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Dormancy breakage of *Stylosanthes humilis* seeds by aluminium

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Abstract

Physiological dormancy of scarified seeds of Townsville stylo (*Stylosanthes humilis* HBK) was released by acidic aluminium (Al^{3+}) solution. Antiethylenic substances inhibited germination of low-pH-stimulated dormant seeds, with a correspondingly low ethylene production and low activity of 1-aminocyclopropane-1-carboxylate (ACC) oxidase in seeds. On the other hand, antiethylenic substances did not decrease the germination of Al^{3+}-stimulated seeds, but ACC oxidase activity and ethylene production by the seeds was decreased to a large extent. These data provide evidence that dormancy breakage by Al^{3+} differs from that caused by low pH and is not associated with ethylene production. Similarly to Al^{3+} action, methyl viologen (MV), a reactive oxygen species-generating compound, broke dormancy of Townsville stylo seeds. Sodium selenate and *N*-acetyl cysteine, antioxidant compounds, largely decreased germination of MV- and Al^{3+}-stimulated dormant seeds. Altogether these data point to oxidative radicals constituting key molecules in the chain of events triggered by Al^{3+} leading to dormancy breakage.

Keywords: aluminium, dormancy, ethylene, seeds, reactive oxygen species, *Stylosanthes humilis*

Introduction

Townsville stylo (*Stylosanthes humilis*) is a tropical forage legume known to exhibit good persistence and productivity in acid soil (Williams *et al.*, 1984; Li *et al.*, 2009). Its tolerance to low-fertility soils grants it the potential to improve native pastures in tropical and subtropical regions (Noble *et al.*, 2000). As with some other legumes, seeds of Townsville stylo exhibit dormancy, resulting from a relatively hard seed coat and also, when freshly harvested, a physiological imposed dormancy. Any stressing factor such as low-pH solution (Pelacani *et al.*, 2005a, b), divalent heavy metals (Delatorre and Barros, 1996) and selenium compounds at high concentrations (Pinheiro *et al.*, 2008a), which induce ethylene production by seeds, promotes the breakage of physiological dormancy of seeds of that leguminous species.

In some biological systems, many stressors can induce the generation of reactive oxygen species (ROS) (Apel and Hirt, 2004), that have been established as key signalling molecules. At low concentrations, ROS have been proposed to play a fundamental role in seed germination and dormancy (Bailly, 2004; Oracz *et al.*, 2007; Müller *et al.*, 2009). On the other hand, at high concentrations and in certain situations, ROS can be toxic (Bailey-Serres and Mittler, 2006). The effects of ROS in plant systems can be simulated by the ROS-generating compound methyl viologen (MV) (Foyer *et al.*, 1994). Thus, MV has been largely used as a powerful tool to assess the role of ROS as signalling molecules. Since the effects of reactive oxygen molecules at the cellular level are mediated by their production and removal via antioxidant activity (Neill *et al.*, 2002), the use of free-radical quenchers may help to identify the role of ROS in plant systems. Selenium compounds and *N*-acetyl cysteine (NAC) are interesting in this matter, since it has been demonstrated that in response to oxidative stress, selenium compounds at low concentration (Seppänen *et al.*, 2003) and NAC (Lobréaux *et al.*, 1995) perform protective functions by scavenging the free radicals.

Aluminium, a potent stressor, induces ROS production and ethylene biosynthesis in plants (Cakmak and Horst, 1991; Massot *et al.*, 2002). Because both ROS and ethylene could contribute to seed dormancy breakage, it remains to be determined whether Al^{3+} acts through the induction of ethylene production or through the formation of free radicals. In order to separate those effects of the trivalent ions, Al^{3+}-stimulated seeds were treated with antiethylenic

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compounds or were provided with the ROS-scavenging compounds sodium selenate and NAC. Furthermore, seeds were treated with MV in an attempt to investigate whether or not ROS play any role in seed dormancy breakage.

Materials and methods

Plant material and germination assays

Plants of Townsville stylo were grown in 3.0 litre plastic pots in a greenhouse in Viçosa (20°45′S, 42°15′W), Minas Gerais, Brazil. Mature pods were harvested and stored in the laboratory (25–30°C). Seeds of several postharvest ages and with correspondingly different dormancy stages were always available for the assays. Seeds were dehusked, scarified with fine sandpaper (no. 150) for a few seconds, sterilized with 0.5% NaOCl for 10 min and thoroughly washed with distilled water. In order to infiltrate the test solutions into seeds they were submitted to 3 min vacuum (84 kPa) followed by 4 min without vacuum and an additional 3 min vacuum. Fifty seeds were placed in glass Petri dishes, 90 mm diameter, or in 50-ml Erlenmeyer flasks containing two layers of Whatman no. 1 filter paper moistened with 10 and 5 ml of test solution, respectively. Petri dishes and flasks with the filter paper were previously heated at 105°C for 4 h. Petri dishes and flasks containing seeds were placed in the dark in a day/night growth chamber (Forma Scientific Inc., Ohio, USA) at 30°C. A seed was considered as germinated upon protrusion of its radicle. Comparison of germination percentage and efficiency amongst the compounds was based on the determination of t_{50}, i.e. the time required for 50% germination to occur.

