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Cellular responses of two Latin-American cultivars of *Lotus corniculatus* to low pH and Al stress

Research Article

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Abstract: Toxic effects of acidic root medium and aluminium were evaluated in two forage cultivars of *Lotus corniculatus* differing in their tolerance to Al stress. The structural response of most of the root cells exposed to low pH without Al^{3+} differed markedly from that induced by the combined stress. Conspicuous alteration of the nucleus was present only at low pH 4.0 and disintegration of the cytoplasmic components was more drastic than in the roots exposed to acidic solution containing Al^{3+}. Cells exposed to low pH without Al, did not produce wall thickenings. Severely damaged cytoplasm and localized death in some cortical cells or groups of cells contrasting with almost intact cells exposed to Al^{3+} stress were found. In this respect, a strong correlation between the occurrence of cell wall thickenings and a better preserved structure of the cytoplasm was observed. The frequency of cell damage in the more tolerant cultivar UFRGS was generally lower, significantly more cortical cells capable of maintaining their resting membrane potential were present than in the sensitive INIA Draco. The difference in their tolerance is related rather to the exudation of citrate and oxalate that was higher in UFRGS than to the accumulation of tannins, which increased after Al treatment in both cultivars.

Keywords: *Aluminium • Acidity • Lotus corniculatus • Root tip • Cell ultrastructure*

1. Introduction

Extensively cultivated pastures are an important base for the beef and sheep feeding in Latin American countries. Introduction of legumes in these pastures improves their productivity and sustainable development due to their contribution to soil nitrogen content derived from nitrogen fixation. Among the forage legumes, *Lotus* species have several advantages, as they do not cause bloat, are adapted to infertile soils, and are moderately tolerant to lower pH or soil salinity [1]. The principal world regions where *Lotus* is sown are Latin America and North America, but it also occurs in some areas of Europe, Australia and Africa. About 95% of this area is planted with *Lotus corniculatus* [2].

Soil acidity is a world-wide agricultural problem in up to 50% of the world's potentially arable land [3]. Acid soils are characterized by an excess of H^+, Mn^{2+}, Al^{3+}, and concomitant depletion of alkali and alkaline earth metals such as Ca^{2+} and Mg^{2+}.

At low soil pH, aluminium is considered the main constraint to plant productivity. It is solubilised to the cytotoxic form $Al(H_2O)_6^{3+}$ (Al^{3+}) below pH 5.0. Soluble aluminium impairs the structure and function of the cell wall and plasma membrane (including its role in water and mineral uptake). Intracellularly it interferes with a number of metabolic pathways and inhibits plant root growth [4,5]. The Al toxicity symptoms can be observed particularly in the root-tips and in lateral roots that become thickened and turn brown [6].

Recent studies of cell responses to Al^{3+} confirmed alterations of cytoplasmic organelles such as mitochondria and ER, increased vacuolation, and formation of cell wall thickenings [7-9]. Cell wall thickening has been related to the accumulation of Al [7], deposition of callose [10,11], pectins [12], and also

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lignin in the most sensitive species [13]. Hydroxyproline-rich glycoprotein accumulation also resulted in thicker cell walls [14].

In nature, plants usually encounter Al stress at low pH and it is similarly administered also to experimental plants. However, it is necessary to recognize the effects of the Al ion and acidity separately, in order to better understand the mechanisms of acidity effects, but also because there are low-pH soils with high organic matter content, in which Al concentrations are not inhibitory. Several studies have shown the low pH rhizotoxicity to induce cytoplasmic acidification, plasma membrane depolarization, disturbance of permeability and alteration of ion fluxes and enzyme activities [15-17]. Stability of the cell wall pectic polysaccharide network was suggested as the primary target of proton toxicity [18]. Recently, Larger [19] reported on the effect of low pH on the expression of genes responsible for cell wall modifying enzymes. Cell death, as revaeled by Evans blue uptake, was higher at low pH than at low Al concentration, suggesting that low concentration of Al may alleviate the proton toxicity at low pH [20]. It seems that modifications of cell ultrastructure induced by elevated Al^{3+} concentration in an acidic root medium and those induced by low pH stress alone have not yet been compared.

