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BRIEF COMMUNICATION

Cell membrane integrity, callose accumulation, and root growth in aluminum-stressed sorghum seedlings

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Abstract

Aluminum stress usually reduces plant root growth due to the accumulation of Al in specific zones of the root apex. The objectives of this study were to determine the localization of Al in the root apex of *Sorghum bicolor* (L.) Moech. and its effects on membrane integrity, callose accumulation, and root growth in selected cultivars. Seedlings were grown in a nutrient solution containing 0, 27, or 39 μM Al^{3+} for 24, 48, and 120 h. The Al stress significantly reduced root growth, especially after 48 and 120 h of exposure. A higher Al accumulation, determined by fluorescence microscopy after staining with a Morin dye, occurred in the root extension zone of the sensitive cultivar than in the tolerant cultivar. The membrane damage and callose accumulation were also higher in the sensitive than resistant cultivar. It was concluded that the Al stress significantly reduced root growth through the accumulation of Al in the root extension zone, callose accumulation, and impairment of plasma membrane integrity.

Additional key words: aluminum tolerance, root extension zone.

Aluminum stress is a major limitation to plant growth and productivity on acid soils. The effect of soil acidity on world food production is significant because 50 % of the world arable lands are acidic (Von Uexküll and Mutert 1995). The basic response to Al toxicity in plants is a reduction of root growth (Kochian *et al.* 2005), an irregular cell division (Silva *et al.* 2012), a disturbance of the plasma membrane (Ahn and Matsumoto 2006), and an induction of callose deposition in root apices (Alvim *et al.* 2012).

Aluminum tolerance in many crops is related to exclusion of Al from root tips, mainly due to immobilization of Al by exuded organic acids (Kochian *et al.* 2005). Various studies have shown that the Al-activated exudation of citric acid from root tips plays a key role in tolerance to Al stress in sorghum (Magalhaes *et al.* 2007, Cheprot *et al*. 2014) and other crops (Panda and Matsumoto 2007). Also exudation of malic and transaconitic acids is correlated with tolerance to Al stress in sorghum (Goncalves *et al*. 2005). Sivaguru and Horst (1998) showed that the root distal transition zone is the primary target of Al, although the elongation zone is also very sensitive to Al injury (Silva *et al*. 2012). Al stress increases the synthesis of callose by a membrane-bound glucan synthase (Massot *et al.* 1999, Pirselova and Matusikova 2013) whose activity increases when the plasma membrane is disrupted. The callose deposition has been shown to be an early sign of Al toxicity in plants and is considered a reliable indicator of Al sensitivity (Smith *et al.* 2011). To our knowledge, the relationship between the Al inhibition of root growth and the callose deposition in sorghum is not documented.

The objectives of this study were to determine the localization of Al in root tips and its effect on membrane

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integrity, callose accumulation, and root growth in selected sorghum cultivars. The study was carried out on the premise that Al-tolerant sorghum cultivars minimize root tip injury in the presence of Al stress.

The sorghum cultivars used in this study were selected from a large sorghum collection after establishing their response to Al in a nutrient solution (Too 2011). MCSR P5, MCSR 124, MCSR 106, ICSR 110, and MCSR 15 are resistant to Al stress, whereas MCSR 60 is moderately resistant, and Seredo, MCSR L5, and MCSR M45b are sensitive to the Al stress. MCSR P5, MCSR L5, and MCSR M45b are Kenyan inbred lines, whereas Seredo is a commercial cultivar grown in East Africa. ICSR 110 is an Al-resistant line from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT). MCSR 124, MCSR 106, MCSR 15, and MCSR 60 are recombinant inbred lines developed from a cross between Seredo and ICSR 110.

Sorghum seeds were surface sterilized and germinated as described by Ringo *et al*. (2010). Seedlings with similar root lengths were subjected to 0, 27, and 39 μM Al^{3+} in nutrient solution for 24, 48 and 120 h with gentle and continuous aeration as described by Magalhaes *et al.* (2004). Temperature was maintained at 28 °C, a photo-period was 7 h, and a photon flux density 200 μmol m^{-2} s^{-1}. The experiment was laid out in a completely randomized design with three replicates for each treatment and seven seedlings per replicate. After 24, 48, or 120 h under the control conditions or Al stress, the root growth was determined by the measurement of seminal root length.

