanding of the observed relationships is required, these results seem useful for elucidating the mechanisms underlying genotype-related Al tolerance and for breeding purposes.

It was repeatedly shown in experiments with bean (Shen et al. 2002), soybean (Silva et al. 2001), wheat (Foy 1996; Khabaz-Saberi and Rengel 2010) and maize (Stass et al. 2006) that root Al content is lower in Al tolerant genotypes. The Al-sensitive pea mutant E107 (*brz*) actively accumulated Al in roots, seriously inhibiting root growth (Guinel and LaRue 1993). On the other hand, opposite observations were obtained when comparing cowpea (Jemo et al. 2007), wheat (Kikui et al. 2007) or rice (Jan and Pettersson 1989) genotypes. Aluminum tolerance did not correlate with root Al content in sorghum genotypes (Bernal and Clark 1997). Similarly, in our experiments Al tolerance was not correlated with root or shoot Al contents in pea, despite of significant genotypic differences in these traits. While we agree that low root Al content is important to counteract Al toxicity in various plant species, but propose that this mechanism does not play a crucial role in Al tolerance of pea. In our previous experiments with pea, growth and metabolic responses to Cd toxicity did not correlate with root Cd content (Metwally et al. 2005), but genotypic differences in Cd tolerance were related to decreased Cd transport from root to shoot (Belimov et al. 2003). However, the pea mutant SGECd^t, characterized by increased tolerance to Cd and Co, and decreased tolerance to Hg, accumulated more Cd, equal Co, but less Hg in roots and shoots (Belimov et al. 2015a). Thus, our present results give new evidence concerning partial independence of traits related to tolerance and uptake of toxic metals by plants.

For the first time we showed that Al tolerance was negatively correlated with residual Al concentration in the nutrient solution, suggesting that Al tolerant genotypes were better able to actively exclude Al from the root zone, probably due to precipitation with phosphates and other compounds. Further studies are needed to investigate the nature and the role of such compounds in more detail. However, one mechanism of the observed phenomenon can be associated with changes in solution pH, because it is known that Al mobility decreases with increased pH (Kochian 1995) and in our experiments the final solution pH positively correlated with Al tolerance of pea genotypes. Previously, the Al-tolerant pea cultivar Success had higher solution pH compared to the Al sensitive cultivar Tulunsky Green (Klimashevsky et al. 1972). Similarly, solution pH and Al tolerance were positively correlated in winter (Taylor and Foy 1985a) and spring (Taylor and Foy 1985b) wheat cultivars, although it was not possible to explain genotypic differences in Al tolerance solely by the ability to maintain a high solution pH (Taylor 1988). Increased H^+ influx by roots, leading to increased root surface pH, was observed in Al-tolerant *A. thaliana* mutant *alr*-104 (Degenhardt et al. 1998). However in other reports Al tolerance did not correlate with solution pH in wheat (Miyasaka et al. 1989) and barley (Wagatsuma and Yamasaku 1985) cultivars. Such contradictory results indicate that lowering pH is not a sole reason for decreased mobile Al concentration in the root zone, and that the latter parameter depends on plant genotype. Indeed, in our case solution pH of Al tolerant pea genotype 0836 was similar to those of Al sensitive genotypes, but the final Al concentration in the solution was similar to Al tolerant genotypes (Fig. 5).

Aluminium tolerant genotypes of cowpea (Jemo et al. 2007), soybean (Silva et al. 2001; Liao et al. 2006), bean (Shen et al. 2002) and lima bean (Mimmo et al. 2013) were characterized by high root exudation of the organic acid anions such as malate and/or citrate. Addition of malate or citrate to the nutrient solution increased Al tolerance of wheat (Kikui et al. 2007) or bean (Shen et al. 2002), respectively. Treatment of pea with malate and citrate restored activity of membrane-associated Mg^{2+}-ATPase in the presence of toxic Al (Matsumoto and Yamaya 1986). These reports showed that active exudation of malate and citrate to complex Al ions in root zone is an important mechanism of Al tolerance. However, Al treatment of Al tolerant cowpea genotypes did not increase citrate