Aluminium effects on dormancy

Dormant seeds were transferred to Petri dishes containing $AlCl_3$ (10^{-4}–10^{-1} M) dissolved in 50 mM HEPES at pH 3.0; this was attained with 0.5 M HCl. Under $AlCl_3$ concentrations employed, and also due to the very high chemical activity of Al, HEPES was the only buffer compound tested in which Al^{3+} did not precipitate in the solutions. pH was measured in the Petri dishes at different times in order to assure the maintenance of the pH medium.

In order to search for any kind of relationship between Al^{3+} and ethylene production, the effects of inhibitors of ethylene biosynthesis and action on Al^{3+}-induced seed dormancy breakage were examined. The inhibitors of ethylene biosynthesis, 2-aminoethoxyvinylglycine (AVG, 10^{-4} M) and $Co(NO_3)_2$ (10^{-3} M), and abscisic acid (ABA, 10^{-8}–10^{-5} M), which in Townsville stylo seeds also inhibit the activity of 1-aminocyclopropane-1-carboxylic acid (ACC) (Vieira and Barros, 1994), were provided in 50 mM HEPES, pH 3.0 alone or also containing $AlCl_3$ (10^{-2} M). For the treatment with the gaseous compound 1-methylcyclopropene (1-MCP), an inhibitor of ethylene action, the gas was produced from Ethylbloc™ (active ingredient 0.14%), as described by Desikan *et al.* (2006). From the stock flask, known amounts of gas were syringed out and injected into other Erlenmeyer flasks containing imbibed seeds. Flask atmospheres were occasionally stirred using a syringe with the needle inserted through the rubber seals.

A causal association between Al^{3+}-induced ROS production and dormancy breakage was searched for by exposing dormant seeds to combined solutions of Al^{3+} plus sodium selenate (Na_2SeO_4, 10^{-6} M) or NAC (10^{-3} M), free-radical quenching compounds. Dormant seeds were also exposed to a solution of the ROS-generating compound MV (10^{-4} M), alone or solutions of MV plus either SeO_4^{2-} or *N*-acetyl cysteine dissolved in HEPES (50 mM, pH 3.0 or 7.0).

Ethylene measurement

Erlenmeyer flasks containing seeds imbibed in test solutions were stoppered with a rubber serum cap, and kept in the growth chamber under the conditions previously described. Air samples (1 ml) were taken from the flask headspace and injected into a gas chromatograph (Hewlett Packard 5890, Series II), equipped with a stainless-steel column (1.0 m × 6.0 mm) packed with Porapak-N 80–100 mesh. Ethylene quantitation was conducted under the following conditions: nitrogen carrier gas and hydrogen fluxes were 30 ml min^{-1}; air flux was 320 ml min^{-1}. Column, injector and detector temperatures were 60, 110 and 150°C, respectively (Saltveit and Yang, 1987). Ethylene peaks were registered by PeakSimple software (Peak Simple, Version 3.92, SRI Instruments, Torrance, California, USA) coupled to the chromatograph, and quantified by comparison with authentic ethylene standards.

ACC oxidase activity measurement

Petri dishes (15 cm diameter) containing 150 seeds on two layers of filter paper and 16 ml of test solutions were transferred to the growth chamber at 30°C for 48 h, after which seeds were thoroughly washed with distilled water, paper-dried and frozen in liquid nitrogen. ACC oxidase activity was quantified as described by Mohamed *et al.* (2001) with slight modifications. Seeds were ground with a mortar and pestle and 4 ml of extraction buffer containing 0.1 M

Tris (pH 7.0), 10% (w/v) glycerol, 30 mM sodium ascorbate, 1 mM dithiothreitol, 0.1% (v/v) Triton X-100 and 5% (w/v) polyvinyl-pyrrolidone (PVP). The slurry was centrifuged at 28,000 *g* for 20 min and the supernatant was desalted by passing through a Sephadex G-25 column (Pharmacia PD-10; Amersham Pharmacia Biotech, Bucks, UK) eluted with the reaction buffer (without ACC). The desalted extract was used immediately for the assay of ACC oxidase activity. An aliquot (0.2 ml) of the extract was incubated in a reaction buffer containing 1.8 ml extract buffer (without PVP), 50 μM $FeSO_4$, 30 mM $NaHCO_3$ and 1 mM ACC. Tubes were stoppered with serum caps and incubated in a water-bath at 32°C for 1 h. Ethylene produced was determined as described above. No ACC oxidase activity was observed with boiled extract or when extract was omitted from the incubation medium. Protein concentrations in the various extracts were determined according to Bradford (1976), using bovine serum albumin as a standard.

