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Short Communication

Root acidification, a rapid method of screening soybean genotypes for low-phosphorus stress

Krishnapriya Vengavasi and Renu Pandey*

Division of Plant Physiology, ICAR-Indian Agricultural Research Institute, New Delhi 110 012

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Abstract

Carboxylate efflux in response to low-phosphorus (P) is one of the plant's adaptive strategies. Genotypic variation between the type and amount of root-exuded carboxylates determines the differential P acquisition efficiency (PAE) in soybean (*Glycine max* (L.) Merrill.). We compared direct (using HPLC) and indirect methods (total carbon exudation, and use of bromocresol purple dye) for quantifying root acidification. Total carbon exudation did not correlate to carboxylate efflux, whereas a significant linear relationship between exudate pH and carboxylate concentration suggested that measure of root acidification might predict the genotypic potential for low-P induced carboxylate efflux.

Key words: Acidification, bromocresol purple, carboxylate efflux, phosphorus, root exudate

Rhizosphere acidification by means of carboxylate exudation is a major trait of interest in breeding programmes to improve phosphorus (P) acquisition efficiency (PAE). Crop species/genotypes with high root exudation rates have potential to enhance soil P availability and thus are assets of low-input farming systems. P nutrition is extremely important for soybean; being a legume it has an inherent capacity to fix atmospheric nitrogen through symbiotic association. Optimum P supply aids *Rhizobium* species to fix more nitrogen, thereby resulting in high protein content, dry matter and grain yield of legumes. Hence, qualitative and quantitative estimate of root-exuded carboxylates is vital to exploit the diversity in soybean germplasm for PAE. However, the direct methods to characterize and quantify root-exuded carboxylates involve sophisticated instrumentation such as HPLC, GC-MS or ion chromatography (Carvalhais et al. 2011) and cumbersome sample preparation making it unsuitable for large number of samples.

Although precise quantification of carboxylates in contrasting genotypes is essential for subsequent analysis or trait selection but initial germplasm screening using indirect measures may save time, labour and resources. Quantification of total organic carbon (Personeni et al. 2007) or carbon isotope (^{14}C) (Pandey et al. 2013) might serve as a rational estimate of total root exudation. Further, P deficiency-induced acidification has been quantified using pH indicator dyes like bromocresol green and bromocresol purple (BCP) (Lei et al. 2015). The applicable pH range of BCP being 4.5 to 7.5, it has been used to detect acidification of the growing media in several crops (Gollany and Schumacher 1993). Since release of carboxylic acids is accompanied by proton/cation extrusion (Meyer et al. 2009), it was hypothesised that measurement of acidity levels of root exudate might serve as a proxy for carboxylate concentration. Thus, the objective was to identify a simple method to predict genotypic potential for low-P induced carboxylate efflux in a large population.

Soybean seedlings were raised in nutrient solution with sufficient (250 μM) and low (4 μM) P

*Corresponding author's e-mail: renu_iari@rediffmail.com
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concentration in glasshouse. The cotyledonary leaves were removed after transferring the seedlings to nutrient solution to avoid any variation in growth arising due to difference in seed P content. Initially, 116 soybean genotypes were screened by indirect method (Pandey et al. 2013) to quantify root exuded carbon by measuring total ^{14}C exudation and total biomass after drying plants in hot air oven. Based on the relative biomass and relative carbon exudation, these genotypes were classified into four distinct categories as genotypes exhibiting high biomass and high exudation, high biomass and low exudation, low biomass and low exudation and low biomass and high exudation under low-P (data not presented). Out of 116, 20 genotypes representing all categories were selected to quantify carboxylates by direct method using HPLC. Root exudate was collected from 20-day old plant and processed (Pandey et al. 2013). Total carboxylate efflux, expressed as μmol g^{-1} root fresh weight, was the sum of individual acids (oxalic, citric, malic, pyruvic, succinic, lactic and fumaric).

Quantification of exudate acidification by indirect method was designed using BCP dye. For standard calibration, 10 μl of 1% BCP was added to 10 ml of trap solution (0.5 mM $CaCl_2$) of different pH levels ranging from 4.50 to 6.50 (Fig. 1a), and absorbance was measured at 435 and 590 nm. Correlation between absorbance and pH of trap solution was slightly higher at 590 nm ($R^2 = 0.96^{***}$, Fig. 1b) as compared to 435 nm ($R^2 = 0.95^{***}$, Fig. 1c). The absorption maxima of unprotonated BCP being 590 nm (Santi and Schmidt 2009), it was the preferred wavelength to estimate acidification in root exudate. Three 20-day old plants were placed together in a test tube covered with black paper containing 10 ml of trap solution (0.5 mM $CaCl_2$, pH 7.0) + 10 μl of 1% BCP between 0800 to 1200 h. Change in colour of trap solution due to acidification was measured at 590 nm and expressed in pH units as calculated from the calibration curve. The root and shoot tissues were dried and digested in diacid mixture for estimation of P concentration. Total P uptake was calculated from P content and expressed as μg P $plant^{-1}$. PAE (%) was the ratio of total P uptake at low-P to sufficient-P. The experimental design was completely randomized with two-factors. Procedures for descriptive statistics, two- or one-way ANOVA and correlation were carried out in the statistical software R version 3.1.2.

