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Root exudation index: screening organic acid exudation and phosphorus acquisition efficiency in soybean genotypes

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Abstract. High-molecular-weight secretory proteins and low-molecular-weight exudates (carboxylates, phenols, free amino acids and sugars) released from roots of soybean (*Glycine max* (L.) Merr.) differentially influence genotypic phosphorus (P) acquisition efficiency (PAE). We hypothesised that genotypes with higher root exudation potential would exhibit enhanced P acquisition, and screened 116 diverse soybean genotypes by labelling shoots with $^{14}CO_2$. A root exudation index (REI) derived from total ^{14}C in the root exudate at sufficient (250 μM) and low (4 μM) P levels was used to classify genotypes for PAE. Genotypes with REI >2.25 exhibited significantly higher exudation at low than at sufficient P, which in turn increased PAE. Under low P availability, efficient genotypes exude a greater quantity of organic compounds into the rhizosphere. This increases P availability to meet the crop requirement, enabling the crop to produce consistent biomass and seed yield with reduced fertiliser addition. Such maintenance of growth and yield potential by mining the inherent soil P is a favourable trait in genotypes, reducing dependence on P fertilisers. Measuring REI at seedling stage to select P-efficient plants accelerates the screening process by accommodating large numbers of genotypes.

Additional keywords: ^{14}C labelling, cluster analysis, genotype × trait interaction, HPLC, hydroponic culture.

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Introduction

Soybean (*Glycine max* (L.) Merr.) is a legume crop that is valued for its high oil (18–23%) and protein (38–44%) contents (Orf 2010). It is cultivated in ~6% of the world's agricultural land (Goldsmith 2008), with a global acreage of 111 Mha, production of 276 Mt and productivity of 2484 kg ha^{-1} (FAO 2015). Soybean exhibits wide genotypic diversity with respect to morphological, physiological and biochemical traits. Germplasm collections are listed and maintained by major soybean-growing countries including USA, Brazil, Argentina, China and India (Orf 2010). Among several constraints affecting soybean production, its growth and yield performance under various biotic and abiotic stresses is of paramount importance. Breeding for efficient and functional traits in soybean cultivars adapted to low-input farming systems is a sustainable option in the face of rising environmental challenges (Scaboo *et al*. 2010). In this context, exploiting the available genetic diversity would enhance research efforts aimed at improving yield potential in soybean (reviewed by Ainsworth *et al*. 2012).

Phosphorus (P) is one of the essential macronutrients required for normal growth and development of soybean; however, P deficiency in the crop is widespread, with most of the world's arable soils exhibiting high P-retention potential, leading to low bioavailability for plant uptake (Kochian 2012). Fertiliser application to meet crop P requirements is therefore essential to achieve expected yields. Global demand for phosphatic fertilisers has been projected to increase up to 55–60 Mt by 2050 (Fess *et al*. 2011). Moreover, continued and increasing mining leads to depletion of P reserves, which are controlled by a few countries. This threatens to create a situation of peak P production (similar to peak oil) by 2033 (Cordell and White 2011). Hence, the need arises to develop P-efficient soybean genotypes that can produce higher yield per unit P applied.

Phosphorus deficiency reduces soybean productivity through direct and indirect effects on chlorophyll content (Fredeen *et al*. 1989; Lauer *et al*. 1989), photosynthesis (Qiu and Israel 1994), leaf area (Gan *et al*. 2002; Chaudhary *et al*. 2008), nodulation (Tsvetkova and Georgiev 2003) and biomass production (Vandamme *et al*. 2013). Higher biomass allocation to the roots is an adaptive strategy to low availability of P (Tang *et al*. 2009); in particular, roots with shallow architecture enable soybean to absorb more P from the cultivated soil layers (Zhao *et al*. 2004). Deficiency of P also induces root exudation of a variety of carbon-containing compounds including carboxylic acids (Hinsinger *et al*. 2003), acid phosphatases (Wang *et al*. 2009), phytases (Li *et al*. 1997) and amino acids, sugars and phenols (Carvalhais *et al*. 2011) that mobilise P from the bound pools. Studies have shown that the genotypic variation in soybean with respect to P acquisition efficiency (PAE) was mainly attributed to root exudation of organic compounds (Dong *et al*. 2004; Liao *et al*. 2006). Soybean genotypes with increased efflux of carboxylic acids and acid phosphatases may be more efficient in mining the inherent soil P than inefficient genotypes (Wang *et al*. 2010).

Screening of genotypes for high exudation may not be accurate in field-grown plants because of the risk of root damage while uprooting plants for exudate collection. Development of rhizotron facilities for the study of root traits and genotypic exudation potential is expensive, and such facilities are rarely accessible to researchers in developing countries. Collection of root exudates at the seedling stage from hydroponically grown plants is a feasible alternative, as reported in soybean (Liao *et al*. 2006), maize (Carvalhais *et al*. 2011) and green gram (*Vigna radiata*) (Pandey *et al*. 2013). Moreover, sampling at the seedling stage could also accelerate screening by accommodating large number of genotypes. To address the above-stated issues, experiments were conducted with the following objectives: (*i*) to screen and select soybean genotypes for total root carbon exudation by the technique of shoot labelling with $^{14}CO_2$ under low P availability, and (*ii*) to validate the performance of selected genotypes under contrasting soil P availability. With the hypothesis that efficient genotypes exhibiting high total carbon exudation (TCE) and PAE at seedling stage would perform better than inefficient genotypes in terms of growth and yield traits under low soil P availability, we attempted to explore the diversity among soybean germplasm available in India.

Materials and methods

Experiment 1: screening genotypes for TCE and PAE at seedling stage

A set of 116 diverse soybean genotypes procured from Division of Genetics, ICAR–IARI, New Delhi, and ICAR–Indian Institute of Soybean Research, Indore, was used in a screening experiment (see Supplementary materials, table 1S, available at the journal's website). Seeds were surface-sterilised with 0.1% (w/v) $HgCl_2$, wrapped in germination towels moistened with 1 mM $CaCl_2$ and incubated in the dark at room temperature to facilitate germination. Upon emergence of the cotyledonary leaf, seedlings were transferred to nutrient solution with one of two P levels: sufficient P (250 μM) and low P (4 μM). The cotyledonary leaves were removed on third day of transfer to nutrient solution to minimise genotypic variation due to seed P content. Seedlings were supported on a 5-cm-thick Styrofoam sheet at a spacing of 3 cm by 3 cm. Thirty-six seedlings were accommodated in individual containers. Three replications with four seedlings each ($n = 12$) were maintained for all treatment combinations. Styrofoam sheet was placed in a plastic container with 10 L basal nutrient solution. The solution was continuously aerated and renewed every 3 days. The composition of basal nutrient solution was as follows: $MgSO_4$, 2.0 mM; $Mg(NO_3)_2$, 3.0 mM; KNO_3, 2.5 mM; $CaCl_2$, 1.0 mM; $Ca(NO_3)_2.4H_2O$, 4.0 mM; $MnCl_2$, 1.5 μM; $ZnSO_4$, 4.6 μM; $CuSO_4$, 2.0 μM; $NiSO_4$, 1.0 μM; H_3BO_3, 10.0 μM; Na_2MoO_3, 0.2 μM; $CoSO_4$, 1.0 μM; EDTA + FeN_2NaO_8, 100.0 μM. Orthophosphoric acid (1.0 M) was used for creating low and sufficient P levels. The pH of the nutrient solution was maintained at 6.5 (by using either 1.0 N HCl or 1.0 N KOH). The experiment was conducted in greenhouse at the National Phytotron Facility, New Delhi, with day–night temperatures of 30°C–26°C, photoperiod of 12 h at a photon flux density of 850 μmol m^{-2} s^{-1} and relative humidity of 85%.

