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Effects of Mn on anatomy, growth and carbohydrate content of adventitious roots in *Citrus maxima* (Burm.) Merr. shoot explants

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Microshoots of *Citrus maxima* (Burm.) Merr. were cultured *in vitro* for 75 days in a modified MS rooting medium containing 1.5 mg l^{-1} NAA (naphthaleneacetic acid) and 0, 1.37, 2.74, 5.48 or 10.96 mg l^{-1} Mn. Small pieces from two regions, i.e. 5-10 mm and 30-35 mm behind the apex, of adventitious roots were examined by light microscopy. Various root growth parameters and the carbohydrate contents of shoots and roots were also measured. The diameters of root cortex and stele progressively decreased as Mn concentration in the medium increased. Shoot explants grown without Mn (0 mg l^{-1} Mn) formed shorter and thicker roots when compared to those treated with Mn (1.37-10.96 mg l^{-1}). The diameter of roots in the 10.96 mg l^{-1} Mn treatment was smaller compared to the other Mn treatments, although the mean length of roots did not differ among the 1.37, 2.74, and 10.96 mg l^{-1} Mn treatments considerably. The greatest root length was observed in 5.48 mg l^{-1} Mn. However, the total length of roots formed per microshoot in 0 and 10.96 mg l^{-1} Mn was significantly smaller than that in 1.37-5.48 mg l^{-1} Mn. The carbohydrate content (μmol) of the roots was higher in 1.37-5.48 mg l^{-1} Mn than in 0 and 10.96 mg l^{-1} Mn.

Key words: carbohydrates, *Citrus*, manganese, growth, root anatomy.

INTRODUCTION

The effects of many abiotic factors on root anatomy have been so far extensively studied in various plant species. Among these factors, the following ones are included: (i) root aeration (McDonald *et al.*, 2002), (ii) CO_2 concentration (Pritchard *et al.*, 1999), (iii) soil moisture and waterlogging (Grzesiak *et al.*, 1999), (iv) heavy metals (Pb, Cd, Zn, and Cu) (Hossain *et al.*, 2002; Vitoria *et al.*, 2003; Sheldon & Menzies, 2004; Zelko & Lux, 2004; Maruthi Sridhar *et al.*, 2005) and chlorates (oxidizing substances) (Borges *et al.*, 2004), (v) salinity (Rashid *et al.*, 2001; Storey *et al.*, 2003; Reinoso *et al.*, 2004), (vi) light intensity (Wahl *et al.*, 2001), and (vii) soil type in relation to P fertilization (Peek *et al.*, 2003). With regard to Mn, which is an essential element for normal plant growth, the only published work about its effects on the anatomy and morphology of roots is that by Lidon (2002) on rice plants. However, depressed root growth has been reported for both Mn deficiency and toxicity (Abbott, 1967; Lidon, 2002). According to Campbell & Nable (1988), inhibition of root growth in Mn deficient plants is mediated by carbohydrate shortage as well as by direct requirement of Mn for growth. Root cell elongation seems to respond more rapidly to Mn deficiency than root cell division (Marschner, 1995).

The study of the effects of Mn on root growth and anatomy in a soil environment is difficult, because Mn tends to accumulate in the roots, even if its defi-

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ciency symptoms appear in the shoots. Thus, we consider that *in vitro* culture of shoot explants in a rooting medium with different Mn concentrations provides a suitable model for the investigation of the interaction between Mn supply and adventitious root formation and allows examination of root formation, growth, and anatomy. This model was applied in the present work to study the effects of several Mn concentrations on growth, anatomy, and morphometry of adventitious roots, formed at the base of *C. maxima* shoot explants. Furthermore, Mn is known to act as elicitor in the transfer of phosphate groups, and since all carbohydrates in the plant exist in their phosphorylated form, there is a plausible connection of Mn to carbohydrate metabolism (Marschner, 1995). Therefore, another goal of this experiment was to examine whether Mn supply affects the content of carbohydrates in the roots and shoots of plantlets grown under *in vitro* conditions.

