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Growth Patterns in Non-Mycorrhizal and Mycorrhizal Short Roots of *Pinus sylvestris*

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Abstract

The structure of lateral roots, non-mycorrhizal and mycorrhizal short roots of *Pinus sylvestris* were studied by 4',6-diamidino-2-phenylindole (DAPI) staining and 5-bromo-2-deoxyuridine (BrdU) labelling of nuclei in thin sections. The microtubule cytoskeleton of the meristematic and elongating cells of the roots was visualized by using a tubulin antibody. The orientation and shape of the BrdU-labelled nuclei and microtubule cytoskeleton was viewed by laser scanning confocal microscopy and the distance between the meristem and the first maturating xylem elements was measured. The methods used revealed that a lower number of cells is produced for the elongation zone in short roots than in lateral roots. This causes the poor elongation of the short roots. In roots with limited capacity to elongate, the slow-growing ectomycorrhizal fungus *Suillus bovinus* is able to compete with the root extension rate and to grow between cortical cells. In ectomycorrhiza, the elongation pattern of the short root is maintained, but the presence of fungal mycelium around the root apex seems to affect the rate and direction of divisions in the meristem.

Keywords: Ectomycorrhiza, *Pinus sylvestris*, *Suillus bovinus*, root development, BrdU, microtubules, IIF-microscopy

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1. Introduction

In Scots pine seedlings, three different root types may be distinguished: (1) main, (2) lateral and (3) short roots and it is well-known that the short roots are the only root type in which ectomycorrhiza is formed (Robertson 1954; Wilcox 1968). The establishment of the symbiotic relationship with the fungus leads to changes in the short root growth pattern and morphology, which are seen as formation of dichotomous or coralloid mycorrhizal short roots (Piche et al., 1982, 1983; Harley and Smith, 1983; Kottke and Oberwinkler, 1986; Massicotte et al., 1989; Smith and Read, 1997).

In the pine root system, we are interested in the morphogenesis of the short roots, in the factors that facilitate the invasion of the fungal hyphae into the short roots and in the regulation of the short root development after fungal invasion (Timonen et al., 1993, 1996; Niini et al., 1996). In the present work, these questions were approached by comparing the anatomy of lateral roots with that of uninfected and infected short roots. Special attention has been paid to the distribution of replicating and dividing nuclei in the root apex. The distance between the first mature xylem elements and meristematic initials was also measured as an indicator of differentiation and maturation of the vascular tissue. The structure of the microtubule cytoskeleton in the meristem and at the base of the elongation zone of uninfected and mycorrhizal short roots has also been viewed with laser scanning confocal microscopy.

2. Materials and Methods

Non-mycorrhizal and mycorrhizal *Pinus sylvestris* L. seedlings were grown as described in Niini et al. (1996) with the ectomycorrhizal fungus *Suillus bovinus* (L.: Fr.) O. Kuntze. Two-month-old seedlings either without or with well developed mycorrhizas, were used in the experiments.

Incorporation of 5-bromo-2-deoxyuridine (BrdU)

For the treatment of the intact roots, BrdU (Cell proliferation labelling reagent BrdU, RPN 201, Amersham International plc, Little Chalfont, UK) was diluted 1:500 with sterile water and the mycorrhizal and non-mycorrhizal plants were transferred to BrdU-solution. The tubes were placed in the growth chamber for 18 h.

Fixation and sectioning

Samples for the anatomical observations and detection of BrdU-

incorporation were fixed in vacuum with freshly made 4% paraformaldehyde in PBS (pH 7.4) for 3 h. The roots were rinsed three times, 10 min each with PBS (pH 7.4) and washed overnight in PBS (pH 7.4) containing 6.8% sucrose, at +4°C. The tissue was dehydrated in ascending ethanol series and infiltrated with Jung HistoResin Plus infiltration medium overnight at +4°C. Embedding of the samples into gelatine capsules with mixed HistoResin Plus infiltration medium and hardener was performed for 4 h on ice in a nitrogen atmosphere. Samples were stored desiccated until sectioning. Sections (5 μm thick) were manually cut with a LKB 8800 Ultrotome III microtome and allowed to adhere in a drop of water overnight on the SuperFrost*/Plus slides (Menzel-Gläser, Germany).

Anatomical observations

Sections were stained with 0.05% toluidine blue for 2 min, rinsed with water and mounted in glycerol:PBS (pH 8.9) (1:1). The size of the meristem and the distance between the first identifiable tracheid and the border of the meristem were measured microscopically using an image analyzer (GLOBAL LAB IMAGE®).