The extraction and quantification of callose were done according to procedures described by Koehle *et al*. (1985) and Bhuja *et al*. (2004). Root tips were collected in 2-cm^3 Eppendorf tubes and fixed in 96 % (v/v) ethanol for 1 h and afterwards thoroughly rinsed in double distilled water, blotted dry, and weighed. The root tips were then ground in 0.2 cm^3 of 1 M NaOH in a mixer mill (*MM 400*, *Retsch*, Haan, Germany) at a frequency of 25 Hz for 2.5 min and then 0.6 cm^3 of 1 M NaOH was added to each tube and mixed. The homogenates were heated in a water bath at 80 °C for 30 min, cooled down to room temperature, and centrifuged at 16 500 *g* for 3 min. The clear supernatant was used for callose quantification. The reaction mixture contained 0.2 cm^3 of the Pachyman standard, 0.4 cm^3 of 0.1 % (m/v) aniline blue, 0.21 cm^3 of 1 M HCl, 0.59 cm^3 of 1 M glycine (pH 9.5), and 0.2 cm^3 of homogenate. The mixture was incubated in a water bath at 50 °C for 30 min and cooled down to room temperature. Fluorescence was measured using a spectrophotometer *F-2000* (*Hitachi*, Tokyo, Japan) with a 10 nm spectral slit width at 394 nm (the excitation) and 484 nm (the emission). A standard calibration curve was made using a freshly prepared Pachyman solution containing β-1,3-glucan (*Megazyme International*®, Ireland) in 1 M NaOH. The callose content was expressed as micrograms of Pachyman equivalent (PE) per gram fresh root mass.

The localization of Al in root tips was assessed in the cvs. ICSR 110 and MCSR L5 by Morin staining as described by Illéš *et al*. (2006). Young seedlings were grown for 24 h in a nutrient solution with or without 27 μM Al^{3+}. Roots were harvested, rinsed in deionized distilled water, stained in 15 cm^3 of 100 μM Morin in methanol for 30 min and rinsed again. Morin-Al fluorescence of stained root tips was visualized and photographed using a fluorescence microscope (*Leica DMLB*, Wetzlar, Germany) equipped with band pass BP 470 - 490 nm and BP 515 - 560 nm excitation filters and fitted with a *Leica DC 300* digital camera. In the same cultivars, root cell plasma membrane integrity was evaluated using the Evans blue staining technique (Baker and Mock 1994). Freshly harvested roots were stained in 0.25 % (m/v) solution of Evans blue for 15 min and then rinsed in distilled water for 30 min. Cross-sections were made from the 1 - 2 mm zone of the root apex and examined under a light microscope.

Data concerning the root growth and callose content were subjected to the analysis of variance, and means were compared using Tukey's HSD test at 5 % level of significance.

The exposure to 27 μM Al^{3+} for 24 h induced significant ($P < 0.05$) reduction in seedling root growth in MCSR L5, Seredo, ICSR 110, MCSR 60, and MCSR M45b (Table 1). A similar response was observed in these cultivars together with MCSR P5 following 48 h of the Al^{3+} treatment. However, this Al^{3+} treatment caused only a minimal reduction in root growth in MCSR 15, MCSR 106, and MCSR 124. A significant ($P < 0.05$) reduction in root growth after the treatment with 27 μM Al^{3+} for 120 h was registered in MCSR L5, Seredo, MCSR 60, and MCSRM 45b (Table 1), but the inhibitory effect was not observed in MCSR 15, ICSR 110, MCSR 106, MCSR 124, or MCSR P5. ICSR 110 and MCSR 124 had the highest root growth at 120 h of treatment with 27 μM Al^{3+}. The reduction in root growth in all the cultivars was induced by the 39 μM Al^{3+} at all exposure periods.

The 27 μM Al^{3+} treatment significantly ($P < 0.05$) increased callose content in the cultivars MCSR L5, Seredo, and MCSR M45b after 24 h of exposure (Table 1). However, after 48 h only two of the Al-sensitive cultivars (MCSR L5 and MCSR M45b) maintained a high callose production and the callose content decreased in the Al-resistant cultivars ICSR 110, MCSR 15, MCSR 106, and MCSR 124 and decreased even further after 120 h of the Al treatment. Regardless of the exposure period, the 39 μM Al^{3+} treatment induced a significant ($P < 0.05$) increase in the callose content in all the sorghum cultivars (Table 1). Moreover, there was a significant negative correlation ($r^2 > 0.75$) between the Al-induced callose content and root growth after 24, 48, and 120 h of the Al^{3+} treatment.

Fluorescence micrographs of Morin-stained root tips after the Al exposure for 24 h showed no staining root tips of seedlings grown in the Al-free nutrient solution. Fluorescence was lower for the Al-resistant (ICSR 110;

Table 1. The root length and callose content in selected sorghum cultivars after 24, 48, and 120 h of exposure to different concentrations of Al^{3+}. Means ± SE, n = 21; means with different letters are significantly different at $P < 0.05$ according to the Tukey's HSD test.

