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Differential Expression of Aluminium Tolerance Mechanisms in Cowpea Genotypes under Phosphorus Limitation

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Abstract: Research efforts have revealed differences in tolerance to Aluminium (Al) toxicity among cowpea (*Vigna unguiculata*) genotypes grown on acid soils (pH<5.0) with tolerant genotypes exhibiting higher capacity for Al exclusion than the susceptible ones under P-deficient conditions. Here, we tested the hypothesis that genotypic differences in cowpea growth and development might be negatively affected by P limitation, particularly in Al-susceptible genotypes. Root growth responses of two contrasting (Al-resistant and Al-susceptible) cowpea genotypes, Epace 10 (E10) and Santo Inacio (SI) were studied at 0 and 20 μM Al. Root elongation was followed over the first 96 h of Al treatment and during the initial 48 hours elongation was determined daily. In addition, exudation of carboxylates in apical root zone and modifications of rhizoplane pH by the two genotypes were compared in hydroponics' culture experiments that involved +/-Al treatments at mild (14 days) and severe (21 days) phosphorus (P) limitation stages. Differential genotypic responses were further evaluated in a rhizobox experiment, using an acidic Aerenosol from West Africa (Niger) with low expression of Al toxicity. Strong Al-induced inhibition of root growth occurred at 20 μM Al without genotypic difference, suggesting the need to further test genotypic differences at lower concentrations. Under P limitation, E10 exhibited a stronger expression (relative to SI) of root induced chemical changes (increased rhizoplane pH and citrate exudation in apical root zone) to counteract Al toxicity. Therefore, genotypic differences in performance of E10 and SI on acid mineral soils may be associated with different expression of Al tolerance mechanisms, particularly under conditions of limited P supply.

Key words: Aluminium tolerance, phosphorus limitation, acidic aeronosol, nutrient solution, rhizobox experiment, rhizoplane pH, organic exudates, *Vigna unguiculata*

INTRODUCTION

Poor crop growth in acid soils is often related to Al saturation, as it may be difficult to differentiate between the effects of Al toxicity and phosphorus (P)-deficiency stress. Acid soils are in up to 30% of world's ice-free land area (Haynes and Mokolobate, 2001), from the northern cold temperate belt to the southern tropical belt (Von Uexkull and Mutert, 1995). Agriculture is practiced as semi-subsistence farming in the latter belt, where increasing population pressures no longer permit reasonable fallow periods, but forces farmers to manage soil fertility in order to maintain productivity (Myers and De Pauw, 1995).

The low P status of highly weathered acid soils is worrisome, as large amounts of fertilizer P are needed to raise the available P to an adequate level (Sanchez and Uehara, 1980). The restriction of plant growth by excess soluble Al in acid soils may arise from either the direct inhibition of nutrient uptake or disturbance of root cell functions (Kochian, 1995) while tolerance to high Al concentrations by indigenous plants of acid soils can be due to the mechanisms of Al exclusion from root cells and/or intrinsic tolerance to the element (Cuenca *et al*., 1990). Sanchez (1997) noted that the "keys" for improvement of food production in humid tropical Africa and the sub-humid Brazilian savannah cerrado are the use of acid tolerant genotypes and the optimisation of nutrient cycling in soil. The exudation of organic acid anions (particularly, citrate and malate) by plant roots probably provides an efficient mechanism, by which some species or cultivars enhance their abilities to acquire P (Haynes *et al*., 2001; Kamh *et al*., 1999, 2001) and to detoxify Al (Delhaize *et al*., 1993). According to Ma (2000), organic acids are capable of forming chelates with Al that is then detoxified inside and outside the plant. Roots of some crop species (including *V. unguiculata)* have also been reported to have the capability of developing certain adaptation mechanisms (exudation of exudates, protons, bicarbonates, ecto-enzymes, or

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association with micorrhiza or cluster root formation) in acid soil conditions in order to solubilise sparingly soluble P forms (Dinkelaker and Marschner, 1992; Neumann and Martinoia, 2002; Obigbesan *et al*., 2002). The germplasm of cowpea exhibits wide genotypic differences in the tolerance to acid soil conditions. The understanding of the physiological mechanisms of the tolerance is indispensable for further improvement of the adapted genotypes (Kensuke *et al*., 2003).