Detection of BrdU-incorporation

Sections were treated with 0.01% trypsine in 0.1% $CaCl_2$ (pH 7.8) for 5 min at +37°C and thoroughly rinsed with PBS (pH 7.4). Nuclear DNA was denatured with 0.5 N HCl in PBS (pH 7.4) for 30 min at room temperature, and the excess acid rinsed away by washing with PBS (pH 7.4) five times. Blocking was done with 10% whole goat serum (Cappel 55984, Organon Teknika Corporation, Durham, NC, USA), 2% BSA and 0.1% Tween20 in PBS (pH 7.4) for 1 h at room temperature. Sections were then treated with undiluted mouse monoclonal anti-BrdU antibody containing nuclease (RPN 202, Amersham) overnight at +4°C. The sections were washed 5 times with PBS (pH 7.4), then treated with the secondary antibody (biotinylated sheep anti-mouse Ig, RPN 1001, Amersham). The secondary antibody was applied diluted 1:40 with PBS (pH 7.4) and the sections incubated at room temperature for at least 3 h. After rinsing 5 times with PBS (pH 7.4), the sections were treated with the detection agents containing streptavidin-fluorescein (RPN 1232, Amersham) in dilution 1:40 overnight at +4°C. Final rinsing was done with PBS (pH 8.9) and the sections mounted in 1% 1,4-diazabicyclo-[2.2.2]octane (DABCO, Sigma Chemical Co., St. Louis, MO, USA) anti-fade solution containing 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI, Sigma) to stain the chromatin.

Detection of α-tubulin

Pine roots were fixed for 2 h in 4% paraformaldehyde in PBS (pH 7.4), transferred to 15% and 30% sucrose and incubated overnight for each step at +4°C, and infiltrated for 10 min with Tissue-Tek (4583 Miles Inc., Elkhart, IN, USA) before freezing with 2-methylbutane in liquid nitrogen. Cryosections (9 µm thick) were obtained with a Leitz 1720 Digital Kryostat at the temperature of –22°C. After the sections had adhered onto slides, the Tissue-Tek was removed by soaking the slides in PBS (pH 7.4) for 1 h. The samples were then treated with 0.1% Triton X-100 and 0.1% BSA for 1 h at room temperature. Sections were treated with primary antibody (mouse monoclonal anti-α-tubulin (DM1A, N 356, Amersham) diluted 1:500 with PBS (pH 7.4) and incubation carried out overnight at +4°C. After rinsing with PBS (pH 7.4), the sections were treated with secondary antibody, biotinylated sheep anti-mouse Ig, as in BrdU-detection.

Immunofluorescence microscopy

Sections were viewed with both Leitz dialux 20 microscope equipped with appropriate epifluorescence filters and with a Bio-Rad MRC 1024 laser scanning confocal microscope mounted onto an Axiovert 135M inverted microscope (Zeiss, Germany) and equipped with a krypton/argon laser with an exciting laser line at 488 nm and a 515 nm emission filter. Fluorescence images from the confocal microscope were captured using a Zeiss Plan-Apochromat 63× oil immersion objective (1,40 DIC, 0.17) and a detector with 522 DF ± 32 nm emission filter. Confocal images were enhanced with Kalman averaging for 4 scans. Serial optical sectioning was done at 0.5 µm intervals and 5 to 10 optical sections were compiled to three-dimensional images and saved as files. Images were captured as slides on ASA100 film using Microsoft POWERPOINT® and a Polaroid slide shooter. Prints were made directly from files using a Codonics NP-1600 printer. Light microscopical and DAPI-fluorescence pictures were taken on Agfapan ASA400 black and white film.

3. Results

In Scots pine seedlings, two types of short roots are distinguished: one type is formed of long and slender roots with a higher number of root hairs (Fig. 1A). The other type consists of truncated, robust roots with round apex and only a few root hairs (Fig. 1B). The majority of the short roots of pine seedlings belong to the latter type and mycorrhiza is only formed in this root type (Fig. 1C). In culture the number of truncated type of short roots increases at the site where

the fungus is inoculated (Fig. 2) and after fungal invasion the roots dichotomize and form coralloid structures (Figs. 1C and 2).

The comparison of the longitudinal sections of the central tissue in lateral and truncated short roots indicated differences in the organization of cell layers in the roots (Figs. 3A and 4A). In the center of the lateral roots, the zone behind the apical meristem was formed of four to five layers of slightly vertically elongated cells with a big central nucleus (Fig. 3A). The DAPI-staining of these cells showed that they were actively dividing (Fig. 3B). The direction of division and the elongation of the cells in this layer was always vertical in contrast to horizontal or oblique cell divisions and horizontal or isodiametric growth of cells in the apical meristem. In the lateral roots, the zone with cell divisions was followed by a zone with more elongated cells, in which the nuclei became long and narrow and were seldomly observed to divide (Fig. 3A). BrdU-labelling of replicating DNA in nuclei was in agreement with the DAPI-staining of dividing nuclei in the lateral roots. BrdU-label occurred in round nuclei of the apical meristem and in the slightly elongated nuclei behind the apical meristem, up to the zone with elongated cells (Fig. 3C).