Statistical analysis

Statistical design of the assays was based on a completely randomized distribution with five replicates with 50 seeds each for germination test in Petri dishes and ethylene determinations in Erlenmeyer flasks. For quantification of ACC oxidase activity five replicates of 150 seeds each were assayed. Differences in germination data were analysed for statistical significance by analysis of variance (ANOVA). Germination percentage was transformed to arcsin $(\%G/100)^{1/2}$, prior to analysis and all data were checked for normality. Differences among means were tested according to the Scott–Knott test for germination. A one-way analysis of variance test (ANOVA) was used to determine the statistical significance for ethylene and ACC oxidase activity, and Tukey's mean separation procedures were used to determine differences between treatments. All mean comparisons were performed with SPSS (Statistical Package for the Social Sciences) 11.0 for Windows (SPSS Inc., Chicago, Illinois, USA).

Results

Physiological dormancy of Townsville stylo seeds was broken by acidic Al^{3+} solutions (pH 3.0) (Fig. 1); germination enhanced by Al^{3+} still occurred at pH 3.5 but not 4.0 (data not shown). At pH 3.0, Al^{3+} enhanced germination of dormant seeds at all concentrations employed, except for 10^{-1} mM, in which it was severely reduced, the result of a toxic effect, since germination was not restored by thiourea (not shown), a compound used to assess Townsville stylo seed viability (Delatorre *et al.*, 1997). Germination kinetics of imbibed seeds over 72 h showed that dormancy was broken quite rapidly by Al^{3+}. At 10^{-2} M Al^{3+} (pH 3.0) the time taken for 50% germination (t_{50}) to occur was 13.5 h, with a maximum germination (G_{max}) of 90%. Low pH (3.0) alone (control) was not as effective as Al^{3+} in the process, as shown by t_{50} values (47.0 h) and G_{max} (about 58%) (Fig. 1), characterizing a probably different mode of action between the two dormancy-breaking agents.

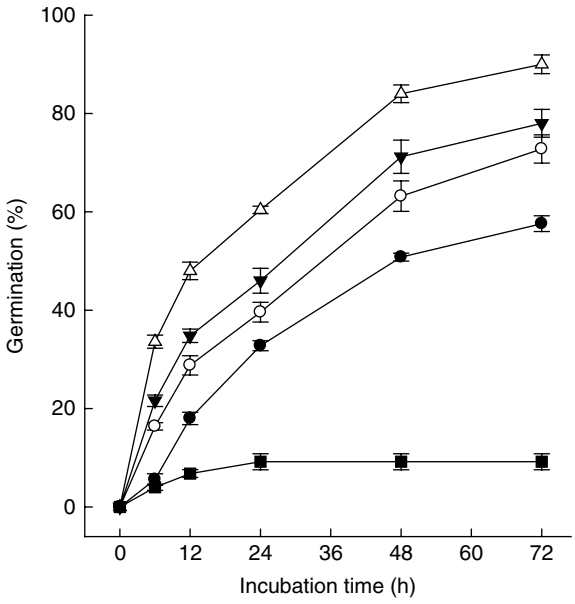

Figure 1. Al^{3+} breaks dormancy of Townsville stylo seeds. Seeds (about 46 post-harvest days old) were exposed to 50 mM HEPES solution (pH 3.0) containing (●) 0, (○) 10^{-4}, (▼) 10^{-3}, (△)10^{-2} or (■) 10^{-1} M $AlCl_3$. Germination in HEPES solution at pH 7.0 was nil, 72 h after the start of imbibition with test solutions. Data points are means of five replicates ± standard error.