Obigbesan *et al*. (2002) examined seven cowpea cultivars in a pot experiment and observed different tolerance on an acidic Alumihaplic Acrisol, pH 3.8 from Brazil, particularly expressed under conditions of P-limitation. Iroh (2004) and Akinrinde *et al*. (2005) selected two of the genotypes (Epace 10 and Santo Inacio) for further analysis of possible tolerance mechanisms. During early growth (31 days after sowing, DAS) on the Alumihaplic Acrisol with three levels of P application, Epace 10 and Santo Inacio were similarly affected by P limitation without differences in plant development (Iroh, 2004). However, at 70 DAS, Epace 10 exhibited higher grain yield at all levels of P supply. Akinrinde *et al*. (2005) further reported high spontaneous AM colonization (up to 60 %) was particularly expressed at limited P supplies but there were no differences between genotypes. Also, shoot concentrations of P, Mg and Ca as putative growth-limiting mineral nutrients in acid soils revealed no genotypic differences. However, particularly at medium and low P supply, Santo Inacio accumulated 2-3 fold higher levels of Al in the shoot tissue (300-600 mg kg^{-1} DM) than Epace 10, suggesting a higher capacity of Epace 10 for Al exclusion under P-deficient conditions (Iroh, 2004). The conclusion has been that genotypic differences in performance of Epace 10 and Santo Inacio on acid mineral soils might be related with different expression of Al tolerance mechanisms, particularly under conditions of limited P supply. This hypothesis needs to be tested by dissecting the influence of Al stress and P deficiency under controlled conditions in nutrient solution and soil culture experiments. Genoptypic differences in cowpea growth and development on the acidic Alumi-haplic Acrisol are not directly affected by the P-nutritional status, but rather by differences in Al-tolerance. Aluminium tolerance may be negatively affected by P limitation, particularly expressed in Santo Inacio.

Kamh *et al*. (2001) explained that maize (*Zea mays* cv. ICAV-109) responded to P deficiency (- P, + Al) by increasing mainly citrate exudation, which was highly reduced by Al but Al stimulated citrate and malate exudation by other cultivars. Nian *et al*. (2003) observed impaired expression of Al tolerance mechanisms (citrate exudation) by soybean (*Glycine max*) under P-deficiency. The genotypic differences in cowpea growth and development under acid soil conditions may also be influenced by differences in their Al-tolerance and not directly by the P-nutritional status. The present study considered the hypothesis that genotypic differences in cowpea growth and development might be negatively affected by P limitation, particularly in the Al-susceptible genotype. In testing this hypothesis, root induced physical changes (elongation and root-shoot ratio) as well as chemical changes for counteracting Al toxicity (increased pH at the rhizoplane and exudation of organic acid anions in apical root zones) would be measured in hydroponics and rhizobox-soil culture studies.

MATERIALS AND METHODS

Plant material and pre-germination: The study involved three experiments (two hydroponics' cultures and one soil culture in rhizoboxes) conducted at the Institute of Plant Nutrition (330) and Institute of Plant Production in the Tropics and Subtropics (380), both Of the University of Hohenheim, Stuttgart (D-70593), Germany between October 2003 and April 2004.

The test crop was cowpea (*Vigna unguiculata,* cvs. EPACE 10 and SANTO INATIO). EPACE 10 (E10) is an improved cultivar while SANTO INATIO (SI) is a local variety from Piaui, Brazil. The first hydroponics' culture was employed to compare growth responses of E10 to Al-toxicity at the seedling stage with that of SI while the second was used to do the comparison at different levels of P-deficiency. The final soil culture in rhizoboxes was to further evaluate the responses of the genotypes to Al-toxicity at different P levels (0 and 100 mg P kg soil) using an acidic Aerenosol (from West Africa) with low expression of Al toxicity.

Prior to each of the studies, seeds of the cowpea genotypes were surface-sterilized for 15 min in a solution containing 15% hydrogen peroxide (H_2O_2), rinsed thoroughly with de-ionised water six times. They were then incubated between filter papers moistened with 2.5 mM $CaSO_4$ for 3 days at 25°C in darkness for germination and allowed further growth in a growth chamber for additional two days.

Hydroponics' cultures

Responses to Al-toxicity at the seedling stage: In comparing cowpea responses to Al-toxicity, 5-day old pre-germinated seedlings of fairly uniform development were incubated in 2.5 L nutrient solutions comprising 0.5 mM $CaCl_3$ (pH 4.5) as control and 0.5 mM $CaCl_2$ (pH 4.5) + 20 μM $AlCl_3$ as treatment, ensuring continuous

aeration (using aquarium pumps) in 3-litre-plastic pots. Ten seedlings were transferred into each of a total number of four experimental pots. There was one pot for each of control and Al treatments in respect of each of the cowpea genotypes. Pots were rearranged daily in order to assure equal access to radiation in the growth chamber, with the following controlled (climatic) conditions: day/night photoperiod, 16/8 h; light intensity, 220 uE m^2 s"; temperature (day/night cycle) 25/20°C; R.H., 70-80%.