The technique of shoot labelling with $^{14}CO_2$ (Pandey *et al*. 2013; Singh *et al*. 2014) was employed to screen the soybean genotypes for TCE. Seedlings that were 20 days old with three fully expanded leaves were placed in 100-mL Erlenmeyer flasks containing 50 mL trap solution (0.5 mM $CaCl_2$, pH 4.5). The flasks were covered with black paper to prevent light from reaching the roots. Plants were kept in an airtight chamber and exposed to a $^{14}CO_2$ environment (specific activity of ^{14}C, 5 μCi $mmol^{-1}$) for 1 h to facilitate assimilation, after which plants were transferred back to their growth conditions as mentioned above. Root exudates were collected in scintillation vials at 24, 48 and 96 h after exposure of plants to $^{14}CO_2$ and evaporated to dryness. After addition of scintillation mixture (Cocktail O; SRL, Mumbai) to the pellets, ^{14}C counts were recorded in a liquid scintillation counter (Tricarb 3110-TR; Perkin Elmer, Waltham, MA, USA). Roots were washed in deionised water and blotted dry and root fresh weight was determined. TCE was expressed as dpm g^{-1} root fresh weight. Root exudation index (REI) of individual genotype was calculated at 24, 48 and 96 h after labelling according to Eqn 1:

$$\mathrm{REI} = \frac{1 - \dfrac{\text{TCE of genotype at low P}}{\text{TCE of genotype at sufficient P}}}{1 - \dfrac{\text{Mean TCE at low P}}{\text{Mean TCE at sufficient P}}} \qquad (1)$$

Root and shoot tissue was kept in a hot-air oven at 60 ± 5°C for drying until constant weight was reached to record total biomass. Tissue P concentration was determined (Murphy and Riley 1962) after digestion of dried tissue with a di-acid mixture (HNO_3 : $HClO_3$ 9 : 4) and expressed as μg g^{-1} dry weight. Uptake of P was calculated by multiplying P concentration by dry weight of root or shoot, and was expressed as μg P $plant^{-1}$. PAE was calculated as the ratio shoot P content at low P : shoot P content at sufficient P and expressed as percentage (López-Arrendondo *et al*. 2014). Six genotypes representing four clusters based on REI derived at 24, 48 and 96 h after ^{14}C-labelling and PAE were selected for evaluation of growth and yield performance under contrasting soil P availability.

Experiment 2: quantifying root-exuded compounds in 20 selected genotypes

Root-exuded compounds (organic acids, proteins, free amino acids, phenols and sugars) were quantified in 20 genotypes selected from Expt 1. Exudates were collected in 20-day-old plants according to Dong *et al*. (2004). The exudate solution was filtered through Whatman Grade 1 filter paper and used for spectrophotometric estimation of phenols (Bray and Thorpe 1954), protein (Lowry *et al*. 1951), free amino acids (Moore and Stein 1948) and sugars (Hedge and Hofreiter 1962).

For analysis of organic acids, 10 mL root exudate was passed through a column (140 mm by 16 mm) filled with cation-exchange resin (Amberlite IR-120 (H^+ form); Sigma-Aldrich, St. Louis, MO, USA). The eluate was then passed through a second column filled with anion-exchange resin (DEAE-cellulose; Sigma-Aldrich). Organic anions retained on the resin were eluted with 2 mL of 1.0 M HCl (HPLC grade; Sigma-Aldrich). The eluate was evaporated to dryness at 45°C and resuspended in 500 μL of 0.005 M H_2SO_4 (HPLC grade;

Sigma-Aldrich). Samples were filtered through 0.4-μm filters before analysis by HPLC (1200 Infinity; Agilent Technologies, Santa Clara, CA, USA). Column temperature of the stationary phase (Hi-Plex H; Agilent Technologies) was 70°C. The mobile phase was 0.005 M H_2SO_4 at a flow rate of 0.6 mL min^{-1}. Acids were detected by refractive index detector with optical temperature of 55°C. The run time for individual samples was 25 min. For peak identification and quantification, calibration was done by using serial dilutions of standards: oxalate, citrate, malate, pyruvate, succinate, lactate and fumarate. Total organic acid exudation was calculated as the sum of individual acids. All root-exuded compounds were expressed in μmol g^{-1} root fresh weight.

Soil P mobilisation was assessed by comparing the molybdate-reactive P extracted by 0.5 mM acid (oxalic, malic, citric, pyruvic, lactic, succinic and fumaric; Sigma-Aldrich) to that of 0.5 mM calcium chloride (Drouillon and Merckx 2003). Phosphorus-solubilisation capacity of the root exudate collected from a representative soybean genotype as described above was also determined. Reagents used for soil extraction were adjusted to pH (7.8) of the soil.

Experiment 3: evaluating growth and yield performance of contrasting soybean genotypes under different soil P availability

Six contrasting soybean genotypes were grown for two consecutive years (27 July–12 November 2012 and 22 July–08 November 2013) under sufficient P (27.0 mg P kg^{-1} soil) and low P (2.0 mg P kg^{-1} soil) availability. The weather parameters recorded during crop growing seasons are presented in Supplementary Material, Fig. 1S. Seeds soaked overnight in deionised water were inoculated with *Bradyrhizobium japonicum* and sown in earthen pots (30 cm diameter) containing 12 kg sandy loam soil. The soil pH (soil : water 1 : 5) was 8.2 and electrical conductivity 0.174 mS m^{-1}. Available P in soil, extracted by Olsen method (Olsen *et al*. 1954), was 2.0 mg P kg^{-1} soil. Single superphosphate at 25.0 mg P_2O_5 kg^{-1} soil was applied to create the sufficient P level, whereas the low P levels received no superphosphate. Recommended doses of nitrogen (N) and potassium (K) for soybean were added to the soil through urea and muriate of potash, respectively. Four seeds were sown in each pot, and upon emergence of first trifoliate leaf, thinning was done to maintain two healthy and uniform plants per pot. Four replications with six pots each were maintained for all treatments. To minimise heterogeneity, pots were repositioned throughout the experimental. Morpho-physiological traits and tissue P status were recorded during anthesis (reproductive stage R6: 40–45 days after sowing).

Plants were uprooted and separated into leaf, stem and root to determine total biomass (g $plant^{-1}$) and root : shoot ratio. Leaf area was measured with a leaf area meter (LI-3000; LI-COR, Lincoln, NE, USA) and expressed as cm^2 $plant^{-1}$. Concentration of P (μg g^{-1} dry weight) in leaf, stem and root, and total P uptake (mg P $plant^{-1}$), were estimated as described previously. Seed yield (g $plant^{-1}$) and its attributes, i.e. number of pods per plant, number of seeds per pod and test weight (g 100 $seed^{-1}$), were recorded at harvest.

Statistical analyses

All experimental designs were completely randomised with two factors: P level and genotype. Procedures for descriptive statistics, two- or one-way analysis of variance (ANOVA) and hierarchical cluster analysis were carried out in the statistical software R version 3.1.2 (R Foundation for Statistical Computing, Vienna). Genotype and genotype × trait interaction analysis was executed in R using the package GGE Biplot GUI version 1.0-8. Clustering based on Ward's method was carried out on the squared Euclidean distance matrix of genotypic REI and PAE values. Duncan's mean range test at $P = 0.05$ was performed on the integrated mean value of traits recorded during 2012 and 2013. Graphs and figures were plotted with GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA, USA) and Microsoft Excel 2010 (Microsoft Corp., Redmond, CA, USA).

Results

Results revealed significantly ($P < 0.001$) higher TCE in soybean under low P than under sufficient P at 24, 48 and 96 h after $^{14}CO_2$ exposure (Table 1). Total biomass, shoot P concentration, root P concentration and total P uptake decreased significantly ($P < 0.001$) at low P. Genotype and genotype × trait analysis revealed that 77.2% of the variability was explained by the first two principal components (PCs) at sufficient P and 72.9% at low P (Fig. 1). Within the first PC, TCE at three time intervals each contributed to 22% of the genotypic variability at both sufficient and low P. Within the second PC, total biomass

Table 1. Mean values of recorded traits in 116 soybean genotypes grown with phosphorus (P) at sufficient (250 μM) and low (4 μM) levels, percentage change at low P, and significance of *F*-values derived from analysis of variance for recorded traits with two factors, P and genotype (G), as treatment effects

TCE, Total carbon exudation; RFW, root fresh weight; DW, dry weight. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Recorded trait	Mean		Change at	*F*-value		
	Sufficient P	Low P	low P (%)	P	G	P × G
TCE (dpm g^{-1} RFW) at:						
24 h	20.45	25.25	+23.5	77.7***	21.0***	6.0***
48 h	20.68	24.79	+19.9	50.2***	17.3***	4.3***
96 h	20.52	24.76	+20.7	46.1***	15.2***	4.3***
Total biomass (mg $plant^{-1}$)	0.207	0.185	–10.6	50.9***	26.5***	6.6***
Shoot P concentration (μg g^{-1} DW)	221	130	–41.2	1480.9***	84.7***	16.2***
Root P concentration (μg g^{-1} DW)	184	110	–40.2	504.3***	8.5***	3.3***
Total P uptake (μg P $plant^{-1}$)	360	239	–33.7	1574.7***	75.0***	14.2***