Tannins may be involved in vacuolar storage of Al. The role of phenolic compounds and of their glycosidic and sulphate conjugates in internal Al detoxification in plants needs further investigation [21]. Phenolics with catecholate groups are strong chelators for Al and they could contribute to detoxification of the low Al amounts that have surpassed the exclusion barrier operating in this Al-tolerant variety [22]. Another possible exclusion barrier to Al is the efflux of one or more organic anions from the root apex. These anions are able to chelate Al^{3+} in the root apoplast or even in the surrounding area of the root tips [23]. Many plants increase the organic acid efflux from roots after exposition to Al^{3+} and a good correlation was often found between the rate of that exudation and the resistance in different genotypes within the same species, supposing the important role of organic acids in the alleviation of the Al^{3+} toxicity [24].

In our previous work with the relative Al-tolerant species, *Lotus corniculatus*, we demonstrated that the Al stress affected membrane permeability of the cortex cells in the root tip, impacting their membrane potential and respiration. The extent of membrane depolarization was closely related to the sensitivity of individual *Lotus* cultivars to Al. More tolerant cultivars showed considerably stronger membrane depolarization (E_M) than the sensitive ones [16]. In the present study, we demonstrate that cells in the root tips of *Lotus corniculatus* cultivars respond with some ultrastructural alterations when exposed to either aluminium administered in low pH cultivation medium, or to the same acidity alone, and that these alternations are not identical. Furthermore, the responses of two cultivars differing in their Al^{3+} are compared.

2. Experimental Procedures

2.1 Plant material and growing conditions

Seeds of two Latin-American cultivars of *Lotus corniculatus*, INIA Draco and UFRGS were obtained from Dr. Monica Rebuffo (INIA La Estanzuela, Colonia, Uruguay) and cultivated in controlled conditions (20°C, 50% relative humidity, 16 h photoperiod and approximately 150 µmol m^{-2} s^{-2} illumination). About 5 cm long shoot cuttings of the plants were taken and transferred to hydroponics to 1/10 strength Hornum media [25] with some modifications (1 mM $CaCl_2$, 0.5 mM KNO_3, 0.5 mM NH_4NO_3 and 0.5 mM KCl). The medium was continuously aerated and replaced every 2 days. After 14 days, the plants were washed with distilled water and transferred to 1 L containers filled with nutrient solution containing 1 mM $CaCl_2$, 0.5 mM KNO_3, 0.5 mM NH_4NO_3 and 0.5 mM KCl (control). For Al-treated plants, the nutrient solution was supplemented with 0.5 mM or 2 mM $AlCl_3$ for 24 hours. The pH was maintained at pH 4.0 or 5.5 (±0.2) throughout the treatment.

2.2 Determination of membrane potential

Measurements of plasma membrane potential (Em) were carried out at 22ºC on the outer cortical cells of 25 mm long apical root segments of *Lotus* by standard microelectrode techniques described earlier [26]. After rinsing the roots with 0.5 mM $CaSO_4$, the root segments were mounted to a Plexiglas holder with a soft rubber ring and mounted in a vertical 5 cm^3 plexiglass cuvette, which was perfused at a flow rate of 10 mL/min with a standard solution containing 0.1 mM KCl, 1 mM $Ca(NO_3)$. The microelectrode was inserted into the outer cortex cells 1-2 mm from the root tip. Insertion of the microelectrode was observed under a microscope.

2.3 Light and electron microscopy

Root tips were fixed with 3% glutaraldehyde in 0.5 M Na-cacodylate buffer pH 7.2 for 1.5 h and post-fixed with 1% OsO_4 in the same buffer overnight, dehydrated in an ethanol series and propylene oxide, and embedded in Spurr´s medium. Semi-thin sections were stained with Toluidine blue and viewed with an Olympus BX61 microscope. Ultrathin sections taken at the distances of 1 to 1.5 mm from the tip of the root cap were contrasted with uranyl acetate and Pb citrate, and investigated with a Tesla BS500 transmission electron microscope.