MATERIALS AND METHODS

Plant material, growth conditions and treatments

One-node explants, approximately 1.5 cm long, that were cut from the same three-year-old tree of *Citrus maxima* (grown under greenhouse conditions), were used. Nodal explants were washed for 15 min in running tap water and then rinsed twice in distilled water. Afterwards, they were surface-sterilized with benomyl (1% w/v) for 20 min, rinsed three times in sterile distilled water for 8 min each time, followed by sodium hypocloride (1.2% w/v) for 20 min, and then rinsed twice in sterile distilled water for 8 min each time. One nodal explant was placed in each glass culture tube (100×25 mm) containing 10 ml of MS medium (Murashige & Skoog, 1962), which had a double concentration of Fe (11.2 mg l^{-1} Fe as FeEDTA). The following organic substances were added to the basic medium (mg l^{-1}): nicotinic acid, 0.5; pyridoxine·HCl, 0.5; thiamine·HCl, 0.1; glycine, 2; myo-inositol, 100; sucrose, 30000; and agar, 6000. The pH of the medium was adjusted to 5.8 prior to addition of the agar. The tubes were sealed with aluminium foil and autoclaved at 121°C for 20 min. The placement of the explants in the culture tubes was conducted under a horizontal laminar flow table, to ensure sterile conditions. Subsequently, the culture tubes with the explants were incubated in a growth chamber illuminated with cool white fluorescent tubes (45 μmol m^{-2} s^{-1}, 400-700 nm) for 16 hrs a day. The temperature of the growth chamber ranged between 21 and 24°C. Four weeks later, new shoots with two to three fully-expanded leaves (shoot explants) were cut off and placed into culture tubes containing 10 ml of rooting medium. The rooting medium was the same as that mentioned previously, but it was further supplemented with 1.5 mg l^{-1} NAA (naphthaleneacetic acid), and 0, 1.37, 2.74, 5.48, or 10.96 mg l^{-1} Mn (as $MnSO_4 \cdot H_2O$). The standard concentration of Mn in the MS medium is 5.48 mg l^{-1}. Twenty-two glass tubes (100×25 mm), having one shoot tip explant each, were used per treatment (Mn concentration in the medium). The pH of the media, the autoclaving process, and the conditions of the growth chamber were the same as those reported above for the culture of nodal explants. The shoot explants remained in the rooting media for 75 days (end of the experiment).

Measurement of growth parameters

At the end of the experiment, the rooted-shoots (plantlets) were harvested, separated into shoots and adventitious roots, and their various growth parameters (5 replications per Mn treatment, each one consisting of 3 plantlets) were measured. These were the fresh weight of shoots and roots, the total fresh weight per plantlet, the length of each root per plantlet, the total length of the roots per plantlet and the mean length per root.

Carbohydrate determination

Fresh shoots and adventitious roots of different Mn treatments were cut into small segments, placed in glass vials containing 10 ml of 80% (v/v) ethanol, and heated at 60°C for 30 min. The extract was filtered and diluted with 80% (v/v) ethanol up to 20 ml (Khan *et al.*, 2000). The concentration of carbohydrates was determined in the diluted extract using the anthrone method (Plummer, 1987; Khan *et al.*, 2000). By multiplying the concentration of carbohydrates (μmol g^{-1} f.w.) of the shoots or roots with the fresh weight of shoots or roots, respectively, the content (absolute quantity) of carbohydrates existing in the shoots and roots at the end of the study, can be calculated. With adding the carbohydrate content of the shoots and roots, the total content (μmol) of carbohydrates per plantlet was assessed.

Microscopy and morphometry

Small pieces from two regions of the adventitious roots (5-10 mm and 30-35 mm behind the root apex)

were initially fixed for 4 hrs with 5% glutaraldehyde in 0.025 M phosphate buffer (pH 7.2). After rinsing in buffer, the specimens were postfixed for 5 hrs with 1% OsO_4 (4°C). For tissue dehydration, an ethanol series (50-100%) was used, and this was followed by resin infiltration and embedment (Spurr, 1969). Semithin sections (1 μm thick) of plastic embedded root segments were cut on a Reichert Om U_2 ultramicrotome, stained with 1% toluidine blue O in 1% borax and photographed in a Zeiss III photomicroscope.

