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A dual role of selenium in the growth control of seedlings of *Stylosanthes humilis*

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ABSTRACT

The growth of seedlings of Townsville sytlo (*Stylosanthes humilis* H.B.K.) is inhibited by aluminium (Al) ions, their elongation being recovered with sodium selenate at 1.0 µM. Methyl viologen and hydrogen peroxide, reactive oxygen species (ROS)-generating compounds, also inhibited seedling elongation and again growth was relieved by selenate. Selenate, thus, seemed to be operating as a ROS quencher, since *N*-acetylcysteine (NAC), an antioxidant compound, also stimulated largely the growth of Al-inhibited seedlings. At a higher concentration (0.1 mM), however, selenate inhibited seedling growth and elongation was recovered by NAC. Ethylene production by selenate plus NAC-treated seedlings was very higher and thus the gaseous hormone was not responsible for the seedling growth inhibition caused by selenate. Hence, it seems that at high levels selenate operates as a ROS-generating compound whose effects were counteracted by NAC. It can be deduced that, at low concentration, selenate behaves as a ROS quencher and at high level as a ROS-promoting species.

Keywords: Aluminium; Ethylene; Growth Inhibition; Reactive Oxygen Species; Selenate; Townsville Stylo

1. INTRODUCTION

Plant growth is greatly affected by several environmental stresses such as drought, extreme temperatures and heavy metals. On acidic soils Al toxicity has been recognized as a major limiting factor of plant productivity [1]. Plants can respond and adapt to Al stress by altering their cellular metabolism and invoking various defense mechanisms [2]. Usually Al toxicity induces accumulation of reactive oxygen species (ROS), that have been established as key signalling molecules controlling a diverse range of physiological functions [3,4]. However, at high concentrations and in certain situations, ROS may be toxic [5].

Since the effects of reactive oxygen molecules at cellular level is mediated by their production and removal via antioxidant activity [6], the use of free radical quenchers may help to identify the role of ROS in plant systems. Selenium (Se) is interesting in this matter because in response to oxidative stresses, Se compounds at low concentrations, perform a protective function by scavenging free radicals [7,8]. On the other hand, excess Se can cause damage to plants, likely by triggering ROS generation [9,10]. This antagonistic property makes Se unique in studies dealing with systems requiring ROS to elicit a physiological response.

Toxicity caused by Se compounds is an ill-understood phenomenon [11]. There are indications that at high levels Se can indiscriminately replace S in certain amino-acids that are incorporated into proteins [11,12]. The formation of Se-aminoacids, in turn, is supposed to enhance ethylene production [13], which can cause damages to plant growth. Some biochemical and physiological studies were conducted with Se-compounds in plant systems [8,14,15], but no physiological co-action between Se and Al has been stablished. In this work the effects of Se at low concentration added to the growth medium as a protector against Al toxicity were investigated. Furthermore, the mode of action of Se, at high concentration, on seedling growth of Townsville stylo was also examined. The experiments were performed with seedlings of Townsville stylo, an annual forage legume cultivated in tropical pastures [16]. The species has been considered as a potential contributor for pasture improvement in tropical zones due to its high-quality forage for livestock, high seed production and wide adaptability to low fertility soils [17].

2. MATERIALS AND METHODS

2.1. Plant Material and General Conditions

Seeds of Townsville stylo (*Stylosanthes humilis* H.B.K.) were obtained from plants cultivated in 3.5 L plastic pots in a greenhouse in Viçosa (20°45' S, 42°15' W), Minas Gerais, Brazil and kept in the laboratory under dry conditions. Non-dormant seeds were freed from their husks, scarified with fine sandpaper (nº 150), sterilized with 0.5 % NaOCl for 10 min, and thoroughly washed with distilled water. Seeds were taken to 15 cm diameter Petri dishes with two layers of Whatman nº 1 filter paper and 16 ml of distilled water (pH 7.0). This assembly was placed in the dark in a day/night growth chamber (Forma Scientific Inc, Ohio, USA), at 30°C, for 18 h. Afterwards, germinated seeds with a protruded radicle about 3 mm long were transferred to 9 cm diameter Petri dishes with two layers of filter paper, and incubated with 10 ml test solutions. Solutions were prepared by dissolving chemicals in 0.5 mM $CaCl_2$ solution, pH 4.0, a condition that prevents proton toxicity and leads to separation of the effects of proton toxicity from the effects of Al toxicity [18]. After 24 h exposure period, root and hypocotyl lengths of the seedlings were determined.