2.4 Determination of organic acid exudates and tannins

The plants were washed and transferred to the Al-free 0.2 mM KCl solution for 3 hours and then the solutions were collected and passed through a column filled with 3 ml of anion-exchange resin AG 1-X8 (100–200 mesh). The organic acids retained in the resin were eluted with 2 M HCl, the eluate was dried using a rotary evaporator at 60°C and stored in the dark and cold until analysis. The residue was then dissolved in 0.5 ml of ultra-pure water and detected by HPLC. The system included the Ecom LCD 3001 pump, UV-VIS detector Ecom LCD 2084, 250x4.6 mm I.D. reversed-phase column Separon SGX C_{18}, 5 μm. Resolution of the organic acids was accomplished using an isocratic system with 25 mM KH_3PO_4 pH=2.5 mobile phase at a flow-rate of 0.7 ml/min.

Tannins were extracted from homogenised, 1 cm long root tips with absolute methanol. Extracts were centrifuged for 5 min at 3000x*g* and then 1 mL aliquots of supernatant were assayed with 5 ml of 1% vanillin + 4% HCl in absolute methanol. Each sample was left in a water bath at 30ºC for exactly 20 min together with the corresponding blank containing 1 mL of supernatant and 5 ml 4% HCl (without vanillin). Absorbance was registered at 500 nm and calculated to catechin equivalents.

3. Results

Exposure of plant roots to both low pH and 0.5 and 2 mM aluminium for 24 h in hydroponic cultures caused several damages to the root tissue. The epidermal cells 1-2 mm from the root tip were highly impaired and hardly any cell was able to maintain its resting membrane potential (E_M). Several cells of the outer cortex (1st cortical layer) were also destroyed (Table 1). The amount of cells still able to maintain their membrane potential values was about 70% at the low pH alone, and even lower after Al treatment. The damage to the cortical cells was higher in cultivar INIA Draco than in UFRGS, especially at the lower Al concentration (0.5 mM). The magnitude of E_M was dependent on the pH of the root media, with some depolarization in acidic condition, and is interpreted as a higher H^+ influx across the plasmalemma. Treatment with Al caused further depolarization of the E_M, which was more obvious in sensitive INIA Draco (about 25% on 0.5 mM Al and 34% on 2 mM Al) than in UFRGS (about 9 and 18%, respectively).

The most common structural response to aluminium was increased vacuolar volume in the young meristematic cells of all root tissues in both *Lotus* cultivars. This can be clearly distinguished in longitudinal sections of the root tips (Figure 1). The protoplasts of meristematic cells were occupied predominantly with dense cytoplasm under control conditions (Figure 1A,C), while their lumen was more transparent due to the development of large vacuoles in the same developmental region (Figure 1B,D).

Electron microscopy of Al-treated cells (Figure 2) revealed some additional structural responses. Vacuoles contained electron-dense osmofilic inclusions after Al treatment (Figure 2G). In contrast to the smooth cell walls in control roots (Figure 2A,B), the formation of cell wall protuberances (irregular thickenings), found in the rhizodermis, cortical layers, and to a lesser extent also in the vascular parenchyma (arrowheads in the Figure 2D-G) was a frequent structural response to Al. In the Al-treated roots the presence of callose in the root tissues was confirmed using fluorescence microscopy of Aniline blue-stained sections (data not shown). The structure of the nucleus and organelles in the narrow layer of the cytoplasm of such cells did not show signs of damage (Figure 2D-G). Numerous organelles and vesicles close to the protuberances may indicate delivering polysaccharide material to the wall extensions

Treatment	*INIA Draco*		UFRGS	
	E_M [mV]	Cells with E_M [%]	E_M [mV]	Cells with E_M [%]
pH 5,5	129 ± 8	100,0	131 ± 6	100,0
pH 4,0	117 ± 5	68,7	123 ± 6	70,8
pH 4,0 + 0,5 mM Al	97 ± 5	49,6	119 ± 5	62,7
pH 4,0 + 2,0 mM Al	85 ± 4	36,3	108 ± 7	44,1

Table 1. Comparison of the resting membrane potential of the 1st layer of the root cortical cells 1-2 mm from the root tip and percentage of the cells able to maintain their membrane potential, in two cultivars of *Lotus corniculatus* after treatment with low pH and alumimium.

