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Structural changes in radish seedlings exposed to cadmium

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Abstract

Radish (*Raphanus sativus* L. cv. Redondo Vermelho) seedlings were analysed by light and scanning electron microscopy to characterize the structural changes caused by the exposure to 0.5 or 1.0 mM cadmium chloride for 24, 48 and 72 h. The analyses showed changes in the anatomical and morphological characteristics of roots, stems and leaves of two-week-old seedlings. In all tissues, pressure potential was decreased. Premature death with the disintegration of the epidermis and an increase in the number of root hairs was observed in roots exposed to Cd. The stem of seedlings exposed to Cd exhibited more cells layers in the cambial region. The main effects observed in leaves in response to Cd were stomatal closure, lack of cell wall thickening and alterations in the shape of the chloroplasts. It is suggested that the structural changes observed in seedlings treated with Cd were mainly caused by a Cd-induced decrease in water uptake.

Additional key words: cell wall, heavy metals, *Raphanus sativus*, stomata.

Introduction

Contamination of the environment with heavy metals is becoming a serious global problem. A critical aspect of the pollution with heavy metals is related to the fact that these elements are not naturally degraded in the environment (Robards and Worsfold 1991). Among the metals, cadmium is probably the most damaging to plant species, when they are cultivated in contaminated soils. Cd is mainly produced by industrial activities, mining, zinc refinement, chemical industries, application of sewage waste and the extensive use of herbicides and fertilizers, among others (McLaughlin and Singh 1999).

Several factors may influence the uptake of Cd by plants such as pH, calcium concentration and other metals in the soil (Zhao *et al.* 2002). Although Cd is not an essential plant nutrient, it can enter a plant very rapidly accumulating mainly in the roots (Vitória *et al.* 2001, Pereira *et al.* 2002) with a variable amount being translocated to the upper parts (Larsson *et al.* 2002, Ramos *et al.* 2002). Cd may form a complex with a group of sulphur rich peptides termed phytochelatins, which are structurally related to glutathione (Bergmann *et al.* 2001, Höfgen *et al.* 2001), prior to sequestration in the vacuole (Rea 1999). Some workers have also indicated that Cd can be stored in the cell walls (Khan *et al.* 1984, Ramos *et al.* 2002).

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Among the well known effects of Cd in plants are the decreased chlorophyll biosynthesis, reduced photosynthetic rate (Prasad 1995), and the reduced growth (Fornazier *et al.* 2002a), although in very low concentration Cd may stimulate the growth of *in vitro* plant cell cultures (Fornazier *et al.* 2002b) or the mycelia of *Aspergillus nidulans* (Guelfi *et al.* 2003). Cd can also inhibit the activity of a range of enzymes involved in plant metabolism (van Assche and Clijsters 1990, Morsch *et al.* 2002). Furthermore, evidence of the production of reactive oxygen species (ROS) and oxidative stress induced by Cd have also been obtained in plants by analyses of lipid peroxidation, chlorophyll breakdown and antioxidant enzymes activity (Vitória *et al.* 2001, Cardoso *et al.* 2002, Ferreira *et al.* 2002, Fornazier *et al.* 2002a,b, Pereira *et al.* 2002, Sandalio *et al.* 2002, Schützendübel and Polle 2002). Cd can inactivate enzymes by reacting with key cysteine residues (Schützendübel and Polle 2002) and by causing the oxidation of proteins to form carbonyl groups (Romero-Puertas *et al.* 2002). It has also been shown that Cd can stimulate senescence by inducing the transition of leaf peroxisomes into glyoxysomes and an increase in protease activity (McCarthy *et al.* 2001). The dynamics of Cd distribution in the intracellular space and inside cells of roots, stems and leaves have also been investigated (Kevrešan *et al.* 2003).

Although a significant amount of information is available concerning the effect of Cd on plant metabolism and growth, little is known about its effect on the cell structure. Cd exposition result in the inhibition of cell growth (Prasad 1995) although such an inhibition may also be a direct or indirect effect of Cd on auxin metabolism or auxin transporters (Barceló and Poschenrieder 1990).