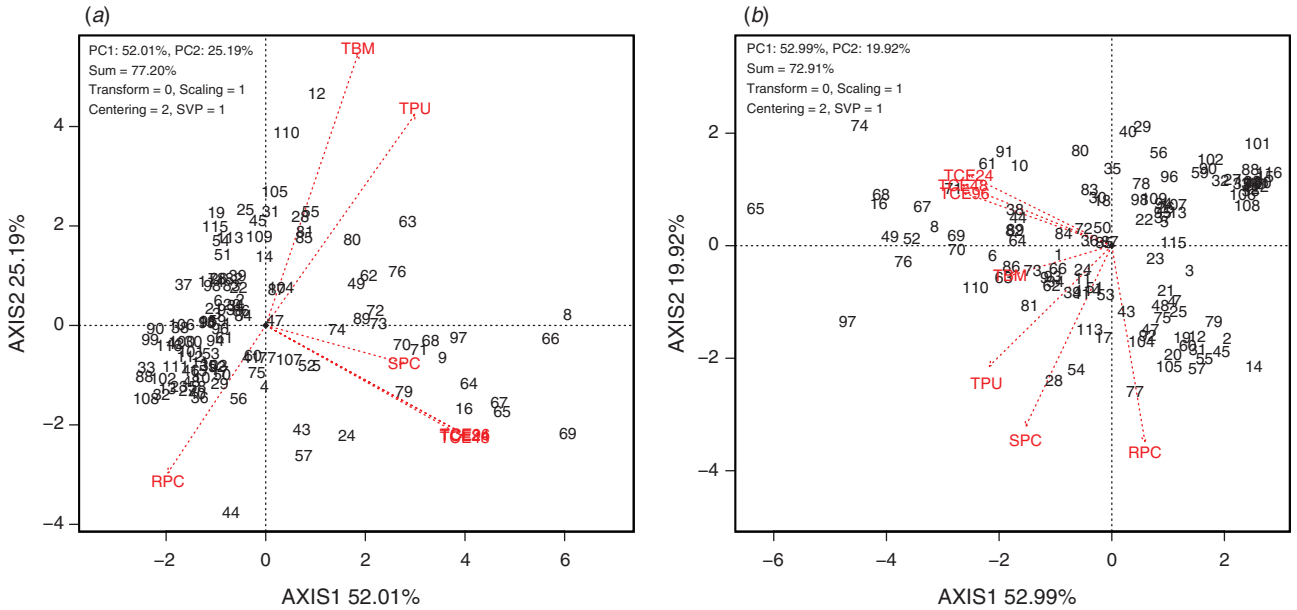

Fig. 1. Genotype and genotype × trait interaction biplot at (*a*) sufficient (250 µM) and (*b*) low (4 µM) phosphorus (P). The data were not transformed (Transform = 0), scaled by standard deviation (Scaling = 0) and genotype-centred (Centering = 2). The biplot was based on trait-focused singular value portioning (SVP = 1) and therefore is appropriate for visualising the relationships among traits. It explained 77.2% of the total variation at sufficient P and 72.9% at low P. Values correspond to genotype ID in Supplementary materials, Table 1S. TCE24, TCE48, TCE96: Total carbon (^{14}C) exudation at 24, 48 and 96 h after labelling; TBM, total biomass; SPC, shoot P concentration; RPC, root P concentration; TPU, total P uptake.

contributed to 41.4% of the variation at sufficient P, whereas root and shoot P concentration contributed to 39.2% and 33.4% of the total genotypic variability, respectively, at low P. Total P uptake also contributed significantly to the genotypic variation at sufficient P (PC1 12.5%, PC2 25.1%) and low P (PC1 15.3%, PC2 14.8%).

Considering the significant variation for TCE and PAE among soybean genotypes, clustering based on REI at 24, 48 and 96 h after $^{14}CO_2$ exposure and PAE revealed four distinct groups: efficient (average REI 3.43, average PAE 145.9%); moderately efficient (average REI 4.08, average PAE 102.3%); moderately inefficient (average REI 2.25, average PAE 71.3%); inefficient (average REI –0.78, average PAE 45.2%) (Fig. 2). Five genotypes belonged to the efficient cluster, 23 were classified as moderately efficient, 46 as moderately inefficient, and 42 as inefficient. In general, REI >2.25 indicated genotypes with significantly higher total carbon exudation at low P than at sufficient P, which in turn resulted in high PAE. Significant variation was observed among the groups with respect to TCE, total biomass, shoot and root P concentration, and total P uptake (Fig. 3). On an average, P deficiency significantly increased the TCE at 24 h (33%), 48 h (70%) and 96 h (55%) in the efficient cluster, whereas inefficient genotypes showed significant reduction in TCE due to P deficiency at all three time intervals (Fig. 3*a–c*). Total biomass decreased significantly at low P in the inefficient cluster (36%), whereas average biomass per plant increased in efficient (73%) and moderately efficient (24%) groups at low P compared with sufficient P (Fig. 3*d*). Shoot P concentration decreased significantly at low P compared with sufficient P in all groups, with maximum reduction in shoot P concentration occurring in the inefficient cluster (28%) (Fig. 3*e*). Deficiency of P significantly influenced root P concentration, with maximum reduction (60%) observed in the efficient cluster, whereas inefficient cluster exhibited the smallest decrease (31%) in root P concentration at low P (Fig. 3*f*). Deficiency of P significantly reduced total P uptake in all groups except the efficient cluster, which exhibited a 37% increase in P uptake per plant at low P over sufficient P (Fig. 3*g*).

Averaged over genotypes, organic acids, followed by proteins, contributed maximum quantity to the total root-exuded compounds at sufficient and low P (Fig. 4). Phenols and sugars constituted a minor proportion of the total root exudates. Significant genotypic variation was evident in the exudation rates of proteins, phenols, sugars and free amino acids (see Supplementary materials, Table 2S). Protein exudation in soybean varied significantly ($P < 0.001$) among genotypes, with EC-232019, NRC-7, EC-325117, G-2344 and EC-592195 exhibiting highest efflux. Lowest rates were observed in CSB-0804, EC-528639 and EC-113396. Low-P-induced protein exudation was exceptionally higher in G-2344 (16-fold), followed by NRC-7 (4-fold) and EC-232019 (2.5-fold). Averaged over P levels, exudation of phenols was significantly ($P < 0.001$) higher in EC-592195, followed by EC-325117, and low-P-induced efflux increased by 2-fold in EC-232019 and EC-592195. In the case of sugars, HIMSO-1521 showed the highest rate of exudation when averaged over P levels, followed by EC-528622 and EC-325117. Genotypes with lowest sugar efflux were MAUS-61, EC-572050 and JS-93-05. Exudation of free amino acids was significantly higher in G-2344 and CO-1 regardless of P levels, whereas lowest efflux was recorded in EC-592195 and HIMSO-1521.