Germination of dormant seeds stimulated by low-pH solution (without Al^{3+}) was depressed by AVG, Co^{2+}, inhibitors of ethylene biosynthesis, and by ABA (Fig. 2). On the other hand, these anti-ethylenic compounds and ABA did not promote a decrease in germination of Al^{3+}-stimulated seeds, suggesting that, unlike the acidic condition alone, ethylene was not involved in the process. The lack of any relationship between ethylene and seed dormancy breakage promoted by Al^{3+} was confirmed by the fact that 1-MCP, an inhibitor of ethylene action, also failed to decrease germination of Al^{3+}-treated seeds, whereas there was a clear dose–response inhibition in seeds imbibed with the acid solution only (Fig. 2).

That Al^{3+} seemed to be acting independently of ethylene on dormancy breakage was further demonstrated by treating dormant seeds with Al^{3+} and inhibitors of ethylene biosynthesis (AVG plus Co^{2+})

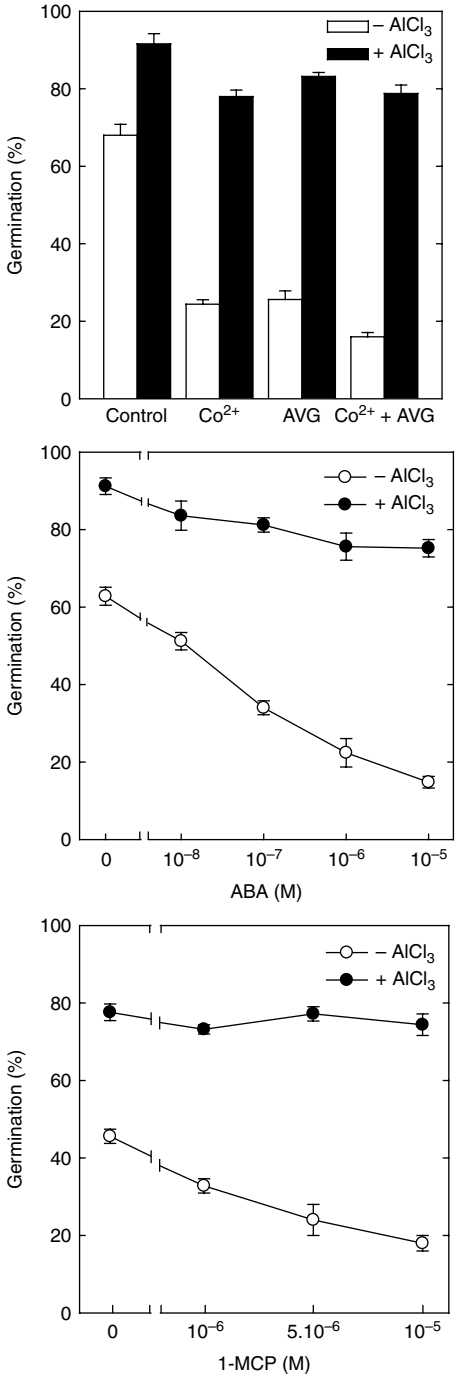

Figure 2. Inhibition of ethylene biosynthesis and action does not prevent germination of Al^{3+}-stimulated dormant seeds. 2-Aminoethoxyvinylglycine (AVG), Co^{2+} and abscisic acid (ABA) were provided to seeds (about 83 d old) in HEPES solution (pH 3.0) alone or also containing $AlCl_3$ (10^{-2} M), pH 3.0. For 1-methylcyclopropene (1-MCP) treatment, seeds (about 22 d old) were imbibed in acid solution (pH 3.0) alone or Al^{3+} solution (pH 3.0) and then 1-MCP gas was injected into sealed Erlenmeyer flasks. Germination in HEPES solution at pH 7.0 was nil (seeds about 22 d old) or 5.2 ± 0.6% (seeds about 83 d old), 72 h from the start of incubation. Data points are means of five replicates ± standard error.

and ABA and monitoring ethylene production and germination (Fig. 3). After exposure for 12 h, when radicles of seeds were just protruding, germination and ethylene production were impaired completely by the inhibitors only in dormant seeds treated with pure acid solution; without the inhibitors ethylene was produced and germination occurred (Fig. 3, inset). Suppression of ethylene synthesis by AVG + Co^{2+} and by ABA did not prevent germination of Al^{3+}-stimulated seeds. After 48 h incubation, when seedling growth was just initiated, the inhibitors largely depressed ethylene production in dormant seeds treated with Al^{3+}, but germination was appreciable, above 74% (Fig. 3). However, germination of seeds treated with the pure acidic solution was greatly reduced by inhibitors of ethylene biosynthesis and ABA, with a correspondingly low ethylene production. In keeping with these responses, the Al^{3+}-induced increase in activity of ACC oxidase was substantially reduced by Co^{2+}, but germination of Al^{3+}-stimulated seeds was not depressed by the inhibitor. On the other hand, Co^{2+} promoted a decrease in activity of ACC oxidase of control (pure acid)-treated seeds, which kept a close relationship with the low germination (Table 1). Since ethylene was not required for Al^{3+}-promoted dormancy breakage, other mechanisms of action of the ion on dormancy breakage of Townsville stylo seeds was likely to be operating.

