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) CO₂ 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-
ciency symptoms appear in the shoots. Thus, we con-
sider that in vitro culture of shoot explants in a root-
ning medium with different Mn concentrations pro-
vides 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 con-
centrations 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 phospho-
rylated form, there is a plausible connection of Mn to
carbohydrate metabolism (Marschner, 1995). There-
fore, another goal of this experiment was to examine
whether Mn supply affects the content of carbohy-
drates 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 run-
ning tap water and then rinsed twice in distilled wa-
ter. Afterwards, they were surface-sterilized with be-
nomyl (1% w/v) for 20 min, rinsed three times in ste-
ryle distilled water for 8 min each time, followed by
sodium hypochloride (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 cul-
ture tube (100 × 25 mm) containing 10 ml of MS me-
dium (Murashige & Skoog, 1962), which had a dou-
ble 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; pyridox-
ine-HCl, 0.5; thiamine-HCl, 0.1; glycine, 2; myo-inosi-
tol, 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 steri-
le conditions. Subsequently, the culture tubes with
the explants were incubated in a growth chamber illu-
minated with cool white fluorescent tubes (45 µmol
m–2 s–1, 400-700 nm) for 16 hrs a day. The tempera-
ture 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 sup-
plemented 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
MnSO₄·H₂O). 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 pro-
cess, 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 param-
eters (5 replications per Mn treatment, each one con-
sisting 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
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 mul-
tiplying 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% OsO4 (4°C). For tissue dehydration, an ethanol series (50-100%) was used, and this was followed by resin infiltration and embedment (Spurr, 1969). Semi-thin sections (1 μm thick) of plastic embedded root segments were cut on a Reichert Om U2 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>
<thead>
<tr>
<th>Parameter</th>
<th>Mn (mg l(^{-1}))</th>
<th>Value (mg)</th>
<th>Parameter</th>
<th>Mn (mg l(^{-1}))</th>
<th>Value (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total fresh weight</td>
<td>0</td>
<td>453.8(^a)</td>
<td>Total fresh weight</td>
<td>0</td>
<td>158.2(^b)</td>
</tr>
<tr>
<td>per plantlet</td>
<td>1.37</td>
<td>481.2(^a)</td>
<td>length of roots</td>
<td>1.37</td>
<td>189.0(^a)</td>
</tr>
<tr>
<td></td>
<td>2.74</td>
<td>494.1(^a)</td>
<td>per plantlet</td>
<td>2.74</td>
<td>204.8(^a)</td>
</tr>
<tr>
<td></td>
<td>5.48</td>
<td>458.6(^a)</td>
<td>per plantlet</td>
<td>5.48</td>
<td>185.9(^a)</td>
</tr>
<tr>
<td></td>
<td>10.96</td>
<td>427.1(^a)</td>
<td>per root</td>
<td>10.96</td>
<td>136.4(^a)</td>
</tr>
</tbody>
</table>

\( N = 5 \), 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 1. Growth parameters of C. maxima plantlets grown under various Mn concentrations**

<table>
<thead>
<tr>
<th>Plant part</th>
<th>Mn concentration in culture medium (mg l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Shoots</td>
<td>12.67(^b)</td>
</tr>
<tr>
<td>Roots</td>
<td>3.51(^b)</td>
</tr>
<tr>
<td>Total</td>
<td>16.18(^b)</td>
</tr>
</tbody>
</table>

\( N = 5 \), 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**
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 (\( \mu \text{mol} \)) of carbohydrates per plantlet was considerably lower in the treatments 0 (16.18 \( \mu \text{mol} \)) and 10.96 mg l\(^{-1}\) Mn (17.32 \( \mu \text{mol} \)), compared to the media containing 1.37-5.48 mg l\(^{-1}\) Mn (19.62-21.12 \( \mu \text{mol} \)). 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-
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 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.
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
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 endodermis 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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REFERENCES