Cultivar	Exposure [h]	Root length [cm]			Callose content [μg(PE) g^{-1}(f.m.)]		
		0 μM Al^{3+}	27 μM Al^{3+}	39 μM Al^{3+}	0 μM Al^{3+}	27 μM Al^{3+}	39 μM Al^{3+}
MCSR L5	24	2.03 ± 0.16a	0.53 ± 0.16h	0.55 ± 0.16h	0.59 ± 0.28l	3.58 ± 0.40c-h	3.66 ± 0.34c-h
	48	3.23 ± 0.28a-d	0.85 ± 0.28j-l	0.58 ± 0.28l	1.09 ± 0.39lm	4.29 ± 0.32bcd	4.44 ± 0.27abc
	120	7.05 ± 0.56bc	1.20 ± 0.56hi	0.63 ± 0.56i	0.64 ± 0.35g	4.43 ± 0.35cd	4.53 ± 0.38bc
Seredo	24	1.24 ± 0.15c-f	0.60 ± 0.19gh	0.62 ± 0.15gh	1.33 ± 0.24j-l	4.52 ± 0.24abc	4.98 ± 0.26ab
	48	2.62 ± 0.25b-f	1.33 ± 0.28i-l	0.81 ± 0.25j-l	1.25 ± 0.25klm	3.71 ± 0.32c-e	5.34 ± 0.26a
	120	5.40 ± 0.56c-e	2.67 ± 0.65gh	0.85 ± 0.65hi	1.35 ± 0.50fg	3.95 ± 0.50cd	6.28 ± 0.71a
MCSR 60	24	1.60 ± 0.16a-d	0.95 ± 0.16e-h	0.83 ± 0.16e-h	1.24 ± 0.28j-l	3.94 ± 0.31c-h	5.10 ± 0.28ab
	48	3.23 ± 0.28a-d	1.73 ± 0.28f-j	1.03 ± 0.28j-l	1.28 ± 0.27j-m	3.59 ± 0.27c-e	5.36 ± 0.35a
	120	7.98 ± 0.56ab	3.85 ± 0.56fg	1.63 ± 0.56hi	1.04 ± 0.41g	2.49 ± 0.41ef	4.24 ± 0.35cd
MCSR M45b	24	1.93 ± 0.19ab	1.07 ± 0.19d-h	0.57 ± 0.19h	0.70 ± 0.28gh	3.02 ± 0.28gh	3.31 ± 0.28e-h
	48	3.60 ± 0.33a	1.43 ± 0.33h-k	0.63 ± 0.33kl	0.89 ± 0.39m	2.96 ± 0.32e-h	3.29 ± 0.32def
	120	6.60 ± 0.56b-d	1.62 ± 0.56hi	0.70 ± 0.56i	1.06 ± 0.38g	4.24 ± 0.35cd	5.61 ± 0.35ab
MCSR 106	24	1.40 ±0.16b-e	0.83 ± 0.16e-h	0.65 ± 0.16gh	1.90 ± 0.31jk	4.25 ± 0.34b-e	5.36 ± 0.34a
	48	2.05 ± 0.28e-i	1.65 ± 0.28g-l	0.95 ± 0.28i-l	1.85 ± 0.35h-l	3.22 ± 0.29e-g	4.77 ± 0.27ab
	120	4.68 ± 0.56ef	4.20 ± 0.65e-g	1.48 ± 0.56hi	1.16 ± 0.38g	3.77 ± 0.35cd	4.29 ± 0.45cd
ICRS 110	24	1.75 ± 0.16a-c	1.10 ± 0.16d-h	0.85 ± 0.16e-h	1.13 ± 0.34j-l	3.14 ± 0.34fgh	3.69 ± 0.34c-h
	48	3.30 ± 0.28a-c	2.33 ± 0.33d-h	1.38 ± 0.28i-l	1.15 ± 0.27klm	2.24 ± 0.27g-k	3.88 ± 0.32b-e
	120	9.30 ± 0.56a	6.00 ± 0.56c-e	2.48 ± 0.56g-i	0.66 ± 0.35g	1.12 ± 0.41g	4.60 ± 0.35bc
MCSR 15	24	1.58 ± 0.16a-d	1.08 ± 0.16d-h	0.95 ± 0.16e-h	1.56 ± 0.40j-l	3.59 ± 0.31c-h	4.37 ± 0.34a-d
	48	2.90 ± 0.28a-e	2.33 ± 0.33d-h	1.28 ± 0.28i-l	1.57 ± 0.39i-m	2.51 ± 0.27f-i	5.37 ± 0.32a
	120	7.08 ± 0.65bc	5.28 ± 0.56c-f	2.50 ± 0.56g-i	1.48 ± 0.41fg	1.61 ± 0.35fg	3.82 ± 0.25cd
MCSR 124	24	1.55 ± 0.16a-d	1.38 ± 0.16c-f	0.85 ± 0.16e-h	2.02 ± 0.40ij	2.86 ± 0.40hi	3.76 ± 0.31c-h
	48	2.80 ± 0.28a-e	2.40 ± 0.33c-g	1.15 ± 0.28i-l	1.98 ± 0.29h-l	2.10 ± 0.35g-k	3.76 ± 0.29c-e
	120	6.58 ± 0.56b-d	6.08 ± 0.56c-e	3.88 ± 0.56fg	1.06 ± 0.35g	1.62 ± 0.35fg	3.46 ± 0.35cde
MCSR P5	24	1.58 ± 0.19a-d	0.83 ± 0.19e-h	0.53 ± 0.19h	0.92 ± 0.28kl	3.35 ± 0.28d-h	4.17 ± 0.28b-f
	48	3.45 ± 0.28ab	1.25 ± 0.28i-l	0.80 ± 0.28j-l	0.99 ± 0.29m	3.69 ± 0.32c-e	3.77 ± 0.27c-e
	120	5.10 ± 0.56d-f	3.60 ± 0.65fg	0.90 ± 0.56hi	1.62 ± 0.41fg	1.70 ± 0.35fg	6.09 ± 0.35a