E_M values are the means ± SD (n=20).

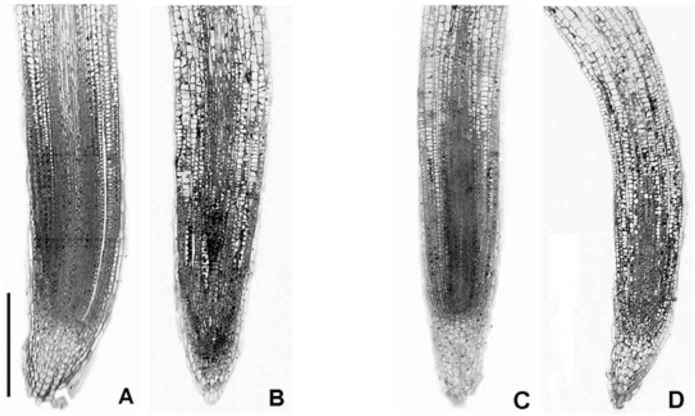

Figure 1. Longitudinal sections of the root tips of Lotus corniculatus, cv INIA Draco (A,B), and UFRGS (C,D) under control (A,C) and 2 mM Al^{3+} stress (B,D). In both cultivars the dense cytoplasm was present in the young meristematic cells of control roots (A, C) while the cellular volume of the Al-treated roots was occupied by large transparent vacuoles within the same region (B, D). Bar represents 0.5 mm.