In a recent report we described the effect of Cd on the antioxidant response, plant growth and Cd accumulation in radish leaves and roots (Vitória *et al.* 2001). In this study we report on the morphological alterations on leaf and root tissues of radish induced by Cd treatment.

Materials and methods

Plants: Radish seeds (*Raphanus sativus*, cv. Redondo Vermelho) were germinated on moistened foam for 1 week at room temperature. Seedlings were transferred to a glasshouse at a temperature of 28 - 34 °C and a 16-h photoperiod with natural irradiance of 2400 μmol m^{-2} s^{-1}. The plants were grown in a hydroponic system with continuous aeration in 1.7 dm^3 pots containing a 50 % diluted Hoagland's nutrient solution (Hoagland and Arnon 1938) for 1 week, when uniform plants were selected and further grown in the same solution, but containing 0 (control), 0.5 and 1 mM $CdCl_2$ for 24, 48 and 72 h. Two-week-old seedlings submitted to the $CdCl_2$ treatments were analysed by light and scanning electron microscopy.

Light microscopy (LM): Samples of roots, stems and leaves were fixed under low vacuum in Karnowsky solution (Karnowsky 1965) for 2 h, at room temperature. Dehydration was carried out in a graded ethanol series, followed by infiltration and embedding in *JB-4* resin. Sections (5 - 6 μm) were obtained in a manual microtome, and stained with toluidine blue (0.05 % m/v in phosphate buffer, pH 4.7), for 5 min, rinsed in distilled water and air-dried. The sections were permanently mounted in *Permount*, and observed and documented using an upright *AxioPlan* (*Zeiss*, Jena, Germany) light microscope.

Scanning electron microscopy (SEM): Leaf and root samples were fixed in a modified Karnowsky solution (Karnowsky 1965), composed of 2.5 % (v/v) glutaraldehyde and 2.5 % (v/v) formaldehyde in 0.05 M sodium cacodylate buffer at pH 7.2. Fixation was carried out for 12 h at 4 °C, under low vacuum, followed by three rinses in sodium cacodylate buffer (0.05 M, at pH 7.2). Post-fixation was carried out in 1 % (m/v) osmium tetroxide in 0.05 M sodium cacodylate buffer, pH 7.2, for 1 h at room temperature in a fume hood. After post-fixation the samples were rinsed 3 times in distilled water and dehydrated in a graded acetone series (30, 50, 70, 90 %), followed by 3 changes in 100 % acetone. The samples were then critical point dried through liquid carbon dioxide. The dried samples were mounted in metal stubs, sputter coated with 20 nm gold, and examined under a *DSM 940A* (*Zeiss*) scanning electron microscope at 20 kV, and the images were then digitalized.

Results

Histological analyses of radish seedlings exposed to varying concentrations of $CdCl_2$ for 24, 48 and 72 h, allowed the observation of structural alterations in roots, stems and leaves. In roots, the alteration could already be observed after 24 h of exposure to 0.5 mM $CdCl_2$ (Fig. 1*C*). The roots of the control untreated seedlings exhibited internally the xylem surrounded by a cambial region, with three to five layers of cells (Fig. 1*A*). The pericycle, endodermis and cortex, composed of turgid highly vacuolated cells and externally a well-defined