Fig. 1*A*) than the Al-sensitive (MCSR L5; Fig. 1*B*) sorghum cultivar especially in cells just behind the root cap.

There was a significant difference in the intensity of Evans blue dye in root tips of the sorghum cultivars with contrasting resistances to Al; staining was lower in root sections of the Al-resistant (Fig. 1*C*) than Al-sensitive (Fig. 1*D*) cultivar.

The Al-sensitive sorghum cultivars had a severe root growth inhibition, whereas no significant root growth inhibition was observed in the Al-resistant cultivars. Reduced root growth under Al stress has been previously reported in sorghum with a less inhibition observed in resistant cultivars (Magalhaes *et al.* 2004, Ringo e*t al*. 2010). All the sorghum cultivars expressed the early (24 h) accumulation of callose in response to the Al exposure. However, the callose content in the Al-resistant cultivars decreased and reached almost undetectable levels at 120 h of exposure to the Al stress. Conversely, the callose content in the Al-sensitive cultivars increased with prolonged exposure to the Al stress. The callose content was negatively correlated with the root growth in the presence of Al. The negative effect of callose on root elongation is probably because of channelling substrates to callose formation (Kaneko *et al.* 1999) and an inhibition of the cell-to-cell translocation of nutrients. Callose inhibits the cell-to-cell transport of substances by blocking plasmodesmata (Sivaguru *et al*. 2000). Callose comprises 1,3-β-D-glucans whose deposition is aggravated by stress (Pirselova and Matusikova 2013). The Al-resistant sorghum cultivars seem to minimize the entry of Al into root cells, and hence prevent cell damage (Ahn and Mastumoto 2006).

In this study, the greater reduction in root growth observed in the Al-sensitive that in Al-resistant sorghum cultivars coupled with an increased deposition of callose was an indication of cell injury as a result of the Al stress. However, the resistant sorghum cultivars appeared to recover from the injury after prolonged exposure to the moderate but not high concentration of Al. Therefore, the early callose accumulation was a sensitive physiological marker for Al injury in sorghum. This finding is in agreement with earlier studies, where Al has been shown to increase callose formation in Al-sensitive genotypes of wheat (Bhuja *et al.* 2004, Silva *et al.* 2012), maize (Narro and Arcos 2010), and rice (Alvim *et al.* 2012). In

contrast, Yang *et al.* (2012) did not record a correlation between the callose content and reduced root growth in Al-stressed common bean. The decrease in callose accumulation over time, particularly in the Al-resistant sorghum cultivars, is probably an evidence of the existence of a more efficient regulatory mechanism of callose synthesis and degradation. Sensitive cultivars seem to be lacking such a mechanism. Recovery studies in wheat (Silva *et al*. 2012) suggest that after the Al induction of callose synthesis, the degradation mechanism in sensitive genotypes collapses. In the current study, some of the Al-resistant sorghum cultivars deposited less callose after a short period of exposure to the Al stress than others. One possible explanation for this is the genetic differences inherent in each cultivar.