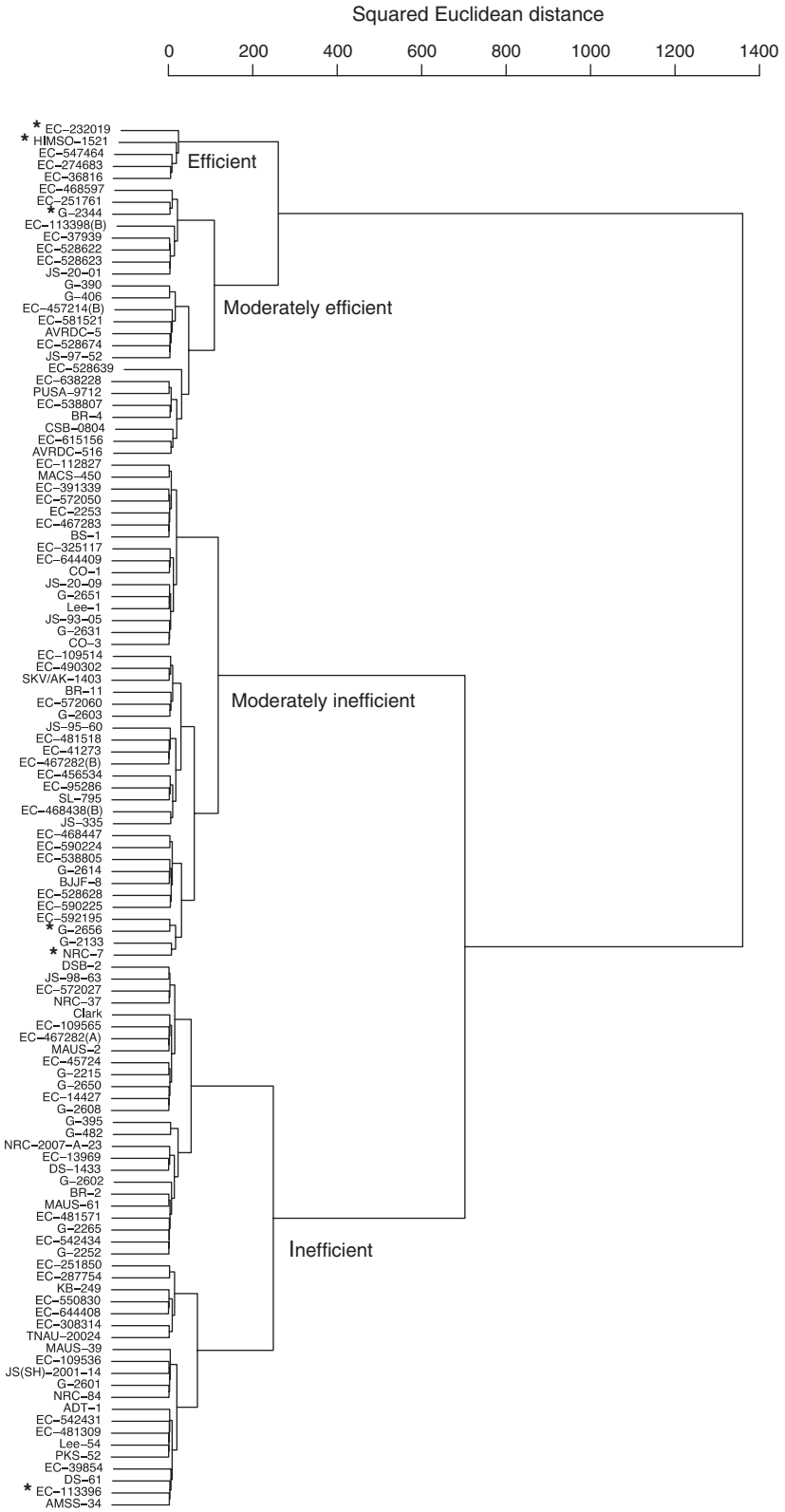

Fig. 2. Clustering of 116 soybean genotypes by Ward's method, using the squared Euclidean distance matrix of genotypic root exudation index at 24, 48 and 96 h after ^{14}C-labelling and values of phosphorus acquisition efficiency. Entries with asterisk represent the six genotypes selected to evaluate growth and yield performance under contrasting soil P availability.

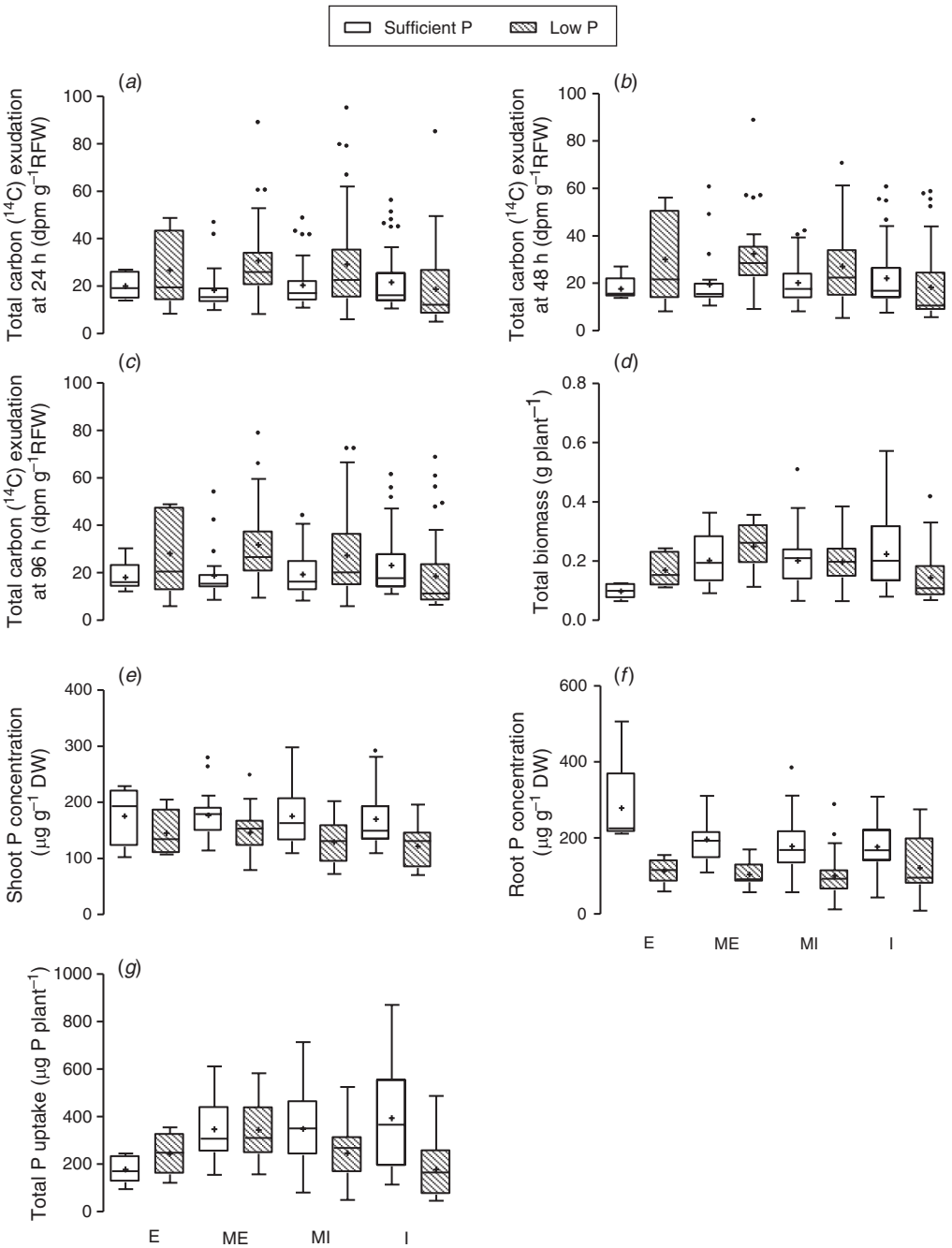

Fig. 3. Variation in 116 soybean genotypes with respect to total carbon (^{14}C) exudation at (*a*) 24, (*b*) 48 and (*c*) 96 h after labelling; (*d*) total biomass; (*e*) shoot phosphorus (P) concentration; (*f*) root P concentration; and (*g*) total P uptake at sufficient (250 μM) and low (4 μM) P. Horizontal line inside boxes, median; +, mean; box hinges, first and third quartiles; whiskers, full range of the data. Solid circles on either side of the whiskers are outliers according to Tukey's test. Genotypes were grouped on the basis of root exudation index and P acquisition efficiency as: E, efficient; ME, moderately efficient; MI, moderately inefficient; I, inefficient. RFW, Root fresh weight; DW, dry weight.

Significant ($P < 0.001$) influence of genotype and P × genotype interaction was observed for organic acid exudation (Table 2). Averaged over P levels, HIMSO-1521, EC-232019, EC-456534, EC-528639 and EC-113396 showed the highest rates of total organic acid efflux. Among high exuders, low-P-induced exudation increased significantly in EC-528639 (97%) and EC-232019 (58%). Eleven genotypes exuded organic acids at levels lower than the mean value of genotype × P interaction, among which NRC-7, CO-1 and EC-592195 produced <20 μmol total organic acid g^{-1} root fresh weight. Irrespective of P level or genotype, organic acids in the root exudate comprised: fumarate > oxalate > lactate > pyruvate >

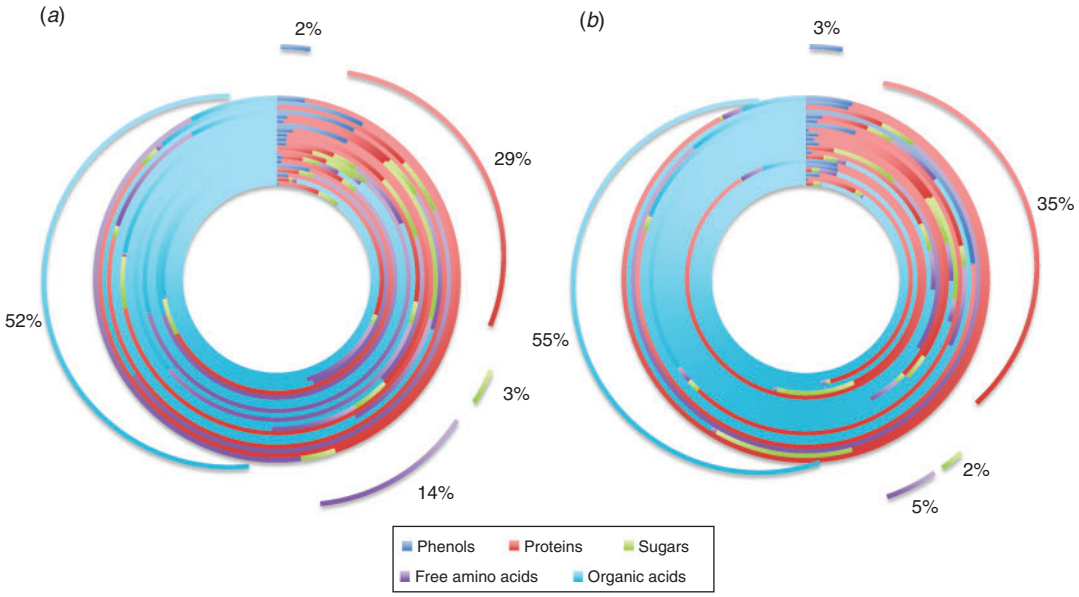

Fig. 4. Doughnut plot showing variation in quantity and type of root-exuded compounds in 20 selected soybean genotypes grown at (*a*) sufficient (250 μM) and (*b*) low (4 μM) phosphorus. Each ring represents a single genotype (data presented in Supplementary materials, table 2S). Outermost 'detached' ring is the mean of 20 genotypes. Data labels denote percentage contribution of individual compound to the total root exudate.

succinate > malate. In high-exuding genotypes (EC-232019 and EC-528639), efflux of oxalate, citrate, fumarate, succinate and lactate was induced under low P. Exudation of succinate and lactate was induced by low P in G-2344, whereas other acids showed significant reduction at low P relative to sufficient P in all genotypes. Soil P mobilisation determined from the molybdate-reactive P extracted by root exudate was >40 mg P kg^{-1} soil, whereas the organic acids such as citrate, oxalate, fumarate and succinate mobilised ~30 mg P kg^{-1} soil (Fig. 5). Malic, pyruvic and lactic acids mobilised >20 mg P kg^{-1} soil, which was significantly higher than that extracted by calcium chloride (10 mg P kg^{-1} soil).