A common feature of several stresses, including Al^{3+} toxicity, is the enhanced production of reactive oxygen species (ROS). Thus, in order to examine a putative involvement of ROS in Al^{3+}-induced dormancy breakage, sodium selenate, an inhibitor of the action of oxidative radicals, was employed. Treatment of dormant seeds with acidic solution containing SeO_4^{2-} had no effect on the germination response or on ethylene emanation. On the other hand, SeO_4^{2-} decreased germination of Al^{3+}-stimulated dormant seeds to the level of the control, without exhibiting any significant effect on either ACC oxidase activity (Table 1) or ethylene production by seeds (Fig. 4). Furthermore, germination speed was substantially different between seeds imbibed with Al^{3+} alone and Al^{3+} plus SeO_4^{2-}. For instance, t_{50} was about 40 h in control seeds, but it took 12 and 38 h for Al^{3+}- and Al^{3+} plus SeO_4^{2-} -treated seeds, respectively (not shown). These observations suggested that Al^{3+} was likely to be operating through eliciting ROS generation. Results from Fig. 5 lend strong support to this hypothesis: MV, a ROS-generating compound, succeeded in breaking seed dormancy above the level of the control (treated with pure acidic solution). Furthermore, antioxidant compounds like SeO_4^{2-} or NAC largely decreased germination of MV- and Al^{3+}-stimulated dormant seeds. Additionally, AVG plus Co^{2+} were unable to inhibit the Al^{3+} and MV responses of dormant seeds (Fig. 5).

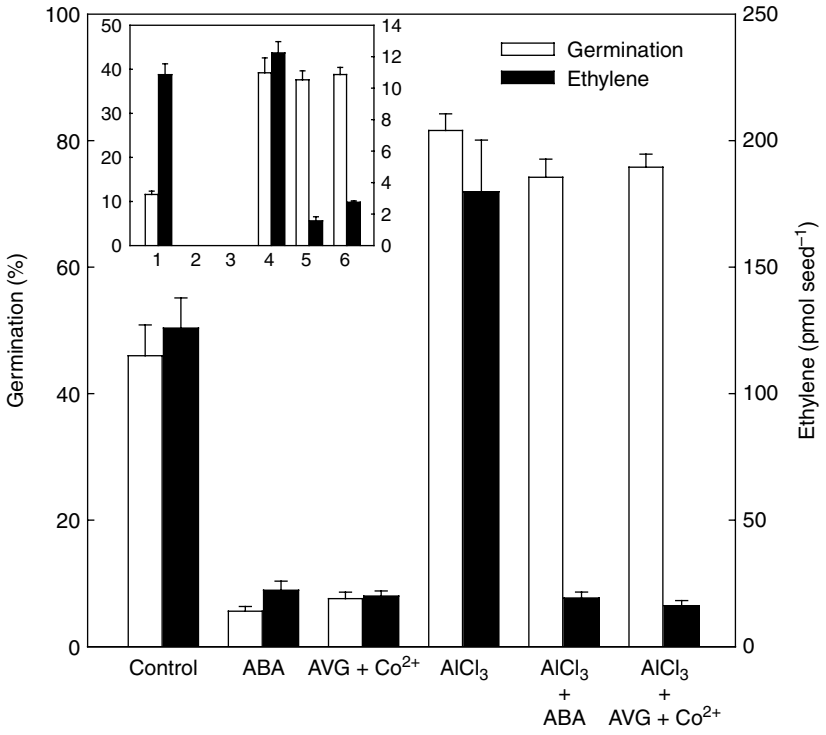

Figure 3. Ethylene production is not associated with dormancy breakage of Al-treated seeds (about 31 d old). 2-Aminoethoxyvinylglycine (AVG; 10^{-4} M) plus Co^{2+}(10^{-3} M) and abscisic acid (ABA; 10^{-5} M) were provided to seeds in HEPES solution at pH 3.0 alone or containing $AlCl_3$ (10^{-2} M) at pH 3.0. Seed germination and ethylene production are shown for 12 h (inset) and 48 h after the start of incubation. Seed germination and ethylene production were nil in HEPES solution pH 7.0, either 12 or 48 h from the start of seed incubation. Axis labels in the inset are the same as those of the main figure; treatments also keep the same order. At 12 h (inset), germination and ethylene production were nil under both ABA (2) and AVG + Co^{2+} (3) treatments. Data points are means of five replicates ± standard error.