Morphometric assessments were conducted on root cross sections (15 light micrographs per Mn treatment, printed at ×210). The area fractions of the root histological components (epidermis, cortex, stele) were estimated by superimposing the micrographs with a transparent acetate sheet bearing a square lattice of point arrays, 10 mm apart, and by applying the point-counting analysis technique (Steer, 1981).

Statistical analysis

The data were subjected to analysis of variance (ANOVA) using the SPSS statistical package (SPSS, INC., Chicago, U.S.A.). For comparison of the means, the Duncan's multiple range test for $p < 0.05$ was employed.

RESULTS

Growth parameters

The fresh weight of plantlets was not significantly influenced by the change of Mn concentration in the medium (Table 1). However, the fresh weight and the total length of roots per shoot explant grown without Mn (0 mg l^{-1}) or in the presence of 10.96 mg l^{-1} Mn were significantly lower than the corresponding values recorded when the medium contained 2.74-5.48 mg l^{-1} Mn (fresh weight of roots) or 1.37-5.48 mg l^{-1}

TABLE 1. Growth parameters of *C. maxima* plantlets grown under various Mn concentrations

Parameter	Mn (mg l^{-1})	Value (mg)	Parameter	Mn (mg l^{-1})	Value (mm)
	0	453.8[a]		0	158.2[b]
Total	1.37	481.2[a]	Total	1.37	189.0[a]
fresh weight	2.74	494.1[a]	length of roots	2.74	204.8[a]
per plantlet	5.48	458.6[a]	per plantlet	5.48	185.9[a]
	10.96	427.1[a]		10.96	136.4[b]
	0	123.1[b]		0	42.2[c]
Fresh	1.37	141.8[ab]	Mean	1.37	56.8[ab]
weight of roots	2.74	148.3[a]	length	2.74	54.9[b]
per plantlet	5.48	149.9[a]	per root	5.48	61.9[a]
	10.96	119.7[b]		10.96	53.3[b]

$N = 5$, each replication represents the mean value of 3 plantlets. The means of each parameter followed by the same letter(s) do not significantly differ from each other for $p < 0.05$ according to Duncan's multiple range test

TABLE 2. Content of carbohydrates (μmol) in fresh roots and shoots of *C. maxima* plantlets grown *in vitro* under various Mn concentrations

Plant part	Mn concentration in culture medium (mg l^{-1})				
	0	1.37	2.74	5.48	10.96
Shoots	12.67[b]	14.74[ab]	16.13[a]	16.46[a]	14.32[ab]
Roots	3.51[b]	4.88[a]	4.99[a]	4.45[a]	3.00[b]
Total	16.18[b]	19.62[a]	21.12[a]	20.91[a]	17.32[b]

$N = 5$, each replication represents the mean value of 3 plantlets. The means of each parameter followed by the same letter(s) do not significantly differ from each other for $p < 0.05$ according to Duncan's multiple range test

Mn (total length of roots). It was also observed that the correlation coefficient between the number of roots formed per shoot explant and the concentration of Mn in the culture medium was significant and negative ($r = -0.275$, $p < 0.05$, $N = 75$). Finally, when the shoot explants were placed in the culture medium with no Mn addition, the mean length per root was significantly decreased compared to the culture medium containing Mn (1.37-10.96 mg l^{-1}).

Carbohydrates

The total content (μmol) of carbohydrates per plantlet was considerably lower in the treatments 0 (16.18 μmol) and 10.96 mg l^{-1} Mn (17.32 μmol), compared to the media containing 1.37-5.48 mg l^{-1} Mn (19.62-21.12 μmol). Similar effects of Mn were observed with regard to the carbohydrate content of the shoots and the roots (Table 2).