Based on important traits such as TCE, total P uptake and total biomass contributing to PAE at seedling stage, six soybean genotypes—HIMSO-1521 and EC-232019 (efficient), G-2344 (moderately efficient), NRC-7 and G-2656 (moderately inefficient) and EC-113396 (inefficient)—were selected for evaluation of their performance under sufficient and low soil P availability.

Soil P availability and genotype significantly influenced leaf area, leaf area ratio and total biomass of soybean genotypes in both growing seasons (Table 3). The P × genotype interaction was significant for all four parameters except root : shoot ratio during 2012 and leaf area ratio during 2013. Averaged over the years, the smallest reduction in leaf area due to low soil P availability was observed in the efficient genotype EC-232019 (24%) (Fig. 6*a*). However, leaf area ratio increased by 36% in the inefficient genotype and >50% in the moderately inefficient genotypes at low P relative to sufficient P (Fig. 6*b*). A decrease in total biomass of ~20% at low P relative to sufficient P was observed in the efficient and moderately efficient genotypes, whereas inefficient and moderately inefficient genotypes showed >50% reduction (Fig. 6*c*). Regardless of P levels, highest root : shoot ratios were exhibited by efficient (EC-232019, 0.38) and moderately efficient (G-2344, 0.29) genotypes (Fig. 6*d*).

The tissue P concentration and P uptake per plant in soybean genotypes showed significant ($P < 0.001$) reduction due to low soil P availability in both years (Table 3). With the exceptions of stem and root P concentrations, all other traits exhibited significant variation due to genotype and P × genotype interaction. Tissue P concentrations reduced drastically (by >40%) with low soil P availability in all genotypes, except for leaf P concentration in the efficient genotype EC-232019 (19%) (Fig. 7*a–c*). Averaged over the years, the smallest reduction in total P uptake per plant at low soil P compared with sufficient soil P availability was observed in the efficient genotype EC-232019 (56%), whereas EC-113396, the inefficient genotype, recorded the maximum decrease (82%) (Fig. 7*d*).

Seed yield and number of pods per plant, with wide genotypic variation, were also significantly reduced by P deficiency during 2012 and 2013 (Table 3). Averaged over the years, low soil P triggered maximum decrease in number of pods per plant of the moderately inefficient genotype G-2656 (39%), whereas no significant reduction was found in the efficient genotype HIMSO-1521 (Table 4). Reduction in seed yield at low soil P relative to sufficient soil P was 6–23% in the efficient group, whereas the inefficient genotype showed reduction of up to 46%. Test weight showed significant ($P < 0.001$) genotypic variation during 2012 and 2013 irrespective of soil P availability (Table 3), with the highest value in the inefficient genotype (Table 4). Averaged over P levels, highest number of pods per plant and highest seed yield per plant were noted in the moderately inefficient group, followed by the efficient genotypes.

Table 2. Concentrations of root-exuded organic acids (μmol g^{-1} root fresh weight) in 20 selected soybean genotypes grown at two phosphorus (P) levels, sufficient (SP, 250 μM) and low (LP, 4 μM)

Data are mean ± s.e. (n = 12); n.d., peaks not detectable. Treatment effects: P and genotype (G). *P < 0.05; **P < 0.01; ***P < 0.001

Genotype	Oxalate		Citrate		Pyruvate		Malate		Succinate		Lactate		Fumarate	
	SP	LP	SP	LP	SP	LP	SP	LP	SP	LP	SP	LP	SP	LP
EC-232019	n.d.	15.1 ± 0.8	n.d.	1.8 ± 0.1	49.1 ± 2.6	0.6 ± 0.0	2.2 ± 0.1	1.6 ± 0.1	2.9 ± 0.2	5.9 ± 0.3	2.5 ± 0.1	3.8 ± 0.2	116.1 ± 6.0	245.8 ± 12.8
EC-528622	n.d.	4.7 ± 0.2	9.5 ± 0.5	0.6 ± 0.0	0.8 ± 0.0	1.3 ± 0.1	0.3 ± 0.0	1.6 ± 0.1	20.7 ± 1.1	5.0 ± 0.3	2.4 ± 0.1	2.5 ± 0.1	7.7 ± 0.4	6.7 ± 0.3
EC-528639	10.5 ± 0.5	2.5 ± 0.1	n.d.	0.9 ± 0.0	0.7 ± 0.0	n.d.	0.4 ± 0.0	0.4 ± 0.0	n.d.	11.1 ± 0.6	7.2 ± 0.4	3.8 ± 0.2	78.9 ± 4.1	173.9 ± 9.0
EC-592195	4.0 ± 0.2	1.7 ± 0.1	0.3 ± 0.0	1.3 ± 0.1	n.d.	n.d.	0.1 ± 0.0	0.2 ± 0.0	2.9 ± 0.2	1.0 ± 0.1	0.5 ± 0.0	0.6 ± 0.0	10.2 ± 0.5	16.3 ± 0.8
G-2344	22.5 ± 1.2	1.1 ± 0.1	1.3 ± 0.1	0.4 ± 0.0	2.4 ± 0.1	0.6 ± 0.0	1.3 ± 0.1	0.3 ± 0.0	5.0 ± 0.3	5.8 ± 0.3	4.1 ± 0.2	5.7 ± 0.3	7.3 ± 0.4	n.d.
CSB-0804	5.4 ± 0.3	3.3 ± 0.2	0.5 ± 0.0	0.2 ± 0.0	1.8 ± 0.1	1.2 ± 0.1	2.9 ± 0.2	0.1 ± 0.0	1.2 ± 0.1	0.8 ± 0.0	1.0 ± 0.1	0.6 ± 0.0	12.9 ± 0.7	33.7 ± 1.7
EC-456534	37.4 ± 1.9	24.9 ± 1.3	3.8 ± 0.2	5.4 ± 0.3	2.4 ± 0.1	44.2 ± 2.3	23.3 ± 1.2	8.6 ± 0.4	6.1 ± 0.3	6.9 ± 0.4	7.1 ± 0.4	93.1 ± 4.8	120.2 ± 6.2	31.1 ± 1.6
CO-1	3.3 ± 0.2	1.6 ± 0.1	0.9 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	n.d.	0.1 ± 0.0	n.d.	1.6 ± 0.1	n.d.	5.5 ± 0.3	1.0 ± 0.1	3.0 ± 0.2	6.9 ± 0.4
HIMSO-1521	173.1 ± 9.0	108.8 ± 5.7	27.4 ± 1.4	37.6 ± 2.0	6.6 ± 0.3	16.3 ± 0.8	6.4 ± 0.3	3.3 ± 0.2	22.6 ± 1.2	33.2 ± 1.7	10.5 ± 0.5	33.1 ± 1.7	146.2 ± 7.6	14.5 ± 0.8
JS-93–05	2.2 ± 0.1	0.8 ± 0.0	0.6 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	n.d.	0.5 ± 0.0	0.3 ± 0.0	0.8 ± 0.0	1.2 ± 0.1	0.4 ± 0.0	1.2 ± 0.1	33.8 ± 1.8	26.6 ± 1.4
EC-109514	7.9 ± 0.4	14.5 ± 0.8	0.2 ± 0.0	2.4 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	n.d.	1.2 ± 0.1	12.0 ± 0.6	1.4 ± 0.1	9.3 ± 0.5	34.4 ± 1.8
EC-325117	4.0 ± 0.2	2.0 ± 0.1	0.1 ± 0.0	1.4 ± 0.1	1.6 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	0.5 ± 0.0	1.1 ± 0.1	0.5 ± 0.0	0.7 ± 0.0	24.9 ± 1.3	19.0 ± 1.0
G-2656	1.4 ± 0.1	12.2 ± 0.6	0.8 ± 0.0	2.8 ± 0.1	2.3 ± 0.1	0.2 ± 0.0	2.2 ± 0.1	0.2 ± 0.0	1.0 ± 0.0	10.4 ± 0.5	1.8 ± 0.1	4.0 ± 0.2	16.5 ± 0.9	28.1 ± 1.5
NRC-7	1.7 ± 0.1	6.1 ± 0.3	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.0	1.2 ± 0.1	0.1 ± 0.0	1.1 ± 0.1	0.4 ± 0.0	0.8 ± 0.0	2.7 ± 0.1	0.8 ± 0.0	3.4 ± 0.2	3.5 ± 0.2
EC-113396	n.d.	3.8 ± 0.2	30.5 ± 1.6	0.5 ± 0.0	1.0 ± 0.1	n.d.	3.9 ± 0.2	0.5 ± 0.0	17.6 ± 0.9	2.6 ± 0.1	1.7 ± 0.1	1.1 ± 0.1	116.6 ± 6.1	102.3 ± 5.3
EC-2253	9.9 ± 0.5	0.7 ± 0.0	1.0 ± 0.1	1.2 ± 0.1	0.6 ± 0.0	1.1 ± 0.1	1.1 ± 0.1	0.5 ± 0.0	9.8 ± 0.5	0.9 ± 0.0	22.5 ± 1.2	0.9 ± 0.0	6.4 ± 0.3	49.7 ± 2.6
EC-467282(B)	1.4 ± 0.1	3.5 ± 0.2	0.4 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.9 ± 0.0	0.1 ± 0.0	1.0 ± 0.1	1.1 ± 0.1	0.8 ± 0.0	1.6 ± 0.1	2.0 ± 0.1	20.1 ± 1.0	10.2 ± 0.5
EC-572050	1.0 ± 0.0	3.0 ± 0.2	0.7 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	1.0 ± 0.1	0.4 ± 0.0	0.1 ± 0.0	1.7 ± 0.1	1.9 ± 0.1	3.1 ± 0.2	1.4 ± 0.1	14.6 ± 0.8	28.1 ± 1.5
MAUS-61	22.4 ± 1.2	6.6 ± 0.3	6.9 ± 0.4	0.6 ± 0.0	0.3 ± 0.0	0.9 ± 0.0	n.d.	0.6 ± 0.0	1.2 ± 0.1	0.7 ± 0.0	2.3 ± 0.1	2.3 ± 0.1	11.5 ± 0.6	31.2 ± 1.6
TNAU-20024	1.5 ± 0.1	10.2 ± 0.5	0.2 ± 0.0	0.5 ± 0.0	1.2 ± 0.1	0.3 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	1.6 ± 0.1	3.9 ± 0.2	0.4 ± 0.0	4.3 ± 0.2	19.5 ± 1.0	12.1 ± 0.6
Mean	18.2 ± 8.9	11.4 ± 5.2	4.7 ± 2.0	3.0 ± 1.8	3.9 ± 2.4	4.7 ± 2.5	2.4 ± 1.2	1.1 ± 0.4	5.5 ± 1.6	5.0 ± 1.7	4.5 ± 1.2	8.2 ± 4.6	39.0 ± 10.3	46.0 ± 13.7
F-value														
P	1104.8***		1495.9***		11.6**		6663.3***		37.9***		3830.2***		366.9***	
G	12347.2***		10561.0***		7009.9***		10452.2***		8331.9***		7202.7***		7559.9***	
P × G	918.2***		2646.9***		7050.1***		2483.9***		2419.3***		6034.5***		2352.7***	