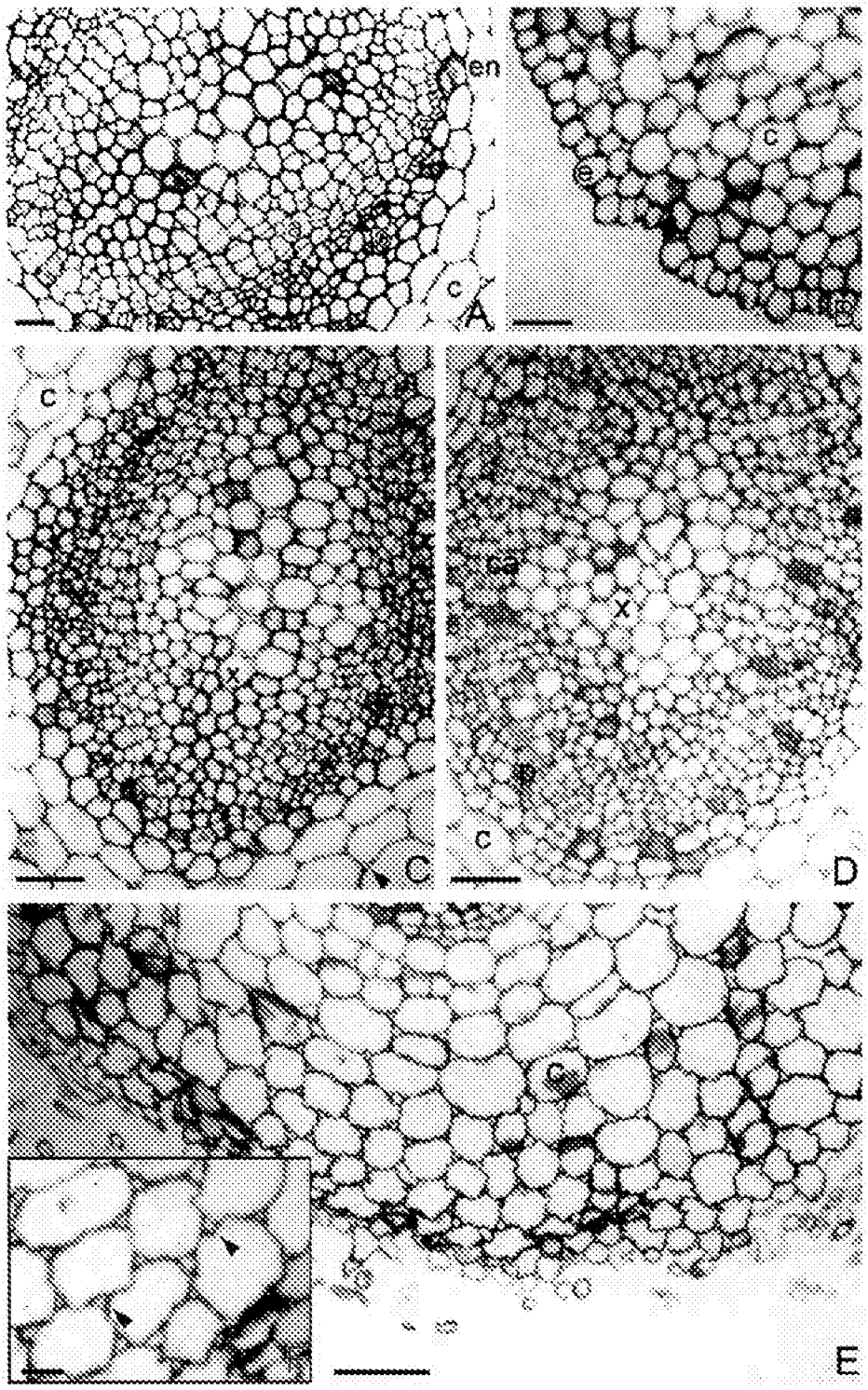

Fig. 1. Transverse sections of radish seedling roots stained with toluidine blue. *A* - control, two weeks after germination, showing the vascular cylinder and a few layers of cortex; *B* - detail of the cortex and epidermis in the control; *C* - exposure for 24 h to 0.5 mM $CdCl_2$, showing increased number of cambial layers, intercellular spaces in the cortex, and loss of pressure potential; *D* - exposure to 0.5 mM $CdCl_2$ for 72 h, showing more intense characteristics compared to *C*; *E* - exposure to 1 mM $CdCl_2$ for 24 h, showing disintegration of the epidermis and cell of external cortical layers, intercellular spaces in the cortex, and loss of pressure potential; *F* - enlarged section of *E*. c - cortex, ca - cambium, e - epiderm, en - endoderm, p - phloem, x - xylem, *arrow* - intercellular space. *Bars* 25 μm (*A*), 80 μm (*B*), 50 μm (*C*, *D*), 100 μm (*E*), 25 μm (*F*).