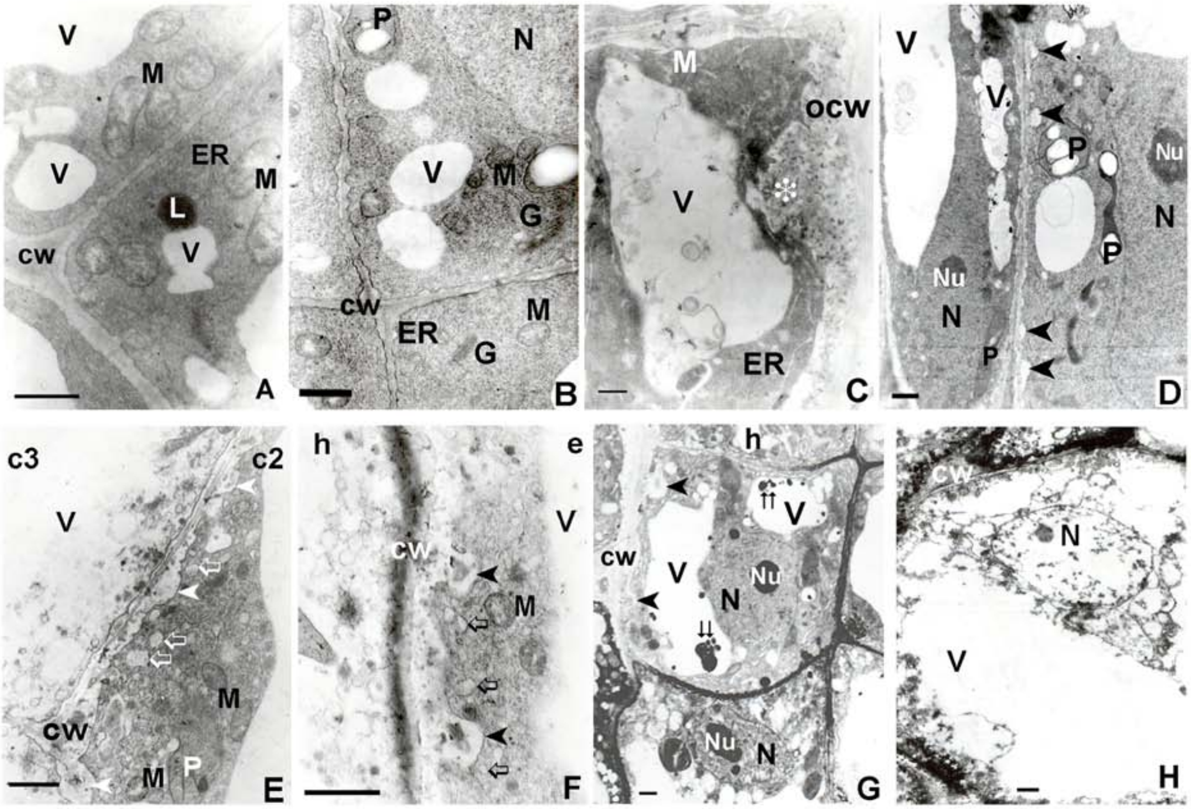

Figure 2. Ultrastructure of root cells of the Lotus cultivars INIA Draco (A, D, G, H) and UFRGS (B, C, E, F) in control (A, B) and after 24 h of 0.5 mM (C) or 2 mM (D – H) AlCl$_3$ treatments. Control root cells had smooth walls and ample organelles in the cytoplasm. Following Al-treatment enormously thickened outer cell walls appeared in the epidermis (asterisk, C) and irregular wall protuberances (arrowheads) in the cells of the epidermis (F), hypodermis (G), cortex (E), and in the vascular parenchyma (D). Numerous organelles and vesicles were close to the wall protuberances (arrows, E, F), and electron dense inclusions appeared in the vacuoles (double arrows, G). Examples are shown of the seriously disturbed cytoplasm in the neighbouring cells lacking wall thickenings in the 3rd cortical layer (c3 in E) and hypodermis (h in F), and of severely disintegrated cytoplasm in the epidermal cell of the INIA Draco cultivar (H). Bars represent 1 μm. N=nucleus, Nu= nucleolus, ER=endoplasmic reticulum, G=Golgi body, M=mitochondria, P=plastid, V=vacuole, L=lipid body, cw =cell wall, ocw=outer rhizodermal cell wall, c2, c3=second and third peripheral cortical layers, h=hypodermis.

(Figure 2E,F). However, the wall protuberances were not uniformly present in the cells throughout the cortex. Cells without such structural modifications occurred and, interestingly, their ultrastructure was severely damaged when compared to the neighbouring cells with distinct wall thickenings (Figure 2E,F). Such cells with marked ultrastructural signs of cell death were more frequent in rhizodermis and they were irregularly distributed within the root cortex. Similar ultrastructural responses were found in both sensitive INIA Draco and tolerant UFRGS cultivars. Slight differences were observed only in a more frequent occurrence of damaged cells with a disintegrated structure of the nucleus and the cytoplasmic organelles, particularly in the rhizodermis but also in more centripetally situated cortical cells of the cultivar INIA Draco (Figure 2H).

Structural responses of *Lotus corniculatus* roots to the acidity at pH 4 without aluminium differed markedly from those induced by Al^{3+} at the same pH in the root medium. Comparing to the control at pH 5.5 (Figure 3A,C), the frequent occurrence of cells with conspicuous structure of the nuclei could be seen already under a light microscope (Figure 3B). Electron microscopy has shown these nuclei with a dark central part, reminiscent of the nucleolus, surrounded by a transparent ring (Figure 3D,E). The nuclear envelope was continuous while the nucleoplasm was disintegrated (Figure 3D,E). Acidity also caused severe damage to the whole cytoplasm, in which only short fragments of cellular membranes and vacuoles could be distinguished (double arrows in Figure 3D). The protoplasts were plasmolysed but neither cell wall thickenings were observed nor callose detected (data not shown) under low pH treatment (Figure 3D). Similar responses to the acidity stress were found in both *Lotus* cultivars.