2.2. Se Effects on Growth

Sodium selenate (Na_2SeO_4) was chosen as to represent the several soluble Se compounds whose effects on dormancy breakage of Townsville stylo seeds are all identical [14]. The effects of Na_2SeO_4, at low concentration (1 µM), on Al-treated seedling were examined by providing the compound to seedlings in the solutions of $AlCl_3$ (1.0, 1.5 and 2 mM). In order to assess for a causal association between Al-induced ROS production and growth inhibition, seedlings were also exposed to combined solutions of $AlCl_3$ plus *N*-acetylcysteine (NAC, 1 mM), a free-radical quenching compound. Seedlings were also exposed to solution of methyl viologen (MV, 10^{-7} - 10^{-4} M) and H_2O_2 (10^{-7} - 10^{-4} M), ROS-inducing substances, alone or to each one combined with Na_2SeO_4. To investigate the effects of the exposure order seeds were treated witch a combined solution of $AlCl_3$ plus Na_2SeO_4, $AlCl_3$ or Na_2SeO_4 for the first 6 h. $AlCl_3$ solutions was then replaced by Na_2SeO_4 and $AlCl_3$; Na_2SeO_4 solutions by $AlCl_3$; seeds were kept in the new media for 18 h.

In order to search for the effects of Na_2SeO_4 at high concentration (0.1 mM) on seedling growth and ethylene production, Se-treated seedlings were also provided with 2-aminoethoxyvinylglycine (AVG, 10 µM) solution, an inhibitor of ethylene biosynthesis. A putative relationship between high Na_2SeO_4 concentration-induced ROS generation and inhibition of seedling growth was also searched for with the employment of NAC (1 mM).

2.3. Root Cell Viability

Cell viability was assessed by staining root tip fragments with fluorescein diacetate (FDA, 10 µM) and propidium iodide (PI, 2.0 µM), according to [19]. After treatment with test solutions, seedlings were washed with distilled water (pH 7.0) and roots tips were stained for 5 min at room temperature with FDA and PI. The viability of cells was observed under a fluorescent microscope (BH2, Olympus, Japan).

2.4. Ethylene Measurement

For ethylene quantification Erlenmeyer flasks (50 ml) containing 10 seedlings imbibed in 3 ml test-solutions were stoppered with rubber serum caps and kept in the growth chamber, under the conditions previously described. Air samples (1 ml) were taken from the flask headspace and injected in 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. Ethylene peaks were registered by a *peak simple* software (Peak Simple, Version 3.92) coupled to the chromatograph, and quantified by comparison with authentic ethylene standards.

2.5. Statistical Analysis

The experiments followed a completely randomized design, with 10 replications per treatment. Experimental units consisted of a Petri dish or an Erlenmeyer flask with 10 seedlings. The Tukey test at 5% was applied to detect differences amongst means.

3. RESULTS

Aluminium inhibited root and hypocotyl growth of Townsville stylo seedlings in a dose-dependent manner (**Figure 1**). Inhibition of growth of Al-treated seedling was alleviated by Na_2SeO_4, at the low concentration employed. Growth recovery by SeO_4^{2-} was about 95%, 81% and 66% in roots inhibited with 1.0, 1.5 and 2.0 mM Al respectively. On the other hand, SeO_4^{2-} completely counteracted the growth inhibition of hypocotyl caused by Al. Selenate seemed to be operating through the quenching of ROS since NAC, an antioxidant compound, similarly recovered partially (roots) or completely (hypocotyls) the growth of Al-inhibited seedlings (**Figure 1**). Whether or not Al and SeO_4^{2-} were supplied to-