Root elongation was measured after 24 h as well as after 48 h (2 days) and 96 h (4 days) to evaluate the effect of Al treatment on root growth of the cowpea genotypes while the pH of the nutrient solution (growth) medium was measured daily with Sb-microelectrode for the assessment of differential root-induced pH changes between the genotypes. Exudates were collected from 1.5 cm apical root zones by dipping the portion into HPLC water (contained in eppendolf vials) for 2 h after the first and the fourth days and subsequently analysed for organic acid contents using the HPLC. Fresh root and shoot biomass were also recorded at the end of the fourth day prior to drying to constant weight in an oven at 70°C. Thus, Root length and Shoot biomass, exudation of carboxylates in 5 mm apical root zones (HPLC) and root-induced pH changes were determined.

Responses to Al-toxicity at different levels of P-deficiency under hydroponics culture: Responses to Al-toxicity were compared at different levels of P-deficiency by cultivating another set of seedlings in +P/-P nutrient solution for 14 days (moderate P-deficiency) and 21 days (severe P deficiency). Subsequently, short-term (24- 48 h) incubation of plants from each P-treatment was carried out in 0.5 mM $CaCl_2$ (pH 4.5) (control) and 0.5 mM CaC l_2 (pH 4.5) + 20 μm $AlCl_3$ (treatment) while measurements included: Root/Shoot growth (Length/Biomass, Leaf area), Exudation of carboxylates in root washings and 20 mm apical root zones (HPLC) as well as Root-induced pH change. One-half of the root for each plant was weighed and dried separately from the shoot while the second half was preserved in 70% ethanol prior to the determination of total root length (at a later date) according to the method described by Tennant (1975).

Rhizobox experiment: In a rhizobox experiment the two genotypes were cultivated at different P levels (0 and 100 mg P kg soil) using an acidic Aerenosol (from West Africa) with low expression of Al toxicity. Details of the description and methodology of rhizobox technique are found in the literature (Dinkelaker and Marschner, 1992). Irrigation was with or without 125 μM Al $(NO_3)_3$ (pH 4.5). After 21 days, growth and biomass production parameters were determined for the evaluation of Al-tolerance of the cowpea cultivars.

Data analysis: All data were analysed by analysis of variance (ANOVA), using Sigmastat software.

RESULTS AND DICUSSION

Comparison of responses to Al-toxicity at the seedling stage (5 DAS)

Root elongation: Aluminium at 20 μM significantly reduced root elongation ($p \leq 0.01$): at 1, 2 and 4 day(s), Relative Root Elongation (RRE), being 72.9, 74.1 and 60.0% that of control plants, respectively across the two cultivars (Table 1). Kidd *et al.* (2001) observed RRE of 46% that of control for maize *(Zea mays)* with 20 μM Al treatment. Marschner (1991) noted that Al - toxicity limits plant growth in acid soils due to series of chemical factors and interactions. However, in the present study, similar Al-induced root-growth inhibition was observed in both genotypes -E10 and SI (Table 1), the insignificant genotypic effect being an indication of the probable need to further test differences at lower concentrations.

Nutrient solution pH changes: Though not sufficient to alleviate Al stress at 20 μM$AlCl_3$, there was a clear trend for pH increase of the nutrient solution by E10 plants with increase in duration of Al-treatment (Table 1). In contrast, acidity of the nutrient solutions in which SI variety plants were cultured tends to increase after exposure to 20 μM $AlCl_3$. The untreated plants of both cultivars also failed to reduce the acidity of the growth medium. Obigbesan *et al.* (2002) obtained higher rhizoplane pH (6.2) in E10 compared with SI (5.1), attributing this to possible relative higher Al-detoxification capability of E10 than SI.

Exudation of organic acid anions: Two main organic acid anions were detected: citrate and malate, which were, in all cases, secreted more in nutrient solutions treated with Al than in those without Al addition. In absolute terms, roots of SI seedlings released more of malate and citrate (with less variability) in the nutrient solutions than E10 seedlings after 1 day growth (Table 2). However, considering the separate releases of the acid anions + or -Al as percent of the total amounts released by each cultivar, Al treatment enhanced relatively more of the secretions in the latter (93.85%) than in the former (88.24%). This is an indication of greater resistance by E10 than SI to Al. After 4 days, secretion of the acid anions by seedlings of the two cultivars was not detected (in control