Low pH in the root medium had hardly any valuable effect on organic acid exudation, whereas aluminium treatment stimulated the efflux of oxalate and citrate from the roots of *Lotus* seedlings. After 24 hours of exposure to 2 mM Al, both cultivars exuded significantly more oxalate and citrate than the control without Al (Figure 4). The magnitude of oxalate and citrate efflux was significantly ($P<0.05$) higher in UFRGS than in INIA Draco. A stronger relative change was found for oxalate exudation, which was higher about 2.5 fold in UFRGS and about 2 fold in INIA Draco in comparison with the samples at low pH without Al treatment. The treatment with lower concentration of aluminium (0.5 mM) resulted in an increase of exudation only for citrate in the more tolerant cultivar UFRGS, but not in the sensitive INIA Draco, whereas the exudation of oxalate was similar to the control in both cultivars.

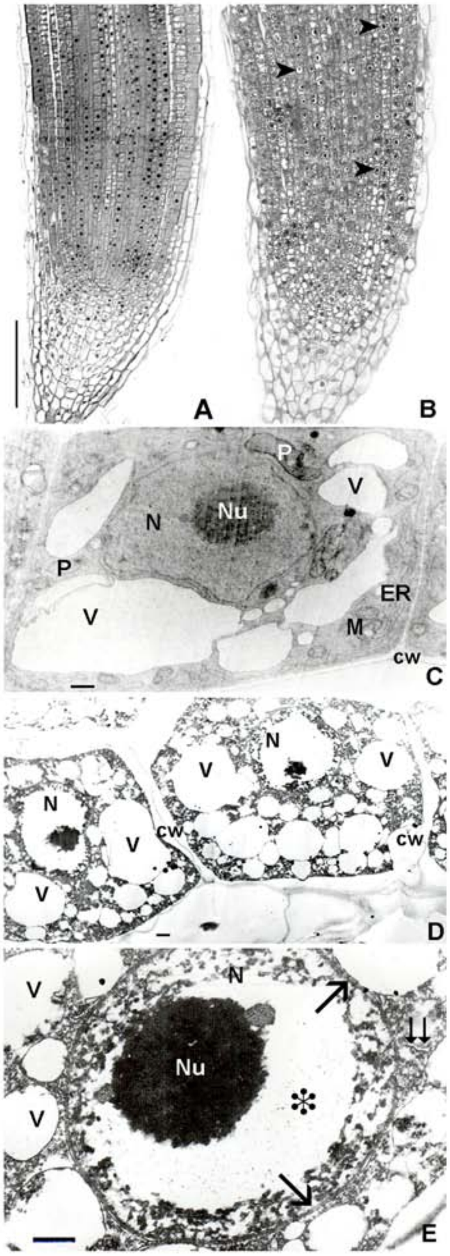

Figure 3. Longitudial sections of the INIA Draco root tips (A,B) and ultrastructure of root cells (C-E) under control pH 5.5 (A,C) and low pH 4.0 without aluminium (B,D,E). Nuclei with disturbed structure (arrowheads, B) occurred in the tissues within the root tip. Protoplasts revealed plasmolysis and the cytoplasm was disorganized (D). The damaged nuclei contained a nucleolus surrounded by an electron transparent ring (asterisk) and disintegrated nucleoplasm at the periphery (E). Note the continuous nuclear membrane (arrows) and fragments of membranes in the cytoplasm (double arrows). Bars represent 0.2 mm (A,B) and 1 μm (C-E). N=nucleus, Nu=nucleolus, ER=endoplasmic reticulum, M=mitochondria, P=plastid, V=vacuole, ocw=outer cell wall of epidermis.

Root tannin content was also unaffected by acidity alone. A significant (approximately 50%) increase in their accumulation was achieved after 24 hours of treatment with 2 mM Al and it was equivalent in the sensitive and more tolerant cultivar (Figure 5).