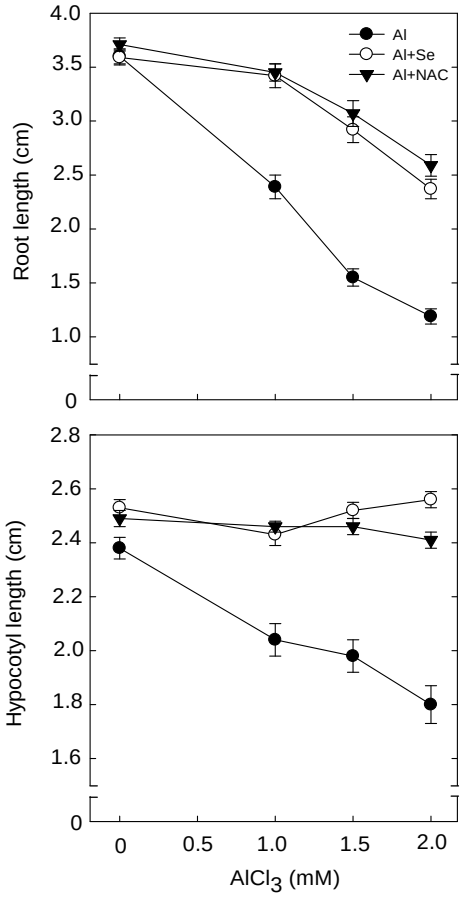

Figure 1. Se at low concentration alleviates $AlCl_3$-induced inhibition of seedling growth. $AlCl_3$ was provided to uniform seedlings in 0.5 mM $CaCl_2$ soluton pH 4,0 or combined with sodium selenate (1 μM) and *N*-acetyl cisteine (NAC 1 mM). Means of 100 seedlings ± standard errors.

gether or separately, one of them anteceding or following the supply of the other, their effects on seedling growth were very similar (**Table 1**). Vital staining also revealed differences in the response of seedlings treated with Al plus SeO_4^{2-} at low concentration **(Figure 2**). Aluminium caused considerable damage to root cells of Townsville stylo seedling. Selenate, on the other hand, caused substantial reduction in the Al damages to roots.

That SeO_4^{2-} seemed to be acting as an antioxidant leading to growth alleviation or restoration of seedling inhibited by Al was further demonstrated by treating seedling with SeO_4^{2-} and methyl viologen (MV) or H_2O_2. Similarly to the Al effects, MV and H_2O_2 inhibited root and hypocotyl elongation in a dose-dependent manner (**Figure 3**). Furthermore, SeO_4^{2-} restores (H_2O_2) or alleviates (MV) the growth of inhibited roots; in hypocotyls SeO_4^{2-} completely overcame the inhibitory effects of both compounds. It is also observed that roots were much more sensitive to Al than hypocotyls (**Figures 1 and 3**).