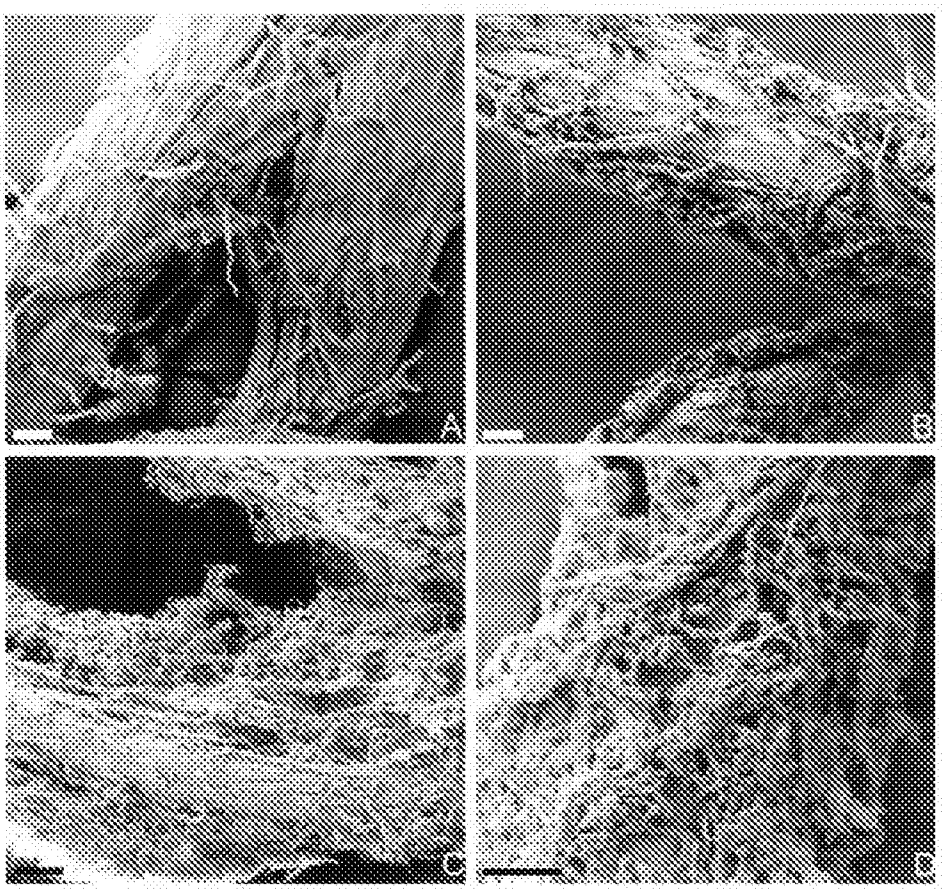

Fig. 2. Scanning electron micrographs of radish seedling roots. *A* - control showing the distribution of root hairs; *B-D* - presence of residues surrounding the root hairs; *B* - exposure for 24 h to 1 mM $CaCl_2$; *C* - exposure for 48 h to 1 mM $CaCl_2$; *D* - a detail of *C*. *Bars* 100 μm (*A, B, D*), 250 μm (*C*).

epidermis (Fig. 1*D*), surrounded the phloem outside the cambium (Fig. 1*A*). However, in the roots of plants exposed to 0.5 mM $CdCl_2$ for 24 h, there was a slight proliferation of cambial cells (Fig. 1*C*), which was intensified after 72 h of exposure in the same concentration of $CdCl_2$ (Fig. 1*D*), with a loss of organization in the cambial region. Exposure to a higher concentration of $CdCl_2$ (1 mM) caused a disintegration of the epidermis and the more external cortical cell layers, and a loss of pressure potential in the cortical cells, which lead to the presence of more conspicuous intercellular air spaces (Fig. 1*E,F*) when compared to the control (Fig. 1*A,B*).