Root anatomy

Comparative cross sections of adventitious roots of *C. maxima* grown in culture media containing 0, 1.37, 2.74, 5.48 or 10.96 mg l^{-1} Mn are illustrated in Figs 1 and 2. Figure 1 concerns the region of the root 5-10 mm behind the root apex, while Fig. 2 the region 30-35 mm behind the root apex. In general, roots exhibited the typical primary anatomical structure of dicots. Thus, from outside toward inside, the root consisted of the epidermis (a single layer of cells), the exodermis (a layer of small cells with thick walls), the cortex (several rows of large parenchyma cells), and the stele (vascular cylinder). The stele was surrounded by the endodermis (en) (innermost layer of cortex) and consisted of the pericycle (pc) and four to six alternate poles of protoxylem (X) and protophloem (P).

Concerning the arrangement of the histological components of the roots, no differences were ob-

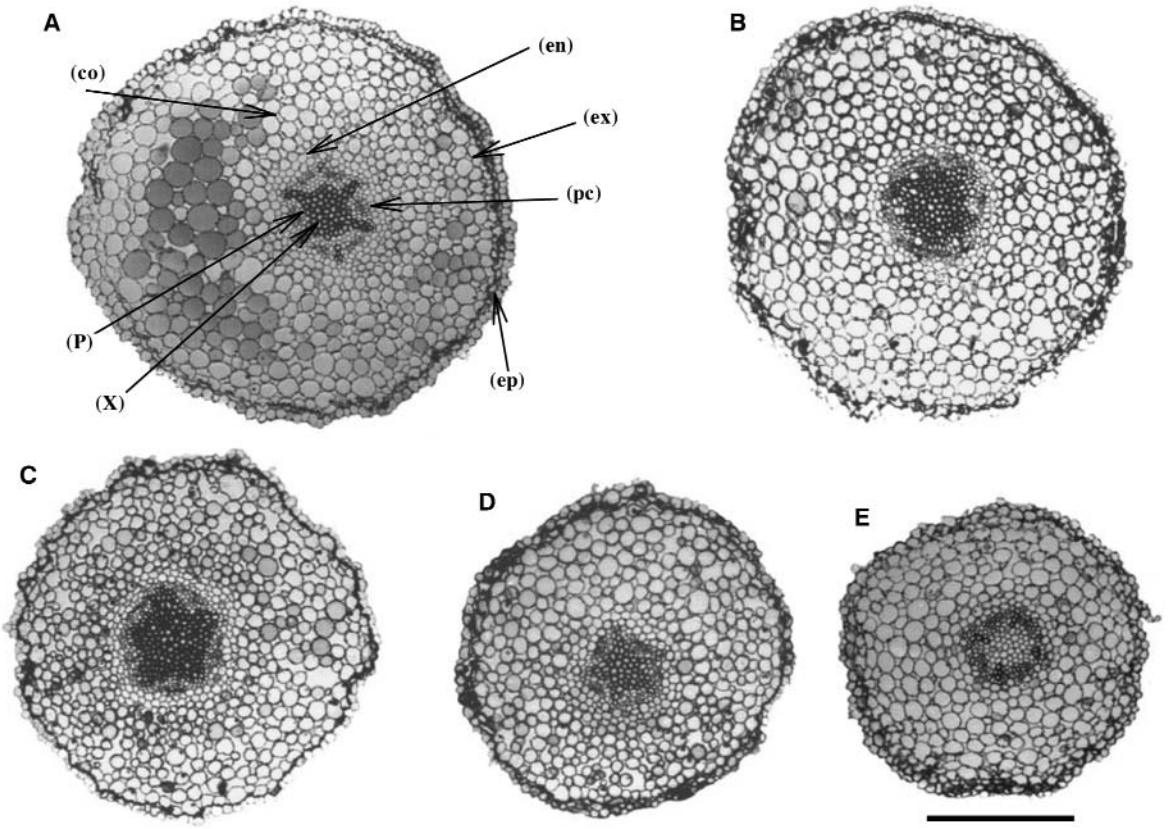

FIG. 1. Cross sections of *C. maxima* adventitious roots cut 5-10 mm behind the root apex and grown under 0.00 (A), 1.37 (B), 2.74 (C), 5.48 (D) or 10.96 (E) mg l^{-1} Mn (scale bar: 200 μm). epidermis (ep), exodermis (ex), cortex (co), endodermis (en), pericycle (pc), phloem (P), xylem (X).