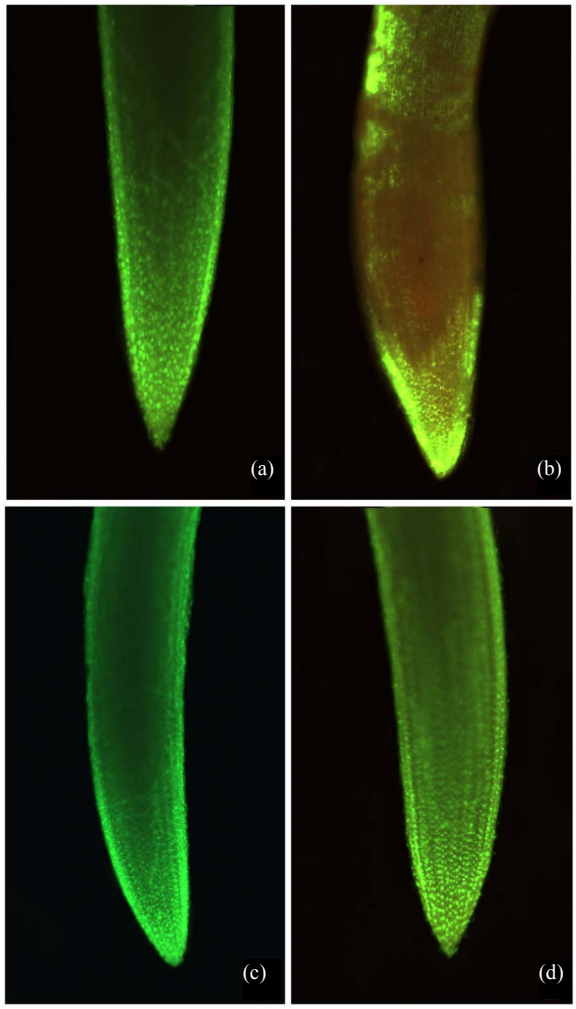

Figure 2. Protective effect of Se against Al-induced damages in root tips of Townsville stylo seedlings. (a) Control; (b) 2 mM $AlCl_3$; (c) 1 μM sodium selenate; and (d) $AlCl_3$ plus sodium selenate. Healthy cells exhibit green fluorescence due to fluorescein diacetate. Propidium iodide produces a red fluorescence of nuclei in damaged cells. Photos are representative of 5 replicates per treatment.

The above results were completely different when SeO_4^{2-} was used in a high concentration (0.1 mM), two orders of magnitude larger than the one employed to counteract the Al effects. At 0.1 mM, SeO_4^{2-} inhibited root and hypocotyl elongation by 48 and 21%, respectively (**Figure 4**). As occurred with aluminium roots were shown to be much more sensitive to high Se level than hypocotyls. Selenate caused an increase in ethylene emanation by seedlings by about 89% (as compared to the control). The inhibitor of ethylene biosynthesis AVG substantially decreased ethylene production by seedlings treated with SeO_4^{2-}, but without any effect on seedling growth (as compared to Se-treated seedlings alone).

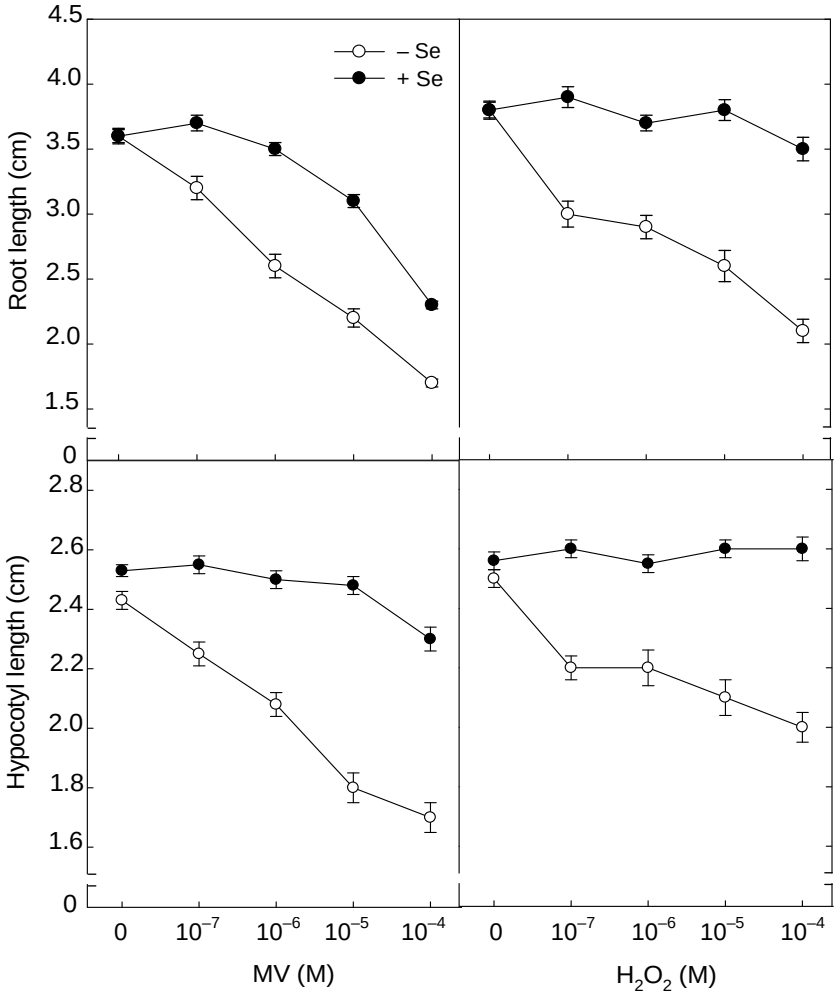

Figure 3. Effects of sodium selenate supplied with methyl viologen (MV, left) or H_2O_2 (right) solutions pH 4.0 on seedling growth. Means of 100 seedlings ± standard errors.