Morphological analysis of the roots under the scanning electron microscope (Fig. 2) showed the presence of root hairs in the control and all treated plants. The use of vacuum during the fixation procedure caused damage to the root hair structure, observed in the control (Fig. 2*A*) and all treatments. An increase in number of root hairs was also observed in roots of plants exposed to Cd, when compared to the untreated control (Fig. 2). In roots exposed to 1 mM $CdCl_2$ for 24 h (Fig. 2*B*) and 48 h (Fig. 2*C,D*), the presence of unknown material attached to the root hairs was observed, as opposed to the untreated control (Fig. 2*A*).

The main alteration observed in the leaf tissue was related to chloroplast shape, initially observed after 24 h of exposure to Cd. Before exposure (Fig. 3*A,B*) the mesophyll cells were highly vacuolated with the presence of small chloroplasts. When cultured in nutritive solution containing either 0.5 mM $CdCl_2$ for 72 h or 1 mM $CdCl_2$ for 24 h, the chloroplasts were more isodiametric (Fig. 3*C,D*). Another structural alteration in the leaf cells observed in plants exposed for 24 h to 1 mM $CdCl_2$ (Fig. 3*D*) is in the cell wall, which was thinner compared to the control. The lack of cell wall thickening was also observed in stem histological sections of seedlings treated with Cd after 24 h of exposure to 1 mM $CdCl_2$ (Fig. 3*D*) when compared to the control (Fig. 4*A,B*).

Epidermal cells observed under the scanning electron microscope (Fig. 5) were turgid and exhibited open stomata in the control (Fig. 5*A,B*). In leaves from plants exposed to cadmium the epidermal cells showed loss of pressure potential and closed stomata (Fig. 5*C,D*). The wilting after 48 h of exposure to Cd, was only observed under the microscope. Wilting was only visible by the naked eye, after 72 h of exposure to Cd.