4. Discussion

Although it is known that *Lotus* species. are more tolerant to acid soils than most other plant species, including legumes [27,28], in several territories, e.g. in South Brazil, strongly acidified mineral soils with high exchangeable Al occur [29]. On the other hand, soils with higher organic matter content may contain lower amounts of aluminium in the soil solution at a given pH. Therefore, one of the milestones of the present work was to distinguish between the toxic effect of Al and the proton excess to the root tips of *Lotus*.

The resting membrane potential is a value of transmembrane voltage that is unavoidable for a number of transport processes and signal transductions. Even though it is tissue and species specific, it is also relatively stable in certain conditions and actively maintained by the cells unless the cell membrane is strongly damaged. The low pH alone as well as the Al treatment caused a strong destruction in the epidermal cells to a similar extent and it was hardly possible to find any cell capable of maintaining its resting potential. Significant damage was also found in most peripheral cortical layer cells. At low pH, no difference was apparent between the cultivars. On the other hand, under Al treatment, significantly more cells maintaining any E_M value were found in cultivar UFRGS than in INIA Draco, confirming the differences in their tolerance to Al. The depolarization of E_M of these cells after Al treatment was caused mainly by the decrease of the diffusion potential E_D and likely reflected cation leakage from the cells, as had been demonstrated previously [16].

To the authors' knowledge, data comparing cell ultrastructural responses to the stress of Al in an acidic solution and the low pH stress are not available in the literature. The structural response of most of the *L. corniculatus* root cells exposed to low pH without Al^{3+} differed markedly from those induced by the combined stress. Firstly, the conspicuous alteration of the nucleus was present only at low pH 4.0. Such a response faintly resembles that of the nuclei in *Vicia faba* roots grown under heavy metal stress on a soil substrate originating from mine tailings (Figure 3C, [30]). Unfortunately, there are no available data on nuclear structure under low pH stress. Secondly, the disintegration of cytoplasmic components was more drastic than in the roots exposed to acidic solution containing Al^{3+}. Thirdly, the cells of the root tissues exposed to a low pH without Al, did not produce wall thickenings or callose. This dissimilar response might be associated with the primary target of proton toxicity, linked to a disturbance of the stability in the pectic polysaccharide network, where Ca plays

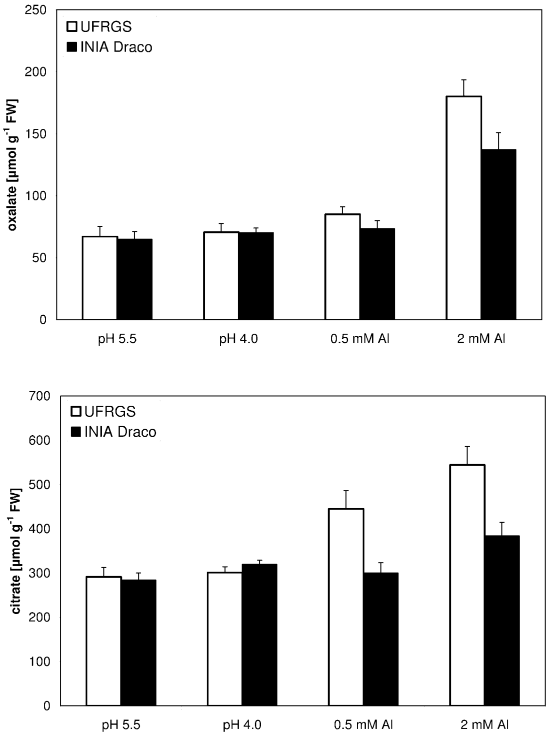

Figure 4. Exudation of oxalate and citrate from roots of Lotus corniculatus cultivars after 24 hour pretreatment with low pH and Al. Results are the means ± SE of two separate experiments each with three biological replicates.