Hence ethylene seemed not to be required for growth inhibition of the SeO_4^{2-}-treated seedlings (**Figure 4**). Selenate-induced inhibition of seedling growth was completely restored by NAC, but ethylene production by SeO_4^{2-}-treated seedling was not decreased by the antioxidant compound, which actually showed to be very high (treatment SeO_4^{2-} plus NAC, **Figure 4**).

4. DISCUSSION

Growth inhibition is a well-known response of plants to toxic concentrations of Al ions [20]. The data described herein demonstrate that SeO_4^{2-}, at low concentration (1.0 µM), can alleviate partial or completely the Al-induced inhibition of root and hypocotyl elongation, respectively (**Figure 1**). Moreover, NAC, an antioxidant compound [21], was also capable of overcoming the inhibited state caused by Al. These results suggest that the SeO_4^{2-}, at low concentration, may act as an antioxidant (possibly as a ROS quencher) to counteract inhibition of root and hypocotyl elongation of Townsville stylo seedlings. In fact, Se can overcome oxidative damages displaying a protective effect against stressing conditions [8, 9]. Selenate addition restored hypocotyl growth to the level of the control under any concentration of Al used. However, root growth inhibition by Al (at 1.5 and 2.0 mM) was not recovered to the control level, indicating that roots were more sensitive to a toxic Al exposure than hypocotyls (see also **Figure 3**).

In order to get further insights into the relationships between Al and SeO_4^{2-} effects on the control of seedling growth, seedlings were treated with Al or Al plus SeO_4^{2-} (dissolved in 0.5 mM $CaCl_2$, pH 4.0) for 24 h and afterwards transferred to the medium without Al. Seedling survival 10 days after transference was about 94%, 90%, 30%, and 80% if they were previously exposed to control (0.5 mM $CaCl_2$, pH 4.0), 1 µM SeO_4^{2-}, 2 mM $AlCl_3$ and $AlCl_3$ plus SeO_4^{2-}, respectively (not shown). In keeping with those responses, Al toxicity effect was