Discussion

Genotype classification and selection based on REI and PAE at seedling stage

Phosphorus-efficient crop species or genotypes exude carbon-containing compounds including carboxylic acids and phenols under low P availability, and these compounds in turn enhance P mobilisation from sparingly soluble sources (Hu *et al*. 2005; Chen *et al*. 2013). Efflux of carbon compounds in legumes under P deficiency is positively correlated with P acquisition (Singh and Pandey 2003). Our results agree with previous reports of the contribution of biomass, tissue P concentration and P uptake to overall P efficiency in wheat (*Triticum aestivum*) (Valizadeh *et al*. 2002), rice (*Oryza sativa*) (Wissuwa and Ae 2001) and *Brassica oleracea* (Hammond *et al*. 2009). Significant genotypic variation for P acquisition was observed among cereals grown with nutrient solutions containing varying levels of P (Osborne and Rengel 2002). Although variation in P utilisation and acquisition compensated each other, soybean was efficient in acquiring more P per unit of carbon invested (Fernández *et al*. 2009). Our results conform to responses of barley (*Hordeum vulgare*) cultivars grown in nutrient solution that differed mainly in exudation of organic acids, the P-efficient cultivar releasing 3-fold higher citric acid and 2-fold higher acetic acid than the P-inefficient cultivar (Gahoonia *et al*. 2000). We did not analyse the different soil P fractions during crop growth; however, Gahoonia *et al*. (2000) reported that a P-efficient barley cultivar absorbed twice as much P from the strongly adsorbed soil P fraction as a P-inefficient cultivar, and this was primarily attributed to enhanced root exudation. Similarly, P-efficient maize (*Zea mays*) lines exhibited higher shoot P content and root growth than inefficient ones (de Sousa *et al*. 2012). Improved adaptability of the efficient genotypes to low P availability in terms of maintaining higher biomass accumulation may be attributed primarily to enhanced P acquisition (Hu *et al*. 2010). Root traits shown by efficient soybean genotypes in response to P availability correspond with reports that P-efficient *Brassica* cultivars have higher root : shoot ratio (Akhtar *et al*. 2008). Greater biomass allocation to the roots governed by net photosynthesis and leaf P status is one of the adaptive responses to P starvation to increase soil exploration for greater P acquisition (Cheng *et al*. 2014).

The present study showed that, among the root exudates of soybean, the proportion of organic acids was highest, followed by proteins and amino acids, with minor quantities of phenols and sugars. Comparable results were observed in maize where P deficiency induced exudation of organic acids, amino acids and carbohydrates (Carvalhais *et al*. 2011). Phosphatases,

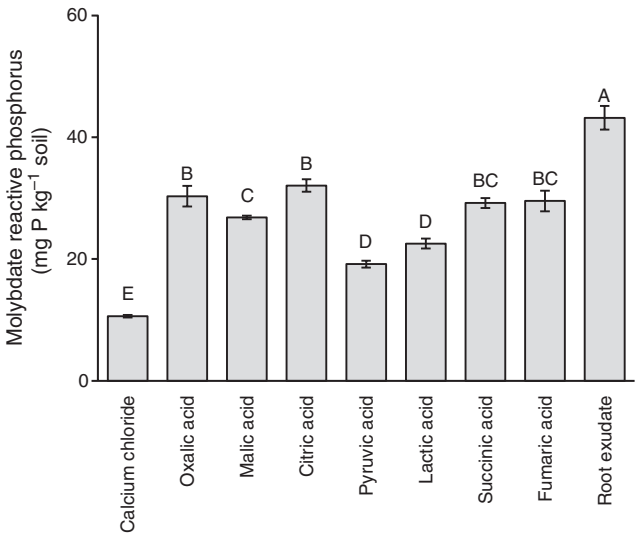

Fig. 5. Variation in soil phosphorus mobilisation by organic acids and root exudate. Data correspond to mean ± s.e. ($n = 3$). Means with the same letter are not significantly different at $P = 0.05$ according to least significant difference test.

Table 3. Mean values of recorded traits in six soybean contrasting genotypes grown with phosphorus (P) availability at sufficient (SP, 27 mg P kg^{-1} soil) and low (LP, 2 mg P kg^{-1} soil) levels, percentage change at low P, and significance of *F*-values derived from analysis of variance for recorded traits with two factors, P and genotype (G), as treatment effects