Table 1: Root elongation and change in pH of culture medium (nutrient solution) by cowpea genotypes (Epace 10 and Santo Inacio) after 1, 2 and 4 day(s) of Al treatment

Treatment		Root elongation (cm) Day(s) after Al treatment			pH Day(s) after Al treatment			
Genotype	Al	1	2	3	Initial (0)	1	2	4
EPACE	Minus Al	2.44	6.96	14.74	4.5	4.3	4.3	4.1
	Plus Al	0.51	1.75	5.54	4.5	4.6	4.7	4.9
SANTO	Minus Al	1.88	5.35	15.93	4.5	4.3	4.3	4.4
INACIO	Plus Al	0.56	1.36	4.85	4.5	4.3	4.5	4.5
LSD (0.05)		1.54	4.36	9.36	0.0	0.24	0.27	0.53

Table 2: Secretion of organic acid anions from 1.5 cm root tips of cowpea plants grown for one and two day(s) with or without Al treated nutrient solutions

Cowpea genotype	Al treatment	Organic acid anions released (μg mL^{-1}) Malate	Citrate
After 1 day			
EPACE10	-Al	0.00	0.12
	+A1	0.55	1.83
SANTO	-Al	0.00	0.44
INACIO	+A1	0.69	3.30
LSD (0.05)			
After 4 days			
EPACE10	-Al	0.00	0.00
	+Al	0.02	0.07
SANTO	-Al	0.00	0.01
INACIO	+A1	0.08	0.08
LSD (0.05)		0.58	2.32

Table 3: Varietal/genotypic differences in shoot biomass yield and root/shoot ratio of cowpea (EPACE 10 and SANTO INACIO) at moderate and severe phosphorus (P) stress levels, with or without aluminium toxicity. [The cowpea cultivars were grown in hydroponics to moderate (14 DAS) or severe (21 DAS) P starvation with or without short-term (20 h) Al treatments]. PAS = Days After Sowing

Treatment		Moderate P limitation Shoot biomass g plant^{-1}	Root-shoot ratiog	Severe P limitation Shoot biomass plant^{-1}	Root-shoot ratio
Genotype	Al				
Minus P					
EPACE10	Minus Al	0.44	0.15	0.88	0.11
	Plus Al	0.46	0.18	0.63	0.16
SANTO	Minus Al	0.29	0.19	0.39	0.22
INACIO	Plus Al	0.29	0.24	0.34	0.28
Plus P					
EPACE10	Minus Al	0.61	0.09	2.36	0.05
	Plus Al	0.72	0.10	1.92	0.05
SANTO	Minus Al	0.57	0.10	1.19	0.07
INACIO	Plus Al	0.48	0.12	1.87	0.05
LSD (0.05)		0.13	0.04	0.64	0.07

Table 4: Leaf area of cowpea varieties (EPACE 10 and SANTO INACIO) grown under severe phosphorus limitation, with or without aluminium toxicity. [The cowpea cultivars were grown in hydroponics to moderate (14 DAS) or severe (21 DAS) P starvation with or without short-term (20 h) Al treatments]. DAS = Days After Sowing

Treatment		Leaf area (cm^2 plant^{-1}) at high P limitation	
Genotype	Al	Minus P	Plus P
EPACE 10	Minus Al	83.50	208.50
	Plus Al	83.50	164.50
SANTO	Minus Al	46.25	235.00
INACIO	Plus Al	51.00	250.75
LSD (0.05)		32.18	60.03

Table 5: Citrate exudation from 1.5 cm apical root zones of cowpea varieties grown under moderate and severe phosphorus limitation, with or without aluminium toxicity [The cowpea cultivars were grown in hydroponics to moderate (14 DAS) or severe (21 DAS) P starvation with or without short-term (20 h) Al treatments]. DAS = Days After Sowing

Treatment		Citrate acid exudation (g mL^{-1}) Moderate P limitation		Severe P limitation	
Genotype	Al	Minus P	Plus P	Minus P	Plus P
EPACE 10	Minus Al	0.04	0.00	0.18	0.14
	Plus Al	0.44	0.71	0.46	0.55
SANTO	Minus Al	0.91	0.02	0.20	0.75
INACIO	Plus Al	0.73	0.89	0.00	0.61
LSD (0.05)		0.60	0.74	0.30	0.42

nutrient solutions) and detected at quite low concentrations (about 0.05 μg mL^{-1}) when grown in Al-treated nutrient solutions (Table 2). This is an indication that as could be expected the seedlings were not very active after 4 days of growth in the predominantly $CaCl_3$ nutrient solution. Changes in root length and nutrient solution pH after various days after Al-treatment as well as Al-induced citrate and malate exudation from 1.5 apical root zones of the cowpea genotypes are illustrated in Table 2. Short-term (20 h) Al-induced citrate (and to a lower extent malate) exudation in apical root zones was also observed but with no genotypic difference. Effort by Obigbesan *et al.* (2002) to differentiate between susceptible and resistant cowpea varieties was unsuccessful, as the plants were not treated with Al-chelaling carboxylates. No relation of organic acid concentrations (μg mL^{-1}) in the rhizosphere soil solution of 5 mm-apical root zones with genotypic differences in tolerance to soil acidity.