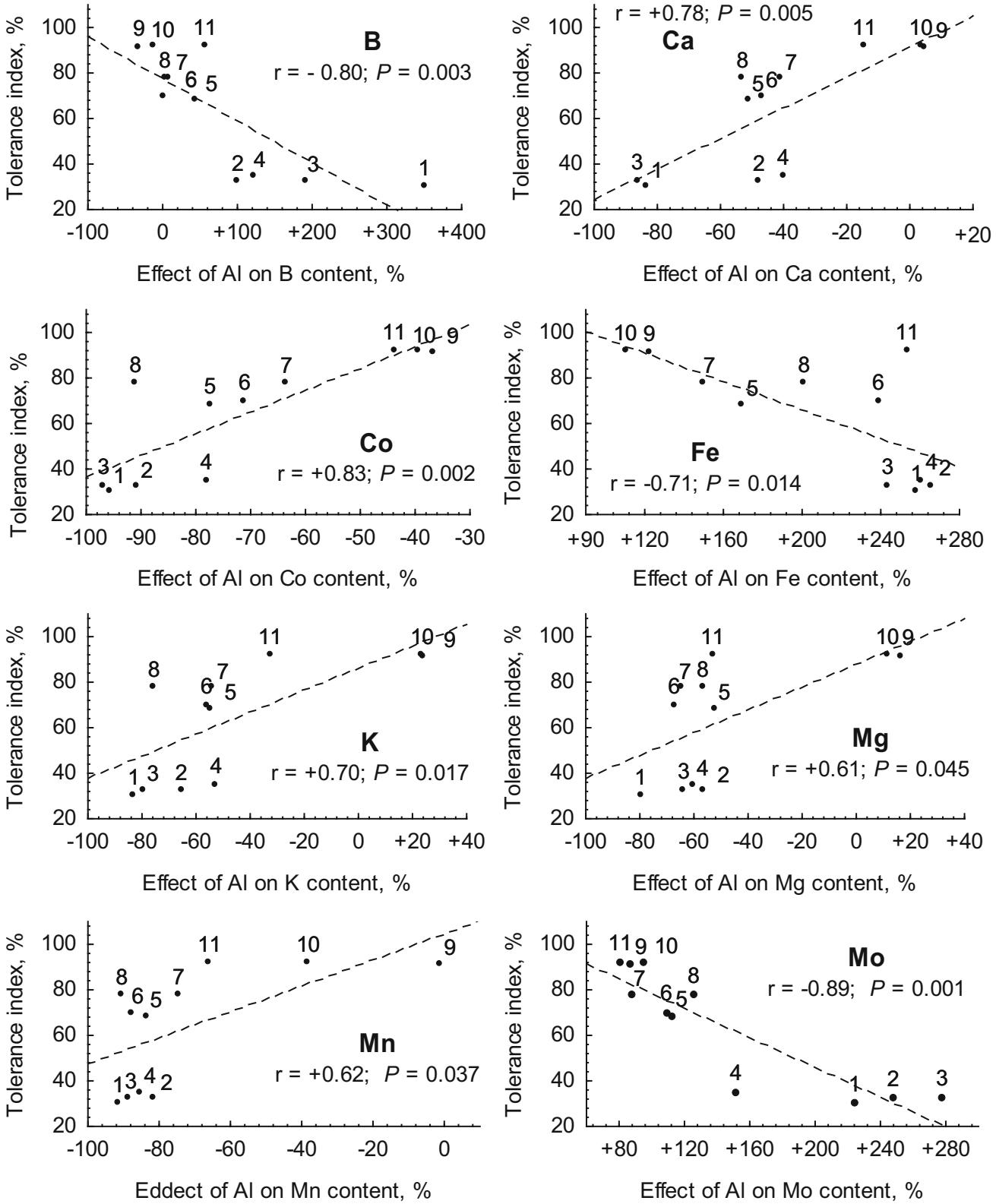


Fig. 7 Correlations between Al tolerance index of roots and effects of Al on nutrient element contents. Pea genotypes: 1—3654; 2—8473; 3—2759; 4—1903; 5—8762; 6—9507; 7—8633; 8—6778; 9—7307; 10—0836; 11—8353. Genotypes are listed in order of increasing root tolerance index. Dashed line shows linear regression. Element symbol, correlation coefficient (r) and probability (P) are shown in each part of the figure (n = 11)