Discussion

The data described herein demonstrate that acidic Al^{3+} solution broke dormancy of scarified Townsville stylo seeds and thus this trivalent ion must constitute another agent to be added to the list of factors that trigger seed dormancy breakage. Low-pH solutions have a marked effect on breaking dormancy of seeds of several species (Pelacani *et al.*, 2005a). In red-rice (*Orysa sativa*), for instance, Footitt and Cohn (1992) showed that embryo acidification constituted a signal for dormancy end. However, the effects of acidic Al^{3+} solutions (range 0.1–10 mM) were consistently much higher than those of low pH alone. Moreover, germination speed was faster in Al^{3+} at pH 3.0 than in pH 3.0 solution alone (control). These data suggest that the mechanisms underlying the germination of Al^{3+}- and low-pH-treated seeds are different.

Ethylene is required for germination of Townsville stylo seeds (Ribeiro and Barros, 2006), and stressors such as divalent heavy metals (Delatorre and Barros, 1996), selenium compounds (Pinheiro *et al.*, 2008a, b) and low-pH solutions (Pelacani *et al.*, 2005a, b) are also good dormancy-breaking agents, likely by triggering ethylene biosynthesis. The mechanism of action of Al^{3+} in promoting dormancy breakage was examined by using inhibitors of ethylene biosynthesis and action. Germination of Al^{3+}-treated seeds was not impaired by AVG and Co^{2+}, inhibitors of ACC synthase and ACC oxidase, respectively, or by ABA. On the other

Table 1. Effects of Co^{2+} and sodium selenate on germination and activity of ACC oxidase of Al^{3+}-stimulated dormant seeds (about 42 d old). Both parameters were assayed at 48 h from the start of incubation. Seed germination and activity of ACC oxidase in HEPES solution at pH 7.0 were 3.8 ± 0.4% and 52.5 ± 18.1 pmol ethylene (mg protein)$^{-1}$ h^{-1}, respectively. Means followed by the same letters in a column do not differ significantly at 5% level by Scott–Knot (germination) or Tukey (ethylene) test. Data are the means ± standard errors of five replicates

Treatment	Germination (%)	Ethylene [pmol (mg protein)$^{-1}$ h^{-1}]
Control (pH 3.0)	55.5 ± 1.6 b	266.8 ± 20.3 a
Co^{2+}	16.6 ± 1.0 c	87.9 ± 15.0 b
Na_2SeO_4	51.1 ± 3.5 b	268.7 ± 14.7 a
$AlCl_3$	89.1 ± 1.6 a	322.9 ± 13.7 a
$AlCl_3$ + Co^{2+}	87.2 ± 1.3 a	64.9 ± 4.3 b
$AlCl_3$ + Na_2SeO_4	58.8 ± 3.4 b	315.1 ± 22.2 a