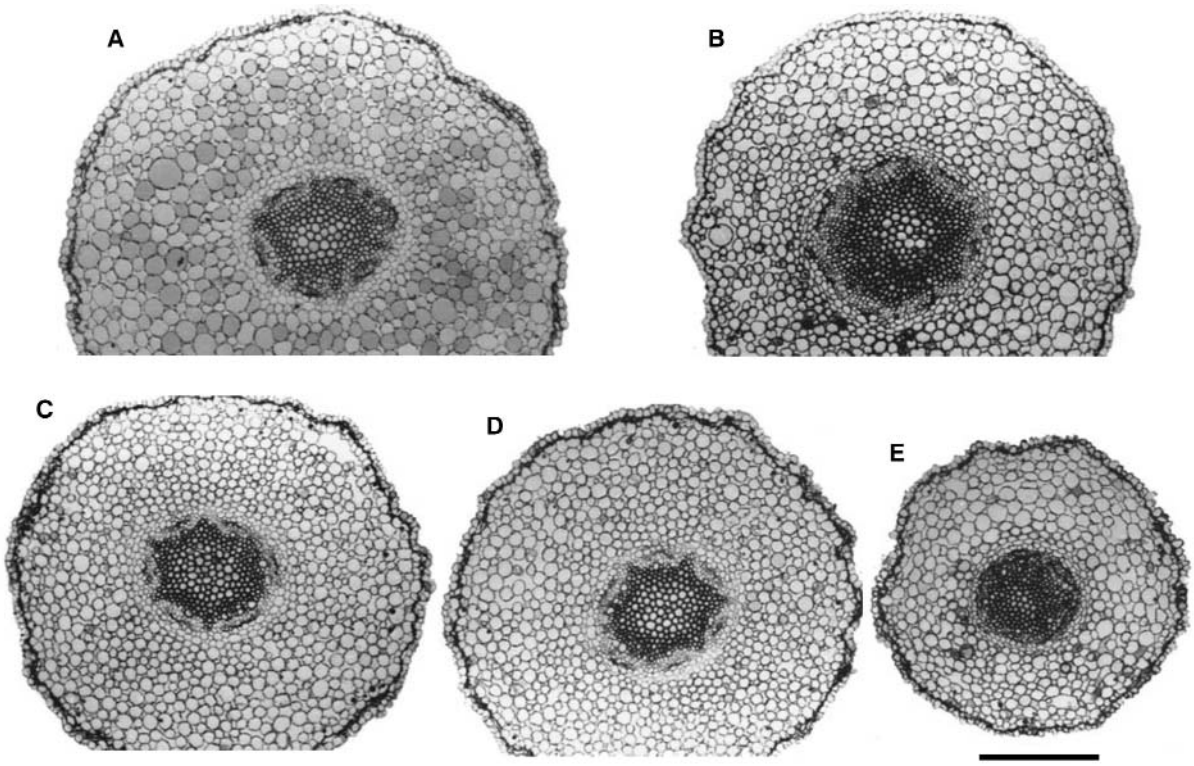

FIG. 2. Cross sections of *C. maxima* adventitious roots cut 30-35 mm behind the root apex and grown under 0.00 (A), 1.37 (B), 2.74 (C), 5.48 (D) or 10.96 (E) mg l^{-1} Mn (scale bar: 200 μm).

served between the two root regions (5-10 mm *vs*. 30-35 mm). However, the diameter of roots was greater in the region 30-35 mm than in the region 5-10 mm behind the root apex. The anatomy of the roots in the various Mn treatments underwent remarkable alterations, particularly referring to the total root diameter as well as the diameters of cortex and stele, which were all progressively decreased as concentration of Mn in the medium increased (Figs 3A and 4A). Based on the relative area (%) of the epidermis, the cortex, and the stele per root cross section, it was revealed that there were no significant differences among the five Mn treatments in none of the two root regions (5-10 mm and 30-35 mm) and for none of the above mentioned characteristics (Figs 3B and 4B). However, in the sections that were closer to the root apex, (5-10 mm behind the root apex), the stele (vascular cylinder) occupied less (%) of the root cross section (8.31-11.23) compared to the sections 30-35 mm behind the root apex (11.41-16.95), irrespectively of the Mn treatment (Figs 3B and 4B).