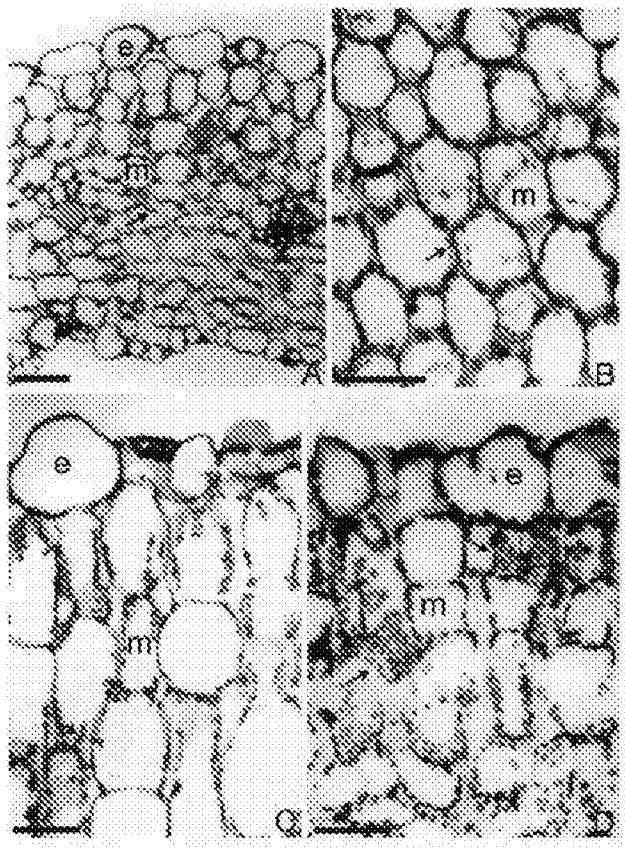

Fig. 3. Transverse sections of radish seedling leaves stained with toluidine blue. *A* - control showing the general structure of the leaf; *B* - detail of the leaf mesophyll cells with small chloroplasts; *C* - exposure to 0.5 mM $CdCl_2$ for 72 h, showing chloroplasts with a distinct shape when compared to *B*; *D* - exposure to 1 mM $CdCl_2$ for 24 h, showing an increase in chloroplast number compared to *B*. e - epidermis, m - mesophyll, *arrow* - chloroplast. *Bars* = 20 μm (*A, B, C*), 50 μm (*D*).

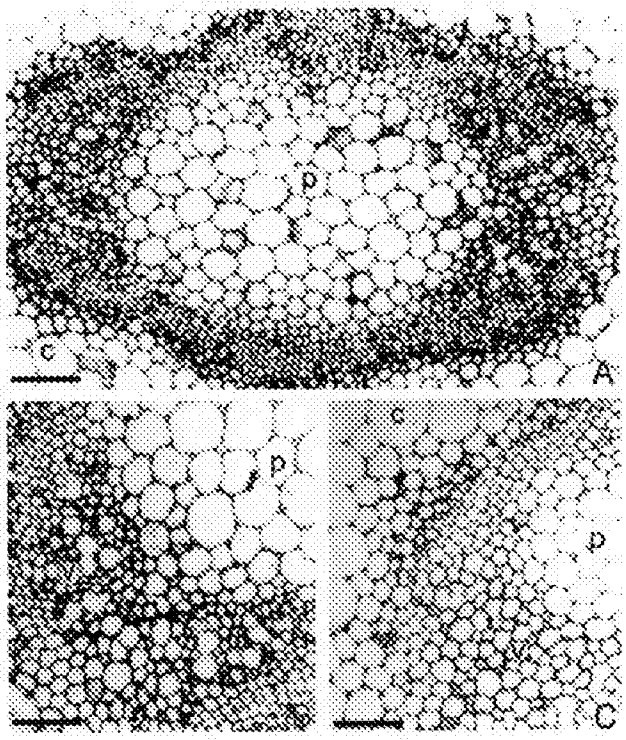

Fig. 4. Transverse section of radish seedling stems stained with toluidine blue. *A* - control showing the vascular cylinder, pith and a few cortical layers; *B* - detail of the stem vascular cylinder in the control; *C* - exposure to 1 mM $CdCl_2$ for 24 h, showing cell wall thinning compared to *B*. c - cortex, p - pith, v - vascular cylinder. *Bars* = 15 μm (*A*), 20 μm (*B, C*).

Discussion

It has been demonstrated in higher plants that Cd enters the plant system rapidly, and is accumulated in high amounts in the root system (Vitória *et al.* 2001, Pereira *et al.* 2002), although Cd may also be translocated to the shoot (Larsson *et al.* 2002, Ramos *et al.* 2002). The increased cell proliferation in the root cambial region of plants exposed to Cd (Fig. 1), may be a strategy which could lead to an increase in water uptake and transport. However, Prasad (1995) reported decreases in water and nutrient uptake due to Cd contamination. Cd was also shown to reduce transpiration and in turn decrease the water uptake by the roots (Barceló and Poschenrieder 1990). Furthermore, the reduction in water uptake was also related to inhibition of growth and root elongation, which have been shown to occur in plants submitted to Cd stress (Boussama *et al.* 1999).

An alteration in root differentiation has been reported in plants submitted to Cd treatment (Schützendübel *et al.* 2001). Schützendübel *et al.* (2001) observed lignin deposition in *Pinus silvestris* plants exposed to high Cd concentrations. In radish, we have observed an alteration in root differentiation in plants exposed to Cd, leading to an increase in the number of root hairs with the length of the period of exposure to Cd (Fig. 2). The presence of the unknown material adhered to the root cell wall may make water uptake difficult, leading to the production of new root hairs. This unknown material is possibly composed of epidermal and cortical dead and decomposed cells, which accumulated externally on the root surface due to the presence of root hairs. Furthermore, the exposure to Cd has also promoted the loss of presssure potential and disintegration of the epidermis and cortical cells (Fig. 1*E,F*), when compared to the control (Fig. 1*B*).