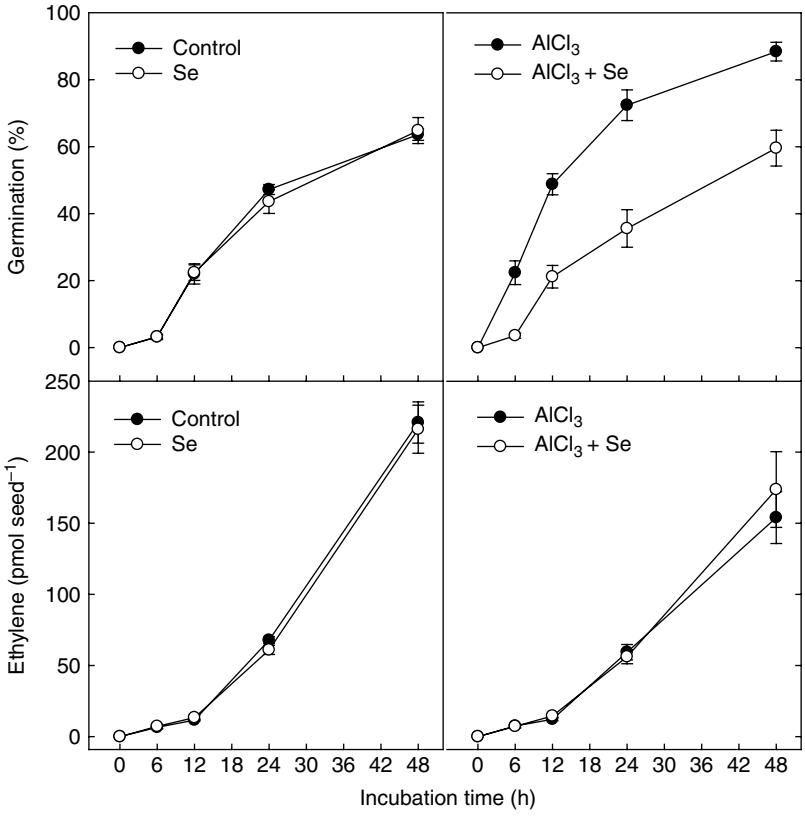

Figure 4. Sodium selenate inhibits germination of Al^{3+}-stimulated dormant seeds without reducing ethylene production. Left: effects of SeO_4^{2-} supplied in HEPES solution pH 3.0 (control) on the germination and ethylene production by dormant seeds aged about 35 d. Right: effects of SeO_4^{2-} supplied in Al^{3+} solution pH 3.0 on the germination and ethylene production by dormant seeds. Seed germination and ethylene production were nil in HEPES solution pH 7.0, at 48 h. Data points are means of five replicates ± standard error.

hand, all inhibitors of ethylene biosynthesis and action and ABA suppressed the effect of the pure acidic solution (minus Al^{3+}) on the germination of dormant seeds (Fig. 2). This suggests that, unlike the acidic solution, Al^{3+} does not act on the breakage of dormancy of Townsville stylo seed through the stimulation of ethylene biosynthesis. Measurements of ethylene production and of ACC oxidase activity in seeds treated with Al^{3+} plus inhibitors of ethylene biosynthesis confirmed this hypothesis (Fig. 3 and Table 1). After 12 h of incubation, germination and ethylene production were impaired completely by the inhibitors in seeds treated with pH 3.0 alone (Fig. 3, inset). In this regard, the inhibitors (ABA and AVG + Co^{2+}) promoted a large decrease in ethylene production by dormant seeds treated with Al^{3+} at pH 3.0, but germination was not affected. Forty-eight hours following seed incubation, comparable amounts of ethylene were accumulated in flasks containing inhibitors plus low-pH stimulated or Al^{3+}-stimulated seeds (Fig. 3); however, germination of all of the Al^{3+}-stimulated seeds was significant, indicating that other factors were modulating the germination of seeds treated with Al^{3+}. In agreement with this result, Co^{2+} inhibited ACC oxidase activity of Al^{3+}-stimulated dormant seeds, without any effect on germination (Table 1). Thus, since under pH 3.0 alone (control) inhibitors of ethylene biosynthesis depressed germination of seeds, with a correspondingly low ACC oxidase activity and ethylene production by seeds, other mechanisms of action of Al^{3+} were likely operative. The occurrence of other modes of action was further confirmed by 1-MCP. Al^{3+} at pH 3.0 and pH 3.0 without Al^{3+} (control) stimulated ethylene production of seeds (Fig. 3). However, impairment of ethylene action by 1-MCP greatly reduced the response of low-pH-treated seeds but not germination promoted by Al^{3+} (Fig. 2). This lends strong support to the hypothesis that dormancy breakage by acidic Al^{3+} solution was not a consequence of its inducing ethylene production.

It has been shown that Al^{3+} induces ROS in several species of plants (Boscolo *et al.*, 2003; Darkó *et al.*, 2004). ROS have emerged as key signalling molecules controlling a diverse range of physiological phenomena in several biological systems (Finkel and Holbrook, 2000; Bailey-Serres and Mittler, 2006). Moreover, a ROS requirement for dormancy