DISCUSSION

The nutritional status of the plants influences considerably the allocation of carbohydrates and thus the partitioning of the dry mass between the shoots and the roots. This is ascribed to the modification of the ability of the plants to produce carbohydrates and/or of the carbohydrates to translocate from the shoots to the roots (Marschner, 1995; Cakmak & Engels, 1999). In the treatments 0 and 10.96 mg l^{-1} Mn, where the total length of roots formed per shoot explant was significantly smaller compared to the other Mn treatments (Table 1), the total quantity of carbohydrates per plantlet was also considerably lower (Table 2), compared to the intermediate treatments of Mn (1.37-5.48 mg l^{-1}). Similar results were recorded concerning the carbohydrates contained in the roots (Table 2). According to Campbell & Nable (1988), the inhibition of root growth in Mn deficient plants is due to the shortage of carbohydrates as well as to the direct requirement of Mn for growth, i.e., metabolism of indole-acetic-acid (IAA). The rate of root elongation seems to respond more rapidly to Mn deficiency

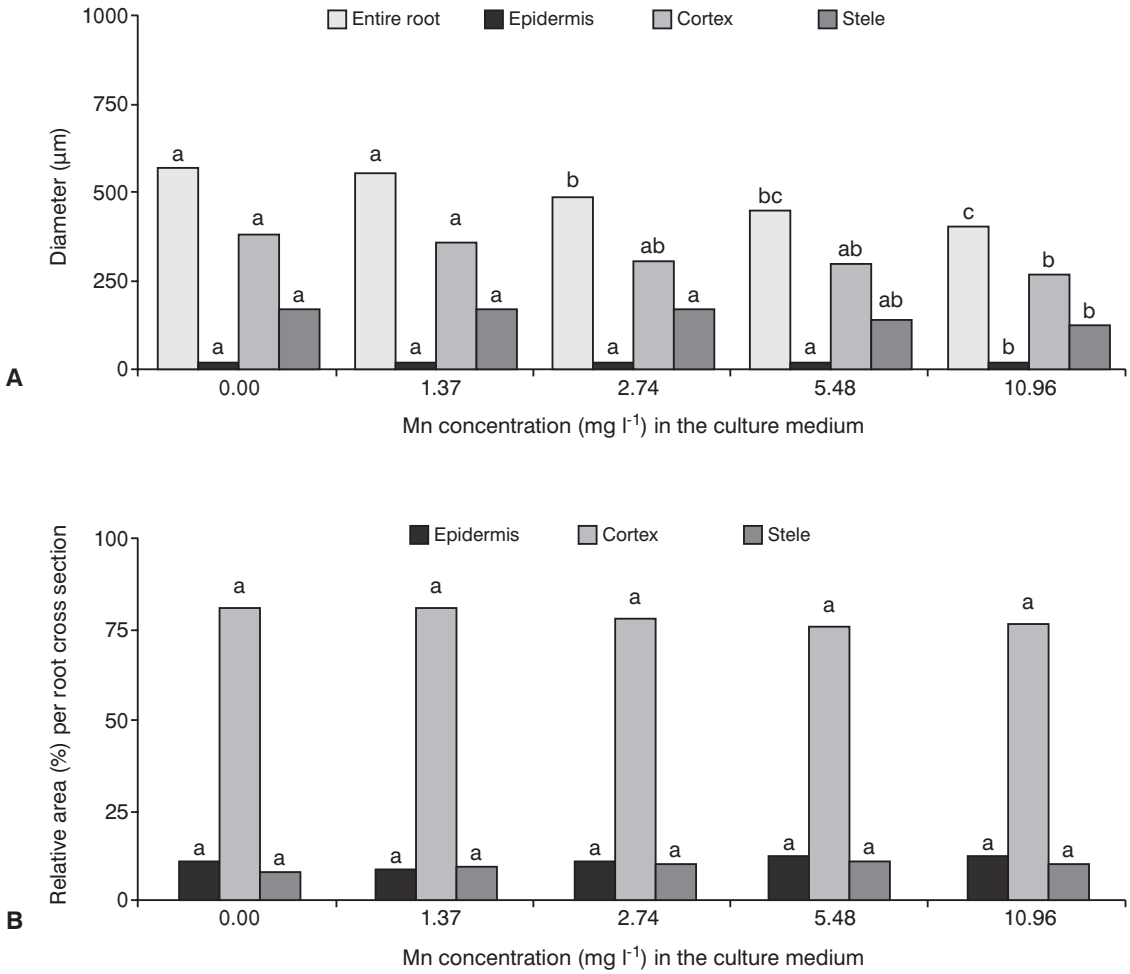

FIG. 3. Morphometric assessments of roots sampled 5-10 mm behind the root apex as affected by Mn concentration (mg l^{-1}) in the culture medium. A. Diameters of epidermis, cortex, stele, and entire root. B. Relative area (%) of epidermis, cortex, and stele per root cross section. The means of each measurement followed by the same letter(s) do not significantly differ from each other ($p < 0.05$, Duncan's multiple range test).