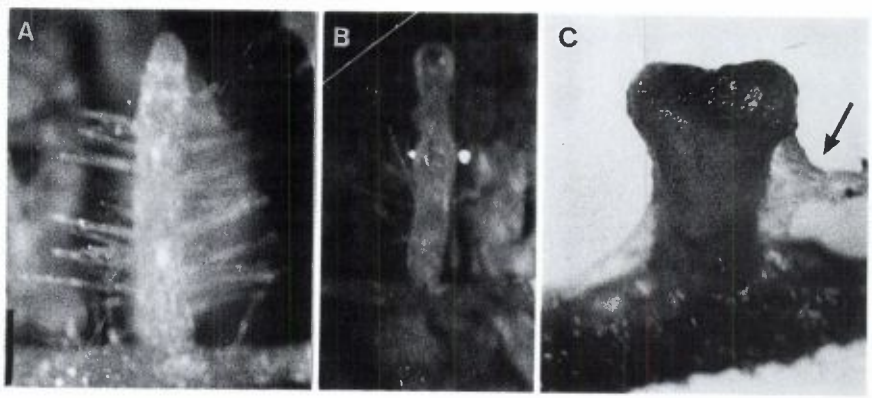

Figure 1. Non-mycorrhizal (A–B) and mycorrhizal (C) short roots. A: Slender short root covered with root hairs. B: Truncated short root with round apex and only few root hairs. C: Dichotomous mycorrhizal short root. Arrow points to the hyphae connecting the fungal mantle with the surrounding mycelium. Bar = 0.5 mm.

The examination of the longitudinal sections of truncated short roots (Fig. 4A) and DAPI staining of the sections (Fig. 4B) indicated that vertical divisions were restricted to one cell layer behind the apical meristem in the roots. The cell layer with vertical divisions was immediately followed by a few layers of tightly packed elongated cells with pointed ends (Fig. 4A). The cell layer with pointed ends was followed by long cells with small nuclei and