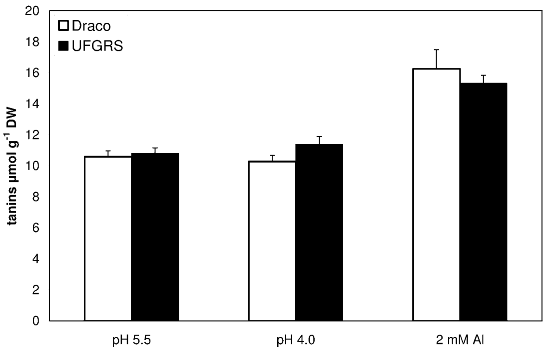

Figure 5. Tannin content in 1 cm segments of the root tips of two Lotus corniculatus cultivars after 24 hour treatment with low pH and Al, presented as µmol of catechin equivalents related to dry weight. Results are the means ± SE of tree biological replicates.

a key role in plant roots, as suggested by Koyama *et al*. [18]. Larger *et al*. [19] provide supporting evidence that the genes involved in cell wall modifications are substantially affected by external pH.

Even though low pH and aluminium are associated in the soil environment of the plant, both the tolerance mechanisms and the response systems of the plant to these cues are substantially separated [19]. Among the effects of the two stresses, differences were found in the mechanisms of root growth inhibition [31], in the effects of cell viability [9,20], or in electrophysiological responses of the plasma membrane [16,17]. Such data indicate that low pH and Al^{3+} toxicity may differentially affect root tissues as well as the structural integrity of their cells, and that the effects of the low pH may be more rapid and more severe.

Severely damaged cytoplasm and a localized death in some cells, or groups of cells, contrasting with the almost intact cells exposed to Al^{3+} stress, were found in root tissues or cell suspensions [8,11,32]. In this respect, a relationship between the occurrence of cell wall thickenings and a better preserved structure of the cytoplasm in *L. corniculatus* root cells was observed in the present work. Severe disintegration of cytoplasmic compartments was present in the cells lacking thickened walls while the cells with cell wall thickenings reveal continuous plasma membrane and organelles with unaltered structure. Al-induced callose synthesis was considered by some authors as one of the mechanisms of Al toxicity inversely correlated with Al resistance [10,33]. Nevertheless, callose accumulated in the cell wall thickenings could also represent a barrier for Al movement into these particular cells. It has been shown that Al-induced callose deposited along the plasmodesmatal sleeve caused effective blockage of the molecular trafficking and seemed to block the symplasmic cell-to-cell communication. Also, other substances like pectins, polypeptides or glycoproteins that can accumulate in the cell walls under Al stress could provide sufficient protection of cytoplasmic organelle integrity for the cells capable of increasing the cell wall thickness [14,34]. The cells without thickenings in epidermal or cortical tissues might participate in sequestering Al and undergo eventual death. Cortex cells with activated lysigeny in root tips can exclude Al. The Al binding capacity of dead cells is very large in comparison with living cells. Consequently, the function of epidermal cell apoptosis may be a mechanism to sequester large amounts of Al, preventing its entry further into the root [7,35].

In the present work, a significant increase of root tannin content was achieved after Al treatment, but not with low pH. In vacuoles of some cells, dense osmophilic deposits were found and osmium is known to bind tightly to condensed tannins. The formation of osmophilic Al-tannin complexes with a protective role, and their colocalization in vacuoles was proposed for *Lotus pedunculatus* by Stoutjesdijk *et al*. [36]. In camphor tree (*Cinamomum camphora*), unique proanthocyanidins - accumulating cells were identified as the innermost layer of the root cap shielding the rhizodermal cells. Their role in protecting the processes of inner root cells from Al toxicity has been suggested [37]. However, because the tannin content increased to a similar extent in both *Lotus corniculatus* cultivars, it is rather unlikely that it contributes to their differences in tolerance. One possible reason for the difference in tolerance between the two cultivars is likely related to the organic acid exudation on the Al-stress conditions. A higher exudation rate was observed in the more tolerant cultivar for both oxalate and citrate. Correlation between the differences in tolerance of cultivars and organic acid exudation was reported from several plant species, such as wheat [38], barley [39], or soybean [40]. Both citrate and oxalate can effectively chelate Al^{3+} and prevent its entry into the root. As tannins and organic acids have a specialised role in Al^{3+} detoxification, they do not accumulate in acidic condition without the presence of Al^{3+}.

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