than does the rate of cell division (Marschner, 1995). Since: i) the ability of plantlets to supply with carbohydrates their continuously growing root system was similar in the treatments 0 and 10.96 mg l^{-1} Mn (there were no significant differences between these two treatments regarding the carbohydrate content of the roots), and ii) the total length of roots per shoot explant did not significantly differ between the two treatments, the considerably smaller mean length per root observed in the treatment 0 mg l^{-1} Mn, than under 10.96 mg l^{-1} Mn (Table 1), was obviously assigned to the increased number of roots formed per shoot explant grown without any Mn addition. Indeed, the number of roots developed at the base of each shoot explant was negatively correlated with the concentration of Mn in the growth medium ($r = -0.275$, $p < 0.05$).

The results of our study also indicated that the increase of Mn concentrations in the growth medium was followed by a proportional reduction of the root diameter. By studying the root cross sections and the relevant morphometric assessments, it is obvious that for the same Mn treatment, the total diameter of the root as well as the diameters of the various root histological components were greater when the root samples were received closer to the root base, i.e., 30-35 mm behind the root apex. In other words, the diameter of the root and the diameters of the epidermis, cortex, and central cylinder increased the closer to the root base the samples were taken. Therefore, the fact that the diameter of roots in the Mn treatment 0 mg l^{-1} was significantly greater than that of