When the hyperaccumulator *Arabidopsis halleri* was submitted to growth in a hydroponic solution containing the heavy metals Cd and Zn, a distribution gradient of Cd

and Zn was observed in the root system (Kupper *et al.* 2000). In *A. halleri*, the cell walls of epidermal cells retained practically all the Cd and Zn ions, suggesting that such an accumulation of these heavy metals may be due to the precipitation of Cd and Zn phosphate in the cell wall of root cells. A similar process, could also be contributing to the formation of the material observed in the root system of radish exposed to Cd, in this work. Moreover, in our previous study with radish seedlings, Vitória *et al.* (2001) reported that Cd accumulated mainly in the roots with low concentration of the metal being translocated to the upper parts of the plants.

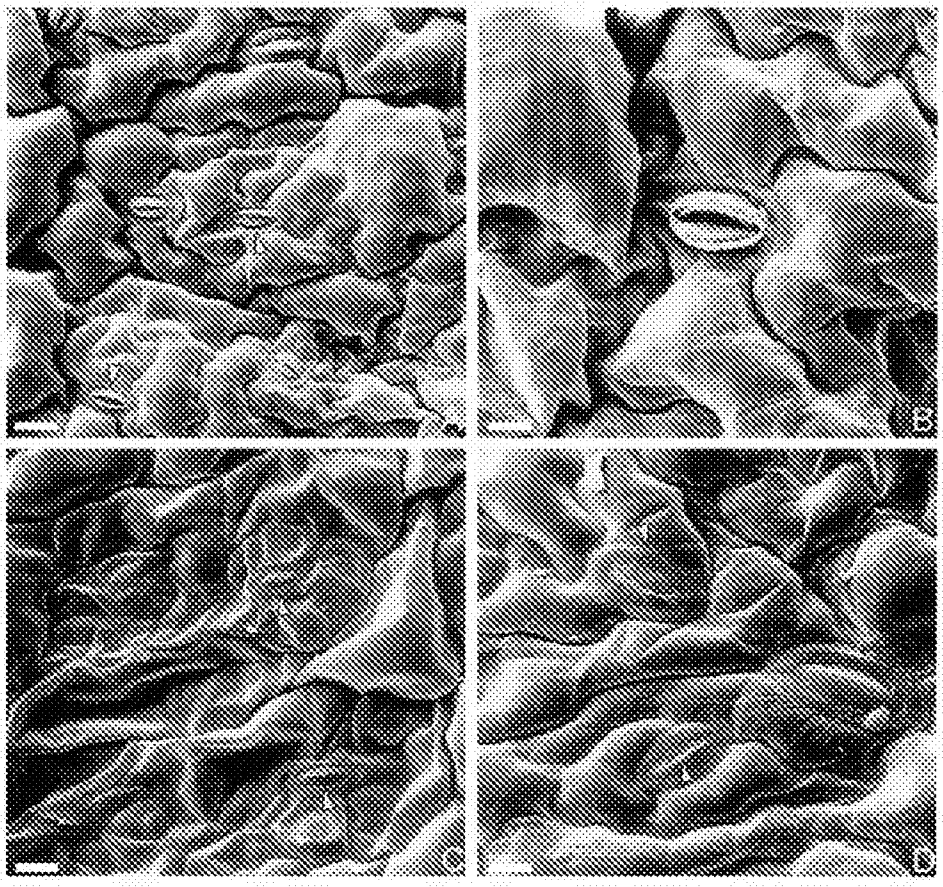

Fig. 5. Scanning electron micrographs of radish seedling leaf epidermis. *A* - control showing the distribution of stomata; *B* - a detail of an opened stoma in the control; *C* - exposure for 48 h to 1 mM $CaCl_2$; *D* - a detail of *C*. *Bars* = 20 μm (*A*, *C*), 5 μm (*B*, *D*).