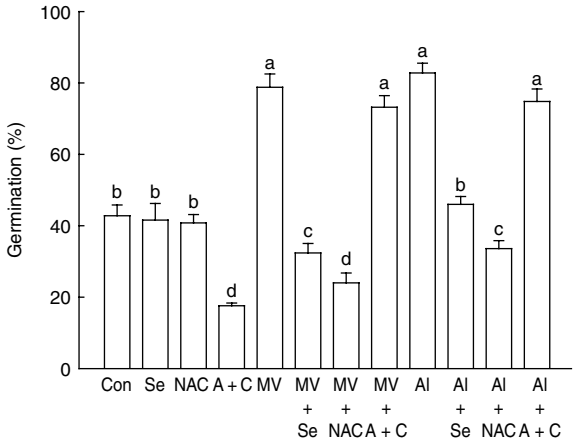

Figure 5. Effects of methyl viologen on germination of dormant seeds (about 12 d old). Con, control (HEPES solution pH 3.0); Se, sodium selenate (10^{-6} M); NAC, *N*-acetyl cysteine (10^{-3} M); (A + C), 2-aminoethoxyvinylglycine (AVG; 10^{-4} M) plus Co^{2+} (10^{-3} M); MV, methyl viologen (10^{-4} M); MV + Se, methyl viologen plus SeO_4^{2-}; MV + NAC, methyl viologen plus *N*-acetyl cysteine; MV + (A + C), methyl viologen plus (AVG + Co^{2+}); Al, $AlCl_3$ (10^{-2} M); Al + Se, $AlCl_3$ plus sodium selenate; Al + NAC, $AlCl_3$ plus *N*-acetyl cysteine; Al + (A + C), $AlCl_3$ plus (AVG + Co^{2+}). Germination in HEPES solution alone, with NAC or Se, at pH 7.0 were nil, at 72 h. Germination in MV pH 7.0 was 23.2 ± 2.8%. Bars with the same letter are not statistically different at the 5% level by Scott–Knott test. Data points are means of five replicates ± standard error.

alleviation was recently demonstrated by Oracz *et al.* (2007) and Müller *et al.* (2009). Hence, it is possible that Al^{3+} triggers dormancy breakage through eliciting the generation of oxidative radicals. This matter was investigated herein by attempting to inhibit the action of oxygen free-radicals with SeO_4^{2-}. At low concentration, this compound has been used as a scavenger for free radicals, protecting plants against damage (Stadtman, 1990; Filek *et al.*, 2008). At low concentration, SeO_4^{2-} was shown to be inactive as a dormancy-breaking agent and it did not stimulate either ACC oxidase activity (Table 1) or ethylene production (Fig. 4) by the seeds. On the other hand, SeO_4^{2-}, at high concentration, stimulates dormancy breakage of seeds of *Stylosanthes humilis* (Pinheiro *et al.*, 2008a, b). In fact, high levels of selenium can indiscriminately replace sulphur and incorporate selenium-amino acids into proteins (Eustice *et al.*, 1981; Brown and Shrift, 1982). The formation of Se-amino acids, in turn, is supposed to enhance ethylene production (Konze *et al.*, 1978), which can promote dormancy breakage of Townsville stylo seeds. When SeO_4^{2-}, at low concentrations was supplied to dormant seeds together with Al^{3+}, the germination percentage was decreased to the level of the control (Fig. 4). Hence a selenium compound at low concentration induced a decrease in germination of Al-stimulated seeds, although it did not inhibit ACC oxidase activity (Table 1) or ethylene production by the seeds (Fig. 4). Together these data provide evidence that Al^{3+}-induced dormancy breakage was likely associated with the action of ROS, but not with ethylene production. In this context SeO_4^{2-} seems to exert a dual effect on the dormancy breakage process: at low concentration it acts as an antioxidant factor, inhibiting germination of Al^{3+}-stimulated seeds; whereas at higher concentration it acts as a dormancy-breaking agent (Pinheiro *et al.*, 2008a, b).

Amounts of free Al^{3+} in Townsville stylo seeds, as measured by plasma atomic emission spectrometry, were very similar whether seeds were treated with either Al^{3+} or Al^{3+} plus SeO_4^{2-} (not shown). Therefore, the antioxidant treatment did not affect Al^{3+} loading to the seeds, but reduced germination of Al^{3+}-stimulated dormant seeds, hence acting on the Al^{3+} action itself. These data are also consistent with the hypothesis that dormancy breakage of seeds by Al^{3+} could require the action of ROS.

A probable role of ROS in dormancy breakage was further examined with the employment of MV. MV, which generates singlet oxygen ($O_2^{\cdot -}$) directly and OH· radicals as secondary activated oxygen species (Babbs *et al.*, 1989; Foyer *et al.*, 1994), constitutes an important tool for investigating the effects of reactive oxygen species in biological systems. Similarly to the effects of Al^{3+}, MV (10^{-4} M) at pH 3.0 broke dormancy of scarified Townsville stylo seeds (Fig. 5). Additionally, SeO_4^{2-} and NAC also decreased germination of both Al^{3+}- and MV-stimulated dormant seeds, whereas AVG plus Co^{2+} failed to inhibit germination of either Al^{3+}- or MV-treated seeds. These strong similarities suggest that the mechanisms underlying the germination of both Al^{3+}- and MV-treated seeds are likely the same.

In summary, the action of Al^{3+} as a dormancy-breaking agent in seeds of Townsville stylo was shown not to depend on ethylene biosynthesis or action. The effect of Al^{3+} on dormancy breakage seemed to be a consequence of a stressing condition triggering ROS generation.

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