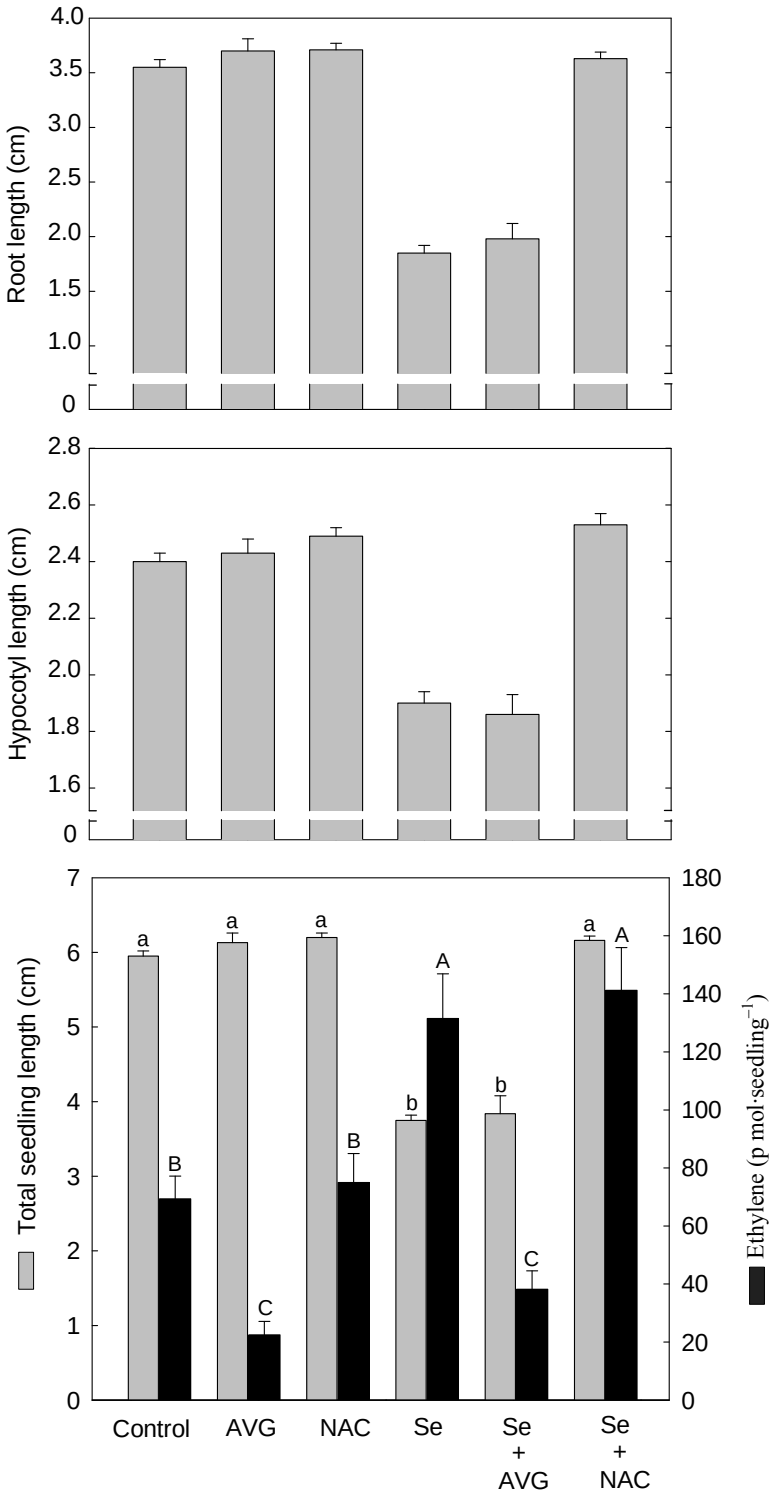

Figure 4. Ethylene production is not associated with growth inhibition of high selenium concentration-treated seedlings. AVG (10 μM) and NAC (1 mM) were provided to seedlings in a $CaCl_2$ solution pH 4.0 alone or also combined with sodium selenate (0.1 mM). Means followed by the same small letter (seedling growth), or followed by the same capital letter (ethylene) do not differ significantly at 5% level. Data shown are means of 100 seedlings ± standard errors.

Table 1. Effects of Na_2SeO_4 provided alone or in combination with $AlCl_3$ on the growth of seedlings of Townsville stylo. Sodium selenate (1 μM), $AlCl_3$ (2 mM) and sodium selenate plus $AlCl_3$ were provided to seedlings in 0.5 mM $CaCl_2$ solution pH 4.0 for 6 h and then transferred to the next solutions (as indicated following the arrows) for 18 h. In each column means do not differ significantly at 5% level, when followed by same letter. Means of 100 seedlings ± standard errors.

Treatment	Root length (cm)	Hypocotyl length (cm)	Seedling length (cm)
Control → Control	3.8 ± 0.09 a	2.4 ± 0.04 a	6.2 ± 0.10 a
Se → Se	3.6 ± 0.08 a	2.5 ± 0.04 a	6.1 ± 0.10 a
$AlCl_3$ → $AlCl_3$	1.2 ± 0.07 c	1.8 ± 0.05 b	3.0 ± 0.09 c
$AlCl_3$ → Control	1.4 ± 0.08 c	1.9 ± 0.05 b	3.3 ± 0.11 c
$AlCl_3$ → Se	2.4 ± 0.10 b	2.5 ± 0.04 a	4.9 ± 0.10 b
Se → $AlCl_3$	2.7 ± 0.12 b	2.3 ± 0.05 a	5.0 ± 0.13 b
$AlCl_3$ + Se → $AlCl_3$ + Se	2.4 ± 0.11 b	2.4 ± 0.06 a	4.7 ± 0.15 b

very high in seedling expose to Al 2 mM, as shown by vital staining of root tips (**Figure 2**). Selenate substantially reduced cell damages caused by Al, also diminishing the Al inhibition of root elongation, evidecing a protective role of Se. The inhibition relief of Al-treated seedling by SeO_4^{2-} was also observed had the seedlings been exposed to SeO_4^{2-} before (Se → Al) or after (Al → Se) the Al supply (**Table 1**). Moreover, pre-treatment of seedlings with SeO_4^{2-} (Se → Al) or with Al (Al → Se) promoted a similar effect on root and hypocotyl elongation as the combined treatment with Al plus SeO_4^{2-}. It follows that in short term Se is capable of impairing or repairing the damages caused by aluminium in the tissues.