The Al-resistant sorghum cultivar had lower

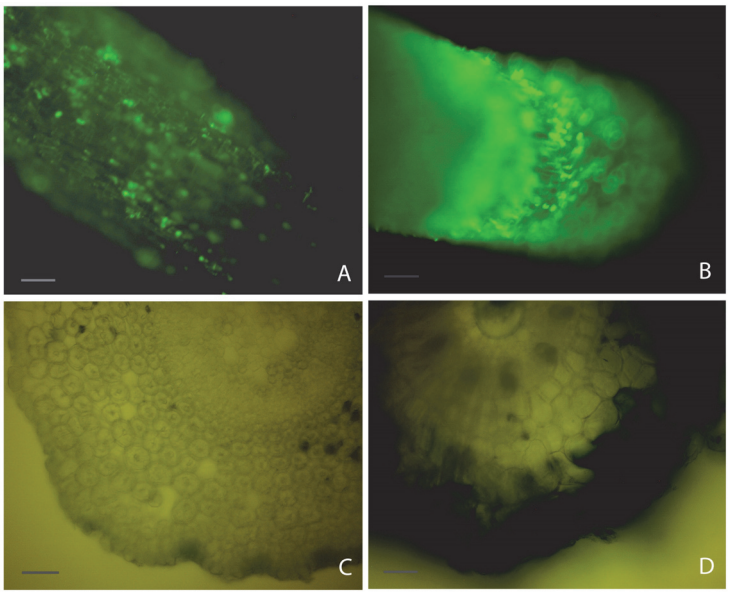

Fig. 1. Staining root tips and sections of resistant (ICSR 110) and sensitive (MCSR L5) sorghum cultivars in response to 27 μM Al^{3+} for 24 h (*A*,*B*) or 48 h (*C*,*D*): *A*,*B* - fluorescence micrographs of Morin-stained root tips of ICSR 110 and MCSR L5, respectively, *scale bars* 100 μm; *C*,*D* - Evans blue stained root sections of ICSR 110 and MCSR L5, respectively, *scale bars* 50 μm.

fluorescence of an Al-Morin complex after the exposure to the Al stress than the sensitive one suggesting that it accumulated less Al in the root tip cells than the Al-sensitive cultivar. This is similar to earlier reports for maize (Garzón *et al.* 2011) and wheat (Silva *et al*. 2012), that tolerant cultivars accumulate less Al in the root apex than sensitive ones. The region of the greatest fluorescence indicated the Al accumulation in the 1 - 2 mm zone from the root tip which is the distal transition zone (DTZ). These results are similar to the findings of Sivaguru and Horst (1998). Aluminium in DTZ presumably interferes with a signalling system involved in the regulation of cell elongation (Sivaguru and Horst 1998). The Al-resistant sorghum cultivar seemed to have a mechanism of excluding Al from the root apex. Although the mechanism of Al exclusion in sorghum was not investigated, organic acid exudation is often a part of Al resistance mechanisms in various plant species and could be operating in sorghum (Kochian *et al*. 2005, Magalhaes *et al*. 2007).

The root cells of the Al-sensitive cultivar were deeply stained with Evans blue which is the evidence for a leaky plasma membrane. Evans blue is a non-permeable dye and it only passes through a compromised plasma membrane to stain the cytoplasmic contents (Baker and Mock 1994, Tamas *et al.* 2006). The Al-induced damage of the membranes is fairly rapid, and appears to be mediated through strong binding to phosphate groups of cell membrane phospholipids (Gunse *et al.* 1997) thus depolarising the cell membrane in root apices (Illéš *et al.* 2006). Al stress causes lipid peroxidation (Martins *et al*. 2013), oxidative stress (Xu *et al.* 2012), and altered lipid composition of the plasma membrane (Peixoto *et al.* 2001) that subsequently can modify membrane properties and function. Therefore, the Al-induced production of reactive oxygen species may be partly responsible for Al inhibition of root growth (Yamamoto *et al.* 2002). This can be the reason why in some plant species, Al-resistant genotypes have enhanced the protection against oxidative stress through an increased antioxidant activity (Cartes *et al.* 2012, Xu *et al*. 2012).

In conclusion, the Al-resistant sorghum cultivars accumulated less Al in the root tips, maintained cell membrane integrity, recovered from the Al-induced callose accumulation over time and had comparatively better root growth compared to the sensitive ones. The sensitive cultivars, on the other hand, absorbed more Al and deposited more callose in their root tips due to compromised membrane integrity. The study has revealed the potential of these Al resistant sorghum genotypes for developing cultivars adapted to acid soils where Al stress is a major constraint to sorghum productivity.

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