Genotypes were selected from contrasting clusters based on root exudation index and P acquisition efficiency. DW, Dry weight. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Recorded trait	2012						2013					
	Mean		Change at	*F*-value			Mean		Change at	*F*-value		
	SP	LP	LP (%)	P	G	P × G	SP	LP	LP (%)	P	G	P × G
Leaf area (cm^2 plant^{-1})	1017	613	–39.8	420.5***	231.5***	58.6***	1791	1073	–40.1	72.8***	18.9**	8.8*
Leaf area ratio	237	212	–10.6	10.9*	19.9**	21.5**	297	457	+53.8	22.1**	2.1	3.2
Total biomass (g plant^{-1})	16.49	11.07	–32.9	124.2***	41.4***	30.0**	15.24	6.77	–55.5	776.3***	66.1***	78.9***
Root : shoot ratio	0.321	0.370	+15.4	5.9	10.2**	1.8	0.203	0.217	+6.7	1.3	8.8*	12.0**
Leaf P conc. (μg g^{-1} DW)	115	62	–46.2	429.1***	31.2**	6.1*	63	32	–49.9	172.3***	11.3**	15.4**
Stem P conc. (μg g^{-1} DW)	80	41	–48.6	150.2***	2.7	0.4	57	21	–62.6	487.1***	23.6**	31.6**
Root P conc. (μg g^{-1} DW)	64	39	–40.1	253.9***	1.8	2.3	60	22	–63.9	289.1***	23.7**	34.9**
Total P uptake (mg P plant^{-1})	7.22	2.62	–63.7	2674.8***	151.3***	121.2***	9.17	1.72	–81.2	575.8***	16.8**	21.6**
No. of pods per plant	40	28	–29.6	95.6***	345.3***	9.2*	60	47	–21.9	12.2*	16.7**	3.2
No. of seeds per pod	2.4	2.2	–4.8	1.2	2.9	2.5	1.7	1.5	–8.5	5.2	7.2*	0.7
Test weight (g 100 seeds^{-1})	5.92	5.69	–3.8	0.6	49.9***	0.7	5.94	5.42	–8.8	10.0*	70.8***	2.2
Seed yield (g plant^{-1})	5.35	3.66	–31.6	28.9***	18.4***	1.2	5.98	3.92	–34.5	35.5***	28.3***	3.2**

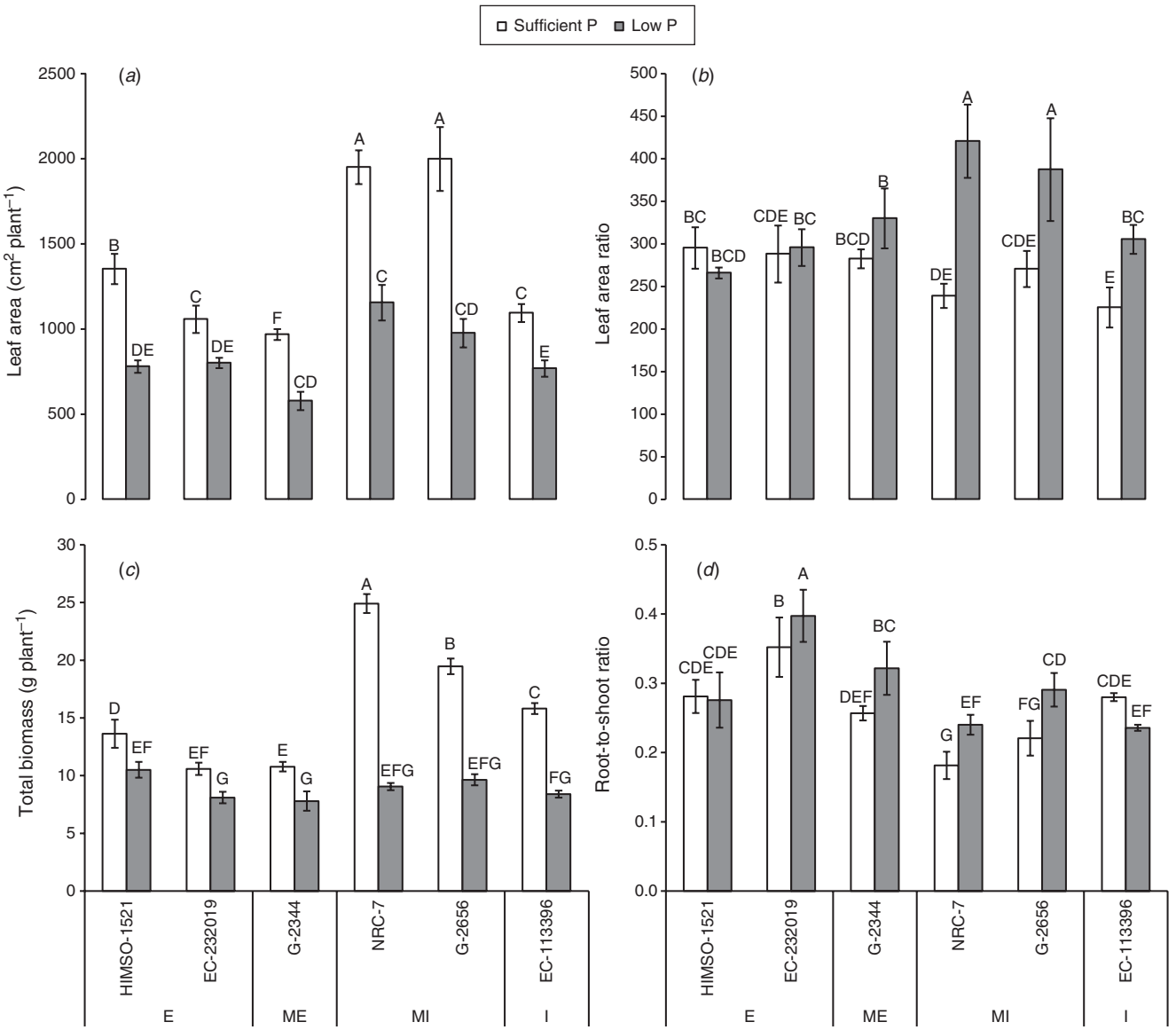

Fig. 6. Influence of phosphorus (P) availability (sufficient, 27 mg P kg^{-1} soil; low, 2 mg P kg^{-1} soil) on (*a*) leaf area, (*b*) leaf area ratio, (*c*) total biomass, and (*d*) root : shoot ratio in six soybean genotypes. Data correspond to mean ± s.e. of 2 years. Means with the same letter are not significantly different at $P = 0.05$ according to Duncan's mean range test. Genotypes were grouped on the basis of root exudation index and P acquisition efficiency as: E, efficient, ME, moderately efficient; MI, moderately inefficient; I, inefficient.

phytases and organic acids are mostly involved in increasing P mobilisation from both organic and inorganic sources (Hinsinger *et al*. 2003). A few studies suggest a positive influence of phenols, amino acids and sugars on P mobilisation (Juszczuk *et al*. 2004; Guppy *et al*. 2005). Piscidic acid released from pigeon pea (*Cajanus cajan*) has a direct role in solubilising P (Ishikawa *et al*. 2002). Root-exuded compounds such as phenols, amino acids and sugars may serve as signalling molecules for arbuscular mycorrhizal fungi (AMF) to colonise the roots (Dakora and Phillips 2002). Sugars and amino acids are preferred assimilates for beneficial microorganisms such as P-solubilising bacteria and AMF, which enhance bioavailability of P in soil (Adhya *et al*. 2015).

Total organic acid exudation increased significantly at low P, by more than 2-fold, in the genotypes G-2656, EC-232019 and NRC-7. Genotype EC-232019 showed substantially more oxalate in the root exudate induced only at low-P stress. Further, succinate and fumarate concentrations increased 2-fold at low P compared with sufficient P in EC-232019. Similarly, HIMSO-1521 exhibited an increase of >2-fold in root exudate concentrations of citrate, succinate, pyruvate and lactate. Exudates comprising fumarate, citrate and malate in P-stressed lucerne (*Medicago sativa*) (Lipton *et al*. 1987) and soybean (Ohwaki and Hirata 1992) support our findings. Oxalate and malate were the major organic acids detected in P-efficient soybean (Dong *et al*. 2004; Liao *et al*. 2006).