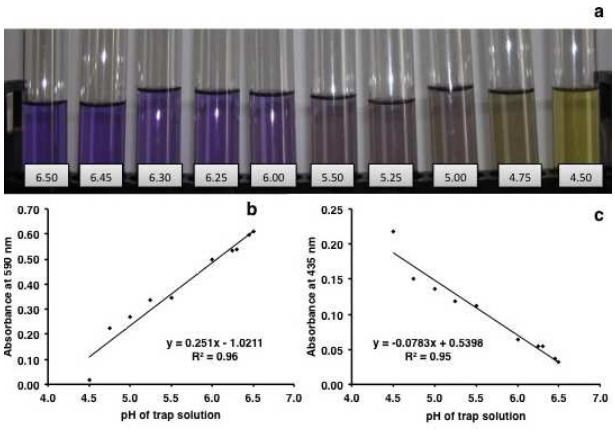

Fig. 1. (a) Standards used for calibrating exudate acidification. Calibration curves of trap solutions and absorbance measured at wavelengths (b) 590 nm and (c) 435 nm

Total carboxylate efflux was significantly influenced by genotype and their interaction with P (Table 1). Low-Pinduced carboxylate exudation increased significantly in genotypes EC-528639 (97%) and EC-232019 (58%).The relative total ^{14}C exudation at low-P over sufficient-P was highest in CSB-0804 (231%), EC-528639 (182%), EC-592195 (219%), G-2656 (139%) and NRC-7 (131%). Similarly, acidification (pH) of root exudate varied significantly among genotypes from 5.51 to 6.93 when averaged over P levels. Out of 20 genotypes, 14 showed pH values >6.59 (P x genotype) while six possessed pH less than the mean. Exudate acidification was highest in genotypes HIMSO-1521 and EC-456534 having mean pH values <6.0 while in EC-592195 and CO-1, it was near neutral (6.93). The total P uptake averaged over genotypes was significantly reduced (70%) at low-P (Fig. 2a). Among genotypes, although total P uptake in EC-232019 was below average, it exhibited least reduction (19%) at low-P. The reduction in total P uptake at low-P was <50% in EC-528622 and CSB-0804, while it was >80% in EC-109514, EC-572050, NRC-7 and EC-467282(B). Genotypes EC-232019, EC-528639, EC-528622 and CSB-0804 recorded >50% PAE while genotypes EC-109514, EC-572050, EC-467282(B), NRC-7 and EC-2253 showed least PAE (Fig. 2b). Earlier reports also showed increased exudation of malate, oxalate and citrate in soybean (Liao et al. 2006), *Vigna mungo* (Jakkeral and Kajjidoni 2011) and *Vigna radiata* genotypes (Pandey et al. 2013) in response to low-P. We also observed highest carboxylate efflux under low-P in P-efficient soybean genotypes EC-232019, EC-528639 and HIMSO-1521, whereas EC-467282(B) with least PAE exhibited least carboxylate exudation.

Proton extrusion causing acidification of rhizosphere or the growing media is a process that

Table 1. Genotypic variation in total carboxylate efflux, total carbon (^{14}C) exudation and acidification of root exudate in soybean grown at sufficient (250 μM) and low (4 μM) P