exudation (Jemo et al. 2007). No correlation was found between Al tolerance and organic acid exudation by oat (Zheng et al. 1998) and maize varieties (Kidd et al. 2001; Pineros et al. 2005) or by closely related signalgrass species (Wenzl et al. 2001), suggesting that other mechanisms were involved in this trait. In our study, root or shoot TIs were not correlated with concentrations of detected organic acids in the nutrient solution, and only five of eleven Al treated genotypes exuded more organic acids than control plants. Moreover, malate was detected only in exudates of one Al tolerant (0836) and one Al sensitive (2759) genotype. At the same time, root Al content was negatively correlated with exudation of pyroglutamate and the sum of all organic acids by Al-treated plants. These results demonstrated that organic acid exudation is not a major determinant of Al tolerance in pea, but is involved in decreasing Al uptake by pea roots. An important observation was that the main components of the exuded organic acids by pea were not malate and citrate, but succinate, pyruvate, acetate and lactate. Interestingly, lactate exudation correlated with final solution pH. The role of these acid anions in plant Al tolerance has not been investigated and it would be worthwhile to elucidate it.

It was reported that Al treatment inhibited uptake of Ca in cowpea (Horst 1987) and wheat (Huang et al. 1995), uptake of Mg in ryegrass (Rengel and Robinson 1989) and uptake of K in pea (Matsumoto and Yamaya 1986). Aluminium toxicity significantly decreased root Ca, Mg, K, Fe, S, Mn and Zn contents in sorghum (Bernal and Clark 1997). On the other hand, Ca and Mg treatments alleviated Al toxicity, probably due to competition for the binding sites in the apoplast, and these nutrient elements were repeatedly shown to be important in enhancing Al tolerance in various plant species (Rengel 1992; Silva et al. 2001; Bose et al. 2011; Kobayashi et al. 2013; Pandey et al. 2013), although pea has not been studied in this respect. Supplemental boron (B) enhanced Al tolerance in several plant species (see references cited by Zhou et al. 2015), including pea (Yu et al. 2009b). For the first time, we have shown that Al toxicity not only inhibits uptake of many nutrient elements and Na, but increases content of several nutrients (P, B, Fe and Mo) and Cr in roots and/or in shoots. Thus, Al treatment perturbed nutrient homeostasis and translocation of elements from root to shoot. Our important observation was that Al tolerance of the selected pea genotypes negatively correlated with percent changes in root and/or shoot element contents caused by Al toxicity. Among these elements, B, Ca and Mg are known to be important agents for plant Al tolerance. The role of other elements such as K, Co, Fe, Mn and Mo in alleviating Al toxicity is worthy of further study. We propose that maintaining nutrient uptake per se is an important mechanism of Al tolerance in pea. This trait is likely related to the ability of tolerant genotypes to counteract negative effects of Al on function and permeability of plasma membranes, which in its turn depends on Ca and Mg nutrition (Kinraide 1998; Kinraide et al. 2004; Silva et al. 2001). The importance of balanced nutrient uptake and transport for pea subjected to toxic metals such as Cd, Co and Hg was previously shown in our experiments with mutant SGECd^{t} (Tsyganov et al. 2007; Belimov et al. 2015a).

Conclusion

Using a representative range of pea genotypes, we revealed significant intraspecific variability in Al tolerance and Al content of plants subjected to Al stress. Moreover, for the first time we found relationships between Al tolerance and provenance or phenotypic traits of plants. The selected pea genotypes are useful model for elucidating the mechanisms underlying genotype-related Al tolerance and for breeding purposes. The absence of correlations between Al tolerance and Al content in plants or organic acid exudation suggests that low uptake, root-to-shoot transport and exclusion of Al from plant tissues and root exudation of organic acids do not play a crucial role in Al tolerance in pea. However, the role of the major organic acids found in pea root exudates (acetate, lactate, pyroglutamate, pyruvate) in response to toxic Al requires further study. The important mechanism of Al tolerance in pea found here is related to active exclusion of Al from the root zone, most probably due to increased solution pH and transformation of mobile Al into insoluble forms via precipitation with phosphates and/or other unknown compounds. The ability to counteract negative effects of Al on uptake and transport of nutrient elements from root to shoot, and thereby maintain nutrient uptake, is a second mechanism of Al tolerance in pea. Thus, genotype dependent and multi-factorial reactions including rhizosphere processes and internal mechanisms modulate Al tolerance in this plant species.

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