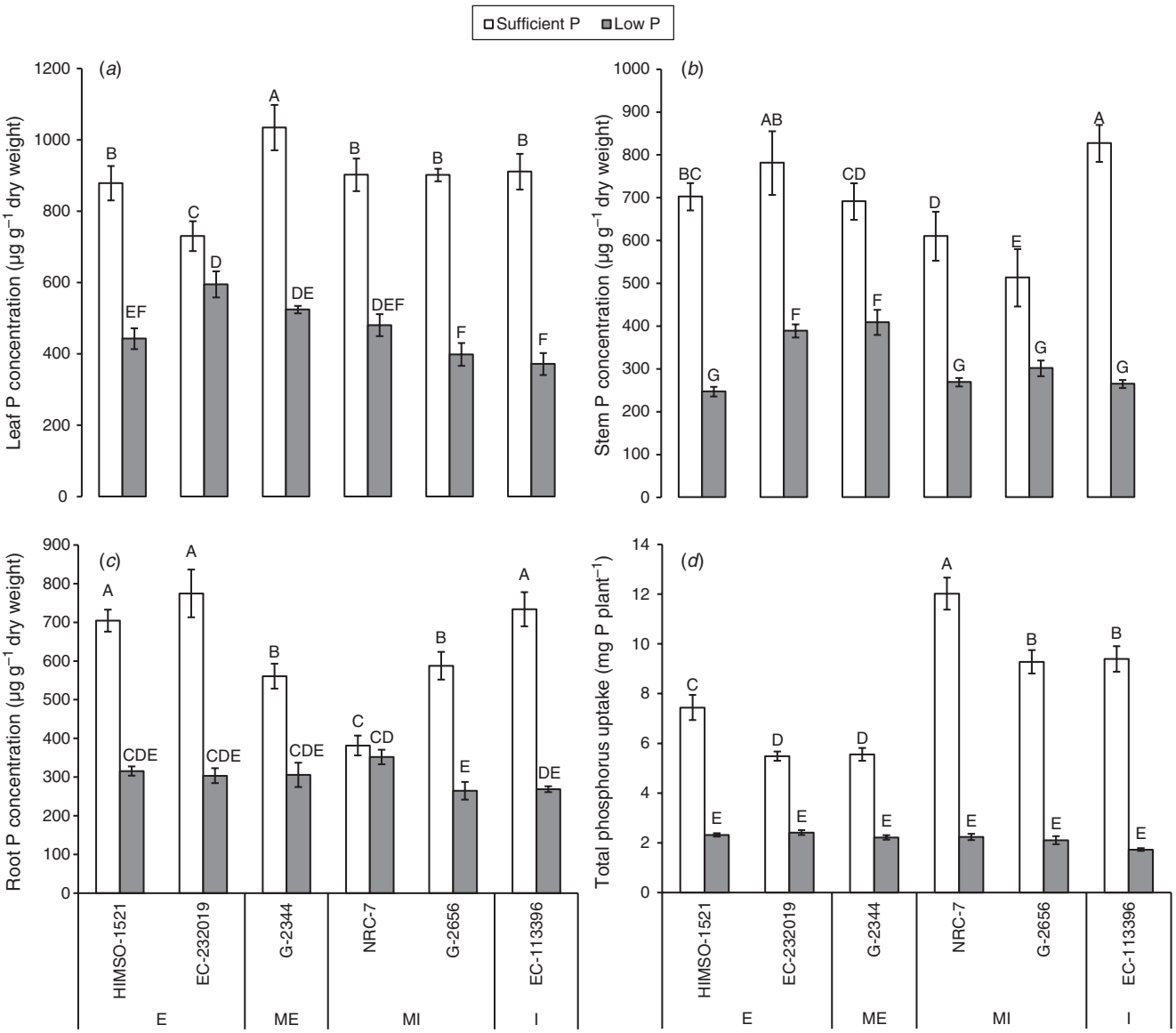

Fig. 7. Influence of phosphorus (P) availability (sufficient, 27 mg P kg^{-1} soil; low, 2 mg P kg^{-1} soil) on (*a*) leaf P concentration, (*b*) stem P concentration, (*c*) root P concentration, and (*d*) total P uptake in six soybean genotypes. Data correspond to mean ± s.e. of 2 years. Means with the same letter are not significantly different at $P = 0.05$ according to Duncan's mean range test. Genotypes were grouped on the basis of root exudation index and P acquisition efficiency as: E, efficient; ME, moderately efficient; MI, moderately inefficient; I, inefficient.

Table 4. Influence of phosphorus (P) availability, sufficient (27 mg P kg^{-1} soil) and low (2 mg P kg^{-1} soil), on seed yield and its attributes in six soybean contrasting genotypes selected from different clusters based on root exudation index and P acquisition efficiency

Genotypes were grouped as: E, efficient; ME, moderately efficient; MI, moderately inefficient; I, inefficient. Data are mean ± s.e. of 2 years. For each parameter, means followed by the same letter are not significantly different at $P = 0.05$ according to Duncan's mean range test

Cluster	Genotype	No. of pods per plant		No. of seeds per pod		Test weight (g 100 seed^{-1})		Seed yield (g plant^{-1})	
		Sufficient P	Low P	Sufficient P	Low P	Sufficient P	Low P	Sufficient P	Low P
E	HIMSO-1521	30 ± 1h	30 ± 3h	2.2 ± 0.1a	2.0 ± 0.1ab	7.41 ± 0.2ab	6.94 ± 0.3bc	5.10 ± 0.3c	3.93 ± 0.5d
	EC-232019	52 ± 6cd	46 ± 6de	2.1 ± 0.1ab	1.9 ± 0.1ab	3.38 ± 0.2g	3.64 ± 0.2g	3.11 ± 1.0def	2.92 ± 0.5ef
ME	G-2344	29 ± 4h	21 ± 1i	1.8 ± 0.1bc	1.9 ± 0.2abc	4.66 ± 0.3ef	4.13 ± 0.3fg	2.27 ± 0.5fg	1.48 ± 0.5g
MI	NRC-7	84 ± 5a	56 ± 2bc	1.8 ± 0.3bc	1.8 ± 0.1bc	6.81 ± 0.4bc	6.14 ± 0.4cd	10.13 ± 0.8a	7.06 ± 0.4B
	G-2656	62 ± 5b	38 ± 4fg	2.1 ± 0.1ab	2.0 ± 0.1ab	5.36 ± 0.1de	5.23 ± 0.5de	7.02 ± 0.8b	3.91 ± 0.8d
I	EC-113396	41 ± 4ef	33 ± 3gh	2.1 ± 0.1ab	1.6 ± 0.1c	7.97 ± 0.3a	7.26 ± 0.2ab	6.36 ± 0.8B	3.44 ± 0.2de

Genotypes G-2656 and NRC-7 exuded a significantly greater quantity of organic acids at low P, in the order: malate > succinate > oxalate > citrate > fumarate > pyruvate > lactate.

In the present experiment, the highest soil-P mobilisation capacity was observed in the root exudate, followed in order by citrate, oxalate, fumarate, succinate, malate, pyruvate and lactate (Fig. 5). Similar studies confirmed the P-mobilising potential of citric acid (Drouillon and Merckx 2003) and oxalic and malic acids (Hocking *et al*. 2000), as well as root exudates collected from pigeon pea (Krishnappa and Aftab Hussain 2014). Organic acids mobilise P bound to metal ligands in the soil; their functionality depends on the number and arrangement of carboxyl and hydroxyl moieties. The complexing capacity for aluminium, iron or calcium follows the decreasing order: tri- ($citrate^{3-}$) > di- ($malate^{2-}$, $oxalate^{2-}$, $fumarate^{2-}$, $succinate^{2-}$) > mono- ($lactate^{-}$) organic acids (Ryan *et al*. 2001).

Genotypic variation in growth and yield responses to contrasting soil P availability

Genotypic variation was observed for soybean growth and yield at contrasting soil P availabilities. Reduction in leaf area and shoot growth under low P availability may be attributed to lower cell proliferation as reported in maize (Assuero *et al*. 2004) or a longer phyllochron as observed in wheat (Rodríguez *et al*. 1998). Shoot biomass was positively correlated with P uptake in wheat under both sufficient and low P availability (Valizadeh *et al*. 2002), indicating that it is an important trait for selection of P-efficient genotypes (Akhtar *et al*. 2008). Significant variation in root traits is also well documented in genotypes of barley (Gahoonia *et al*. 2000), maize (Bayuelo-Jiménez *et al*. 2011) and soybean (Vandamme *et al*. 2013).

Leaf P concentration below a critical value triggers drastic reduction in leaf extension rate and dry matter accumulation (Dingkuhn *et al*. 2006). Hence, the smaller decline in leaf area and total biomass exhibited by efficient soybean genotypes might be attributed to maintenance of higher leaf P concentration than in other genotypes at low soil P levels. Consistent with genotypic variation for seed yield in soybean, P-efficient wheat genotypes exhibited yield traits superior to inefficient ones, attributed to higher biomass and P acquisition under P deficiency (Fageria and Baligar 1999; Gill *et al*. 2004). The smaller reduction in seed yield under P deficiency that is displayed by the efficient soybean genotypes correlated with, and may be governed by, the number of pods per plant.

Among the traits measured at seedling stage, TCE, total P uptake per plant and total biomass per plant contributed to maximum genotypic variability of soybean, and hence, they may be used as selection criteria in breeding programs. Therefore, in addition to favourable biomass-accumulation and P-acquisition strategies, root exudation is a relevant mechanism to mobilise inorganic P in soybean genotypes adapted to low P availability. Soybean genotypes G-2656, NRC-7 and EC-113396 responded to application of P fertiliser with higher biomass and seed yield, whereas genotypes HIMSO-1521, EC-232019 and G-2344 had similar growth potential under both sufficient and low P availability. Under low P availability, the efficient genotypes exude a greater quantity of organic compounds into the rhizosphere, which improves P availability to meet the crop requirement, enabling them to produce consistent biomass and seed yield with reduced application of P fertiliser. Such maintenance of growth and yield potential by mining the inherent soil P is a favourable trait in genotypes, thereby reducing the dependence on P fertilisers. Results from hydroponic experiments led to selection of genotypes with a potential to minimise use of P fertilisers; therefore, the hydroponic screening method may be adopted as a simple and rapid technique to select efficient genotypes from a large set of soybean germplasm as well as other field crops.

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