Genotype	Total carboxylate efflux (μmol g^{-1} root fresh weight)		Total carbon (^{14}C) exudation (dpm g^{-1} root fresh weight)		Acidification of root exudate (pH)	
	Sufficient P	Low P	Sufficient P	Low P	Sufficient P	Low P
EC-232019	172.7 ± 8.98	274.6 ±14.27	27±7.04	38±6.59	6.64±0.19	5.82±0.40
EC-528639	97.6 ± 5.07	192.6 ±10.01	11±1.09	31±2.09	6.10±0.04	5.97±0.21
EC-528622	41.4 ± 2.15	22.5 ± 1.17	19±0.91	26±4.84	6.73±0.47	6.93±0.28
CSB-0804	25.7 ± 1.33	40.0 ± 2.08	16±2.12	53±0.49	6.86±0.36	6.67±0.27
HIMSO-1521	392.8 ±20.41	246.7 ±12.82	19±0.61	8±0.75	4.87±0.25	6.15±0.18
G-2344	43.8 ± 2.28	13.9 ± 0.72	47±7.61	89±0.48	6.76±0.20	6.29±0.13
EC-592195	18.0 ± 0.94	21.1 ± 1.10	21±0.93	67±2.37	6.97±0.30	6.89±0.24
EC-325117	31.9 ± 1.66	24.5 ± 1.27	49±0.11	80±1.40	6.82±0.30	6.87±0.24
JS-93-05	38.5 ± 2.00	30.5 ± 1.58	18±1.07	17±4.12	6.81±0.14	6.76±0.29
G-2656	25.9 ± 1.34	57.8 ± 3.01	33±2.03	79±1.74	6.85±0.28	6.58±0.34
EC-456534	200.2 ±10.40	214.2 ±11.13	19±5.31	12±4.62	5.70±0.20	6.18±0.27
NRC-7	9.0 ± 0.47	13.8 ± 0.72	13±3.01	30±0.55	6.91±0.24	6.86±0.20
EC-113396	171.2 ± 8.90	110.8 ± 5.75	27±0.72	22±2.44	6.69±0.29	5.95±0.31
CO-1	14.8 ± 0.77	9.7 ± 0.50	31±1.56	35±4.35	6.97±0.48	6.88±0.36
TNAU-20024	25.1 ± 1.30	31.8 ± 1.65	12±0.94	5±0.81	6.87±0.14	6.83±0.47
MAUS-61	44.7 ± 2.32	42.8 ± 2.22	23±4.28	21±5.40	6.81±0.29	6.71±0.19
EC-2253	51.3 ± 2.67	54.9 ± 2.85	42±1.15	27±1.12	6.66±0.23	6.61±0.28
EC-467282(B)	25.1 ± 1.31	18.8 ± 0.98	16±0.98	15±2.94	6.90±0.36	6.87±0.04
EC-572050	21.8 ± 1.13	36.0 ± 1.87	15±0.17	12±0.21	6.93±0.48	6.59±0.04
EC-109514	29.7 ± 1.54	54.4 ± 2.82	14±2.30	29±1.78	6.87±0.24	6.46±0.28
CD value	$\alpha = 0.05$	$\alpha = 0.01$	$\alpha = 0.05$	$\alpha = 0.01$	$\alpha = 0.05$	$\alpha = 0.01$
P	0.835	1.108	1.067	1.405	0.179	0.237
G	2.641	3.504	8.127	10.698	0.564	0.749
P x G	3.735	4.956	11.494	15.129	0.798	1.059

Data correspond to mean ± SE (n=12). Phosphorus (P) and genotype (G) are treatment effects

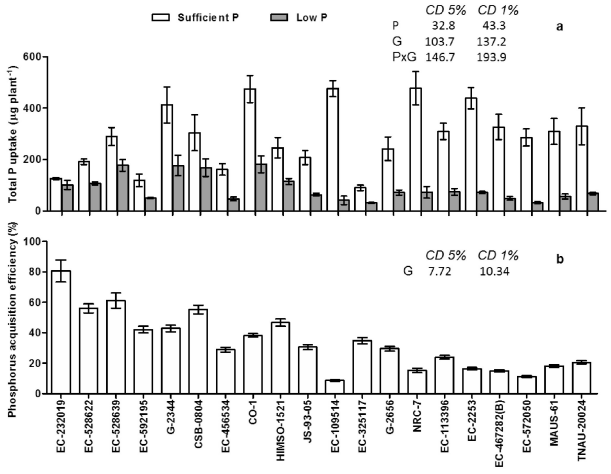

Fig. 2. Genotypic variation in (a) total P uptake and (b) PAE of soybean grown at sufficient (250 μM) and low (4 μM) P.Data correspond to mean ± SE (n=12). Phosphorus (P) and genotype (G) are the treatment effects

accompanies carboxylate exudation. Efflux of carboxylate anions creates a negative potential difference across the plasma membrane resulting in concomitant release of protons or K^+-ions (Meyer et al. 2009). Genotypes with higher carboxylate exudation (EC-232019, EC-528639, EC-572050, EC-109514) exhibited enhanced acidification under low-P (Table 1). Exudate acidification was not observed in EC-467282(B) corresponding to minimum release of carboxylates both under sufficient-P as well as low-P. Quantifying media acidification using BCP aided screening of *Arabidopsis* mutants in response to iron (Santi and Schmidt 2009) and P nutrition (Lei et al. 2015). Correlation studies showed that the pH of exudate was negatively correlated to exudation of total carboxylates including individual acids at both sufficient (r = –0.99***) and low (r = –0.94***) P. Further, a significant linear regression between pH and carboxylate exudation ($R^2 = 0.76$**) suggested exudate

acidification might be used as a rapid and simple measure to predict low-P induced carboxylate efflux during germplasm screening.

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