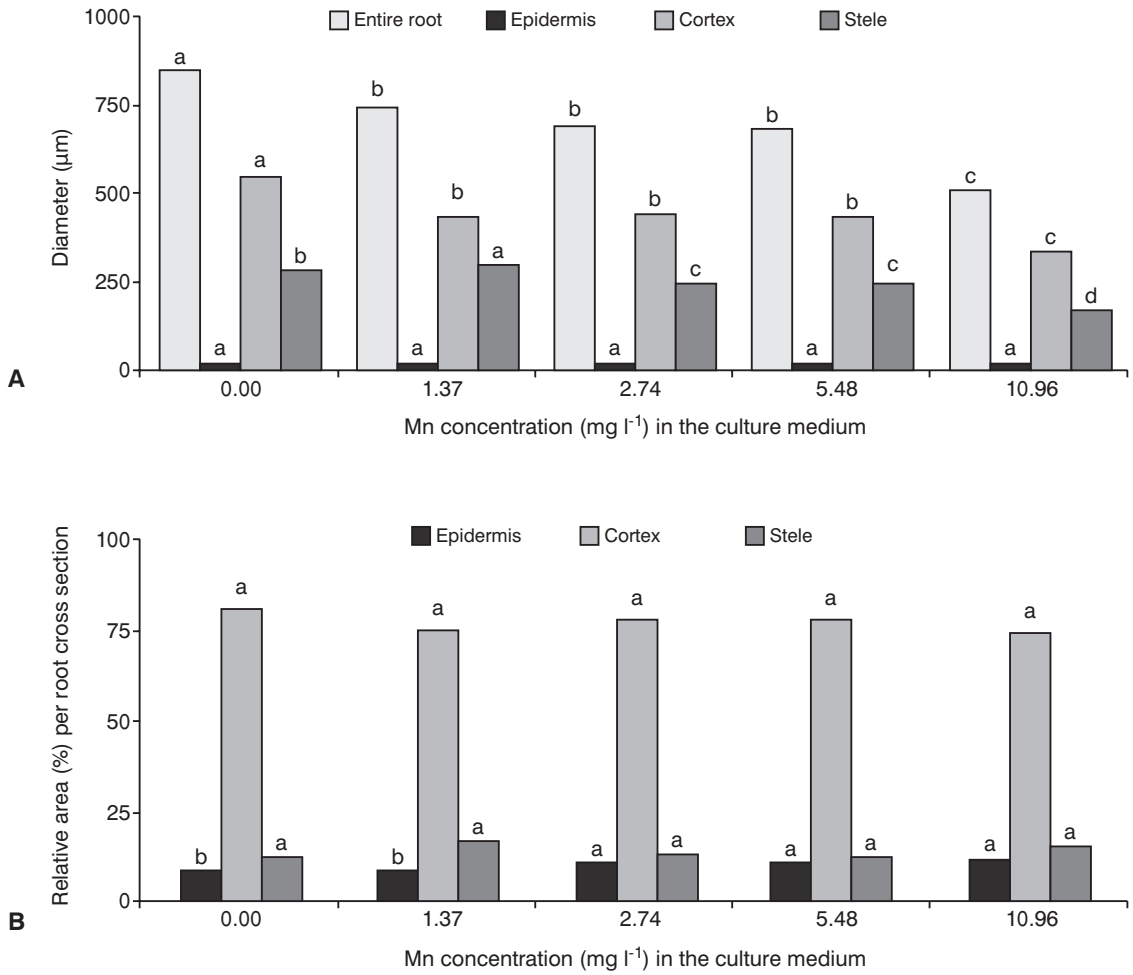

FIG. 4. Morphometric assessments of roots sampled 30-35 mm behind the root apex as affected by Mn concentration (mg l^{-1}) in the culture medium. A. Diameters of epidermis, cortex, stele, and entire root. B. Relative area (%) of epidermis, cortex, and stele per root cross section. The means of each measurement followed by the same letter(s) do not significantly differ from each other ($p < 0.05$, Duncan's multiple range test).

the other treatments, was rather a result of the effect of Mn in the length of roots (in the absence of Mn, roots were shorter). Given that: i) the mean length of roots did not considerably differ between the treatments 1.37, 2.74 and 10.96 mg l^{-1} Mn (Table 1), and ii) the mean root diameter and the number of cells per root cross section were smaller in the treatment 10.96 mg l^{-1} Mn, compared to the treatments 1.37 and 2.74 mg l^{-1} Mn (Figs 1-4), it could be concluded that high concentrations of Mn in the culture medium probably affect negatively the division of root cells. Similarly, Foy *et al.* (1978) reported that the toxicity of Mn results in the reduction of the number and size of nodules in the roots of bean plants. Also, high Mn concentrations (32 mg l^{-1}) in the nutrient solution supplied to the roots of rice plants, although they did not cause any changes in the anatomical structure of roots, resulted in a reduction of the root diameter and in the production of lateral roots (Lidon, 2002). Furthermore, although Mn concentrations did not considerably influence the anatomical pattern of the adventitious roots of the *C. maxima* plantlets, as indicated by the morphometric data, the increase of Mn in the medium contributed to a reduction of the diameter of the roots. A decrease in root diameter was also recorded in other plant species under salinity stress (Rashid *et al.*, 2001; Reinoso *et al.*, 2004).

Microscopic observations revealed the existence of an exodermis (one layer of small cells with thick walls) located between the epidermis and the cortex of the root. Exodermis was also observed in the roots of the sour orange and asparagus plants, while it was

not found in the roots of other species such as peach and soybean plants (Rieger & Litvin, 1999). The exodermis is considered to protect the internal soft parenchymatic tissue (cortical cells) and also to control the uptake of water and mineral nutrients from the nutrient medium, in a way similar to that of the endodermis.

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