Responses to Al-toxicity at different levels of P-deficiency under hydroponics culture: Table 3 indicates that the experimental factors (P-nutritional status, Al treatments and the genotype) did not affect root biomass production with P-starvation (and + or -short-term, 20 h Al-treatments). However, shoot biomass production was limited by P deficiency, particularly in SI but there was no clear genotypic difference in the P-sufficient plants. The

Table 6: Leaf area, biomass production and relative (%) change in root length (between 7 and 10 days after sowing) of cowpea plants grown for 21 days with or without P and Al in a P-deficient acid soil. [Cultivation of cowpea genotypes in rhizohoxes using an acidic Aerenosol from West Africa with low expression of Al toxicity at different P levels (0 and 100 mg P kg soil") with or without repeated Al application (125 μM) via Irrigation water]

Cowpea genotype	Al treatment	Leaf area (cm^2 $plant^{-1}$)	Biomass production (g $plant^{-1}$) Root	Shoot	Root-shoot ratio	Relative (%) change in root length
Minus P						
EPACE 10	-Al	66.50	0.20	0.40	0.49	25.00
	+A1	74.00	0.20	0.41	0.49	70.00
SANTO	-Al	55.20	0.18	0.28	0.62	30.00
INACIO	+A1	63.50	0.14	0.31	0.45	35.00
Plus P						
EPACE 10	-Al	66.70	0.13	0.38	0.33	18.00
	+A1	96.00	0.22	0.48	0.48	10.00
SANTO	-Al	75.70	0.12	0.42	0.31	82.00
INACIO	+A1	92.50	0.19	0.48	0.43	55.00
LSD (0.05)		11.83	0.06	0.03	0.08	21.48

short-term Al treatments also had no significant difference. However, root-shoot ratio increased in P-deficient plants due to inhibition of shoot growth, particularly in SI but no genotypic clear differences in P-sufficient plants and no effect of short-term Al treatments.

Table 4 shows that P-deficiency limited leaf expansion, as was particularly expressed in SI. Again, there was no genotypic clear difference in P-sufficient plants and no effect of short-term Al treatments. Improved shoot development of E10 compared with SI in hydroponics without any P supply suggests a higher P-use efficiency of E10. Furthermore, mild/moderate P-deficiency (14 DAS) stimulated citrate exudation in SI but not in E10 (Table 5). Severe P deficiency (at 21 DAS) inhibited Al-induced citrate exudation in SI but not in E10.

Responses to Al-toxicity at different levels of P-deficiency under soil culture in rhizoboxes: Results of the cultivation of cowpea genotypes in rhizoboxes (with an acidic Aerenosol from West Africa having low expression of Al toxicity) at different P levels (0 and 100 mg P kg soil) +/-repeated Al application [125 μm] with irrigation water are provided in Table 6. Phosphorus deficiency limited shoot biomass production in SI but not in E10 and tended to increase root biomass in both genotypes. Accordingly, P-deficiency increased the root-shoot ratio, particularly in SI. There was a clear trend for increased root biomass production in response to Al treatments in both genotypes. Under P-deficient conditions, E10 but not SI was able to increase the pH at the rhizoplane in the apical root zone above the critical level for Al toxicity (pH 5.5) in response to Al treatments. In the rhizobox experiment, P-deficiency stimulated root elongation in E10, but inhibited it in SI. Treatments with Al stimulated root elongation of E10, particularly under P-deficiency, while in SI root elongation was unaffected (-P) or inhibited (+P) by Al application.

CONCLUSIONS

Under P-deficient conditions, E10 exhibited a stronger expression of root-induced chemical changes to counteract Al toxicity compared with SI. The increase in pH at the rhizoplane and root exudation of citrate in apical root zones are associated with a higher tolerance to Al treatments on the P-deficient acid soil in terms of root elongation. The stronger impairment of Al-toxicity responses in SI under P-limitation may be related to more severe expression of P stress due to a lower P-use efficiency compared with E10.

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