The more conspicuous intercellular air spaces observed in radish seedlings (Fig. 1*E*,*F*) exposed to cadmium is in accordance with results observed by other authors (Barcelo and Poschenrieder 1990), who reported a decrease in water absorption as one of the main effects of this heavy metal in plants. Prasad (1995) reported that the inhibition of stomata opening in plants exposed to cadmium depends on several factors, such as metal concentration, period of exposure and degree of sensitivity of the species to the metal. We have observed in leaves of radish plants exposed to Cd, that the chloroplasts appeared to exhibit differences in shape. In previous reports, the presence of Cd within the leaf tissue was shown to promote a disorganization of the middle lamella ultrastructure mainly in the grana stacks of the chloroplasts (Prasad 1995). The distribution of chloroplasts within the control cells was markedly peripheral, suggesting that the vacuole is in the central region of the cell. On the other hand, in the plants exposed to Cd the distribution of the chloroplasts was also shown to be less uniform, with some organelles located also in the central area of the cell, although most of them were still located in the periphery of the cell. This may be explained by the increased chloroplast volume, which, although not clear in this study, could be pressing the tonoplast in the direction of the centre of the cell, or a reduction in the volume of the vacuole might be taking place. The involvement of the vacuole in the processes of heavy metal stress tolerance is crucial, since the sequestration of these ions into the vacuole would drastically reduce their concentration in the cytoplasm, thus preventing or avoiding the damage to the physiological and biochemical cell processes (Rauser and Ackerley 1987, Prasad 1995). Our results indicate that the integrity of the vacuole appears to have been maintained in the cells submitted to Cd treatment.

The lack of cell wall thickening observed in leaves and stems of seedlings treated with $CdCl_2$ (Figs. 3 and 4), was probably caused by the lack of deposition of cell wall components. However, it has been shown that the Cd remains associated with the cell wall and middle lamella and appears to increase the cross-linking of pectins in the middle lamella, which in the short term could lead to the inhibition of cellular expansion (Poschenrieder *et al.* 1989). Further evidence for the involvement of Cd with alterations in cell wall thickening has been suggested by the induction of premature lignification of the cell walls of Scots pine roots treated with Cd (Schützendübel *et al.* 2001). Lozano *et al.* (1997) also observed Cd ions associated with the cell wall and soluble leaf and root fractions of maize and pea plants grown in the presence of the heavy metal. The accumulation of Cd has also been reported in the cell walls and middle lamella between the endodermis and pericycle of maize (Khan *et al.* 1984) and in the soluble fraction of the cell wall of *Lactuca* sp. (Ramos *et al.* 2002). Hart *et al.* (1998) working with wheat suggested that the binding of Cd to the cell wall components (cellulose, hemicellulose and proteins) can be reduced at low temperatures, indicating that there is a system of Cd transport coupled to cell metabolism.

It is well known that Cd can induce oxidative stress in higher plants, including radish (Vitória *et al.* 2001), which could partially explain some of the results observed in this report. ROS can cause a series of molecular effects in living cells such as damage to lipids, fatty acids, proteins, pigments and nucleic acids leading to cellular effects such as chlorophyll breakdown, chromosome breakage and a general alteration to cell metabolism. Mechanisms can be induced to prevent or alleviate the damage caused by ROS that would otherwise lead to cell death (Azevedo *et al.* 1998).

In conclusion we also suggest that lower absorption of water by radish seedlings exposed to Cd may be an important factor to explain the structural alterations observed in this report. In response to the stress situation induced by Cd, the plant is able to induce mechanisms for the prevention of water loss, such as stomatal closure and higher water absorption suggested by the increase in vascular cambium activity and increase in the number of root hairs.

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