A probable role of SeO_4^{2-}, at low concentration, as an antioxidant agent to alleviate the Al-induced inhibition of seedling growth was also examined with the employment of MV and H_2O_2. MV, which generates singlet oxygen (O_2^-) directly and $OH^{\bullet}$ radicals as secondary activated oxygen species [22,23], and H_2O_2 constitute important tools for investigating the effects of activated oxygen species in biological systems. Similarly to the treatment with Al, both MV and H_2O_2 reduced seedling growth and their inhibitory effect was substantially reversed by SeO_4^{2-}, at the low concentration employed (**Figure 3**). These results suggest that the diminished action of oxygen species could explain the protective role of SeO_4^{2-} in Al-stressed plants.

The results point out to Se as exerting a dual effect on seedling growth process: at low concentration, it could act as an antioxidant enhancing growth of Al-inhibited seedlings, whereas at higher concentration it could act as a growth-inhibiting agent [9,24]. In fact, selenomethionine at high concentration inhibited seedling growth, as shown in [25], and several Se-soluble compounds elicited ethylene production by seeds and seedlings of Townsville stylo [14]. It is known that selenate at high levels, upregulates the genes coding for 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase, the two last enzymes in the pathway to ethylene biosynthesis [26]. Root elongation is also inhibited by ethylene [27] and thus it is likely that SeO_4^{2-}, at high concentration, inhibits seedling growth through eliciting ethylene biosynthesis (**Figure 4**). However, AVG inhibited ethylene production of SeO_4^{2-}-treated seedlings to a great extent, without any effect on seedling growth in comparison to seedlings treated with SeO_4^{2-} solely (**Figure 4**). These findings suggest that other mechanisms of action of SeO_4^{2-} at high concentrations, might be operative, which was supported with the use of NAC. When NAC was supplied to seedling together with SeO_4^{2-}, seedling growth was increased to the level of the control and ethylene production stimulated by SeO_4^{2-} was not inhibited at all by NAC (**Figure 4**). Together these data provide evidence supporting that SeO_4^{2-}-induced inhibition of seedling growth was likely associated with the action of ROS and not with ethylene production.

The results obtained on radicle growth of Towsville stylo seedlings are in contrast to those of bean, another legume [28], and *Arabdopsis thaliana*, a Brassicaceae [27]. In these species radicle growth is inhibited by the large amounts of ethylene induced by Al. In *Arabidopsis* the effects of ethylene were much reduced in the mutants *etr*-1 and *ein*-2, defective in ethylene signalling or with the use of AVG, Co^{2+} and with Ag^+, inhibitors of ethylene biosynthesis and action. By also employing the mutants *aux1*-7 and *pin*2, defective in auxin polar transport, and using naphthylphthalamic acid, winch disrupts the auxin polar transport, the inhibitory effects of Al in radicle growth were also greatly diminished or no longer observed. It was concluded that ethylene constitutes a signal which alters auxin distribution in roots by disrupting *AUX*1 and *PIN*2-mediated auxin polar transport, causing an arrest in root elongation [29]. There remains, however, the possibility of a direct action of Al^{3+} in auxin distribution, thus bypassing the ethylene requirement, [29]. In this context, it can be conclused that the causes for inhibition of root growth are species-specific.

In summary, the action of SeO_4^{2-} on seedling growth of Townsville stylo seedling was shown to depend on its concentration. At low concentration, it promotes the release of the Al growth inhibition, seeming to work as a scavenger of free radicals. At high concentrations, inhibition of seedling growth is not associated with a SeO_4^{2-}-dependent ethylene biosynthesis, but seems associated to the ROS generation.

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