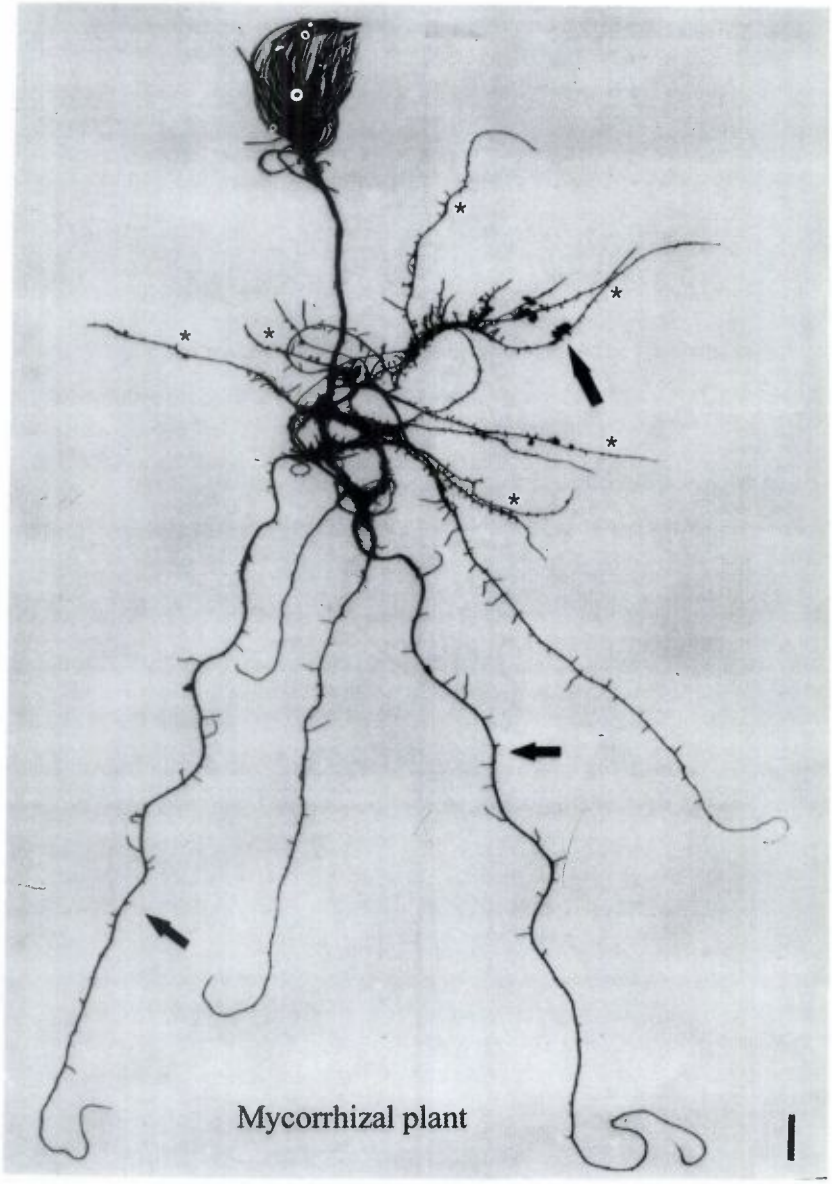

Figure 2. Root system of a two month-old *Pinus sylvestris* seedling grown with *Suillus bovinus* mycelium. Non-mycorrhizal short roots marked by short arrows and coralloid mycorrhizal short roots by long arrow. Note the decrease in the lateral root length and the increase in the number of truncated short roots in the part of the root system that have been in contact with the ectomycorrhizal fungus (stars). In five seedlings like the one presented here the mean of the short root number per one centimeter of lateral root length was 3.4±0.2 and 2.6±0.6 in the mycorrhizal and non-mycorrhizal parts of the root system, respectively. Bar = 1 cm.

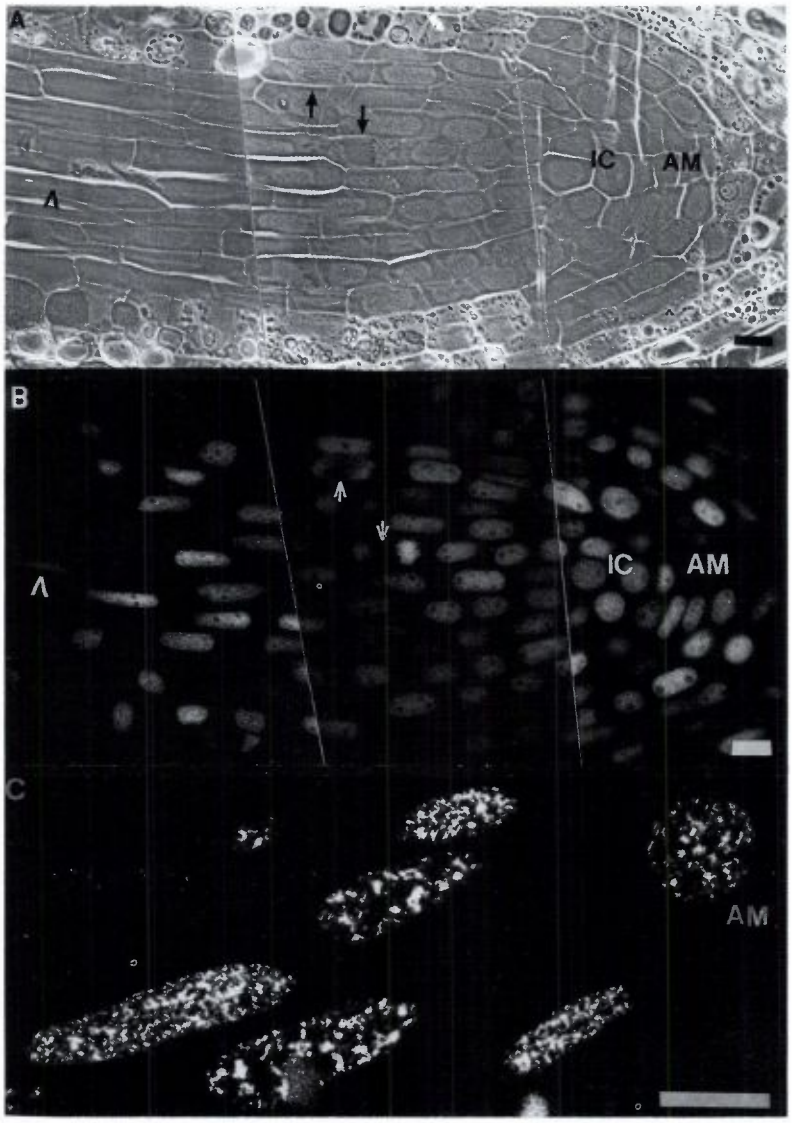

Figure 3. The structure of the apex in the lateral roots of *Pinus sylvestris*. A: Phase contrast microscopy of the apical region of a lateral root. Black arrows point to the nuclei undergoing mitosis in several cell layers behind the apical meristem (AM). Note that the length of the cells increases behind the cell layers with dividing nuclei (arrowhead). B: DAPI-staining of nuclei in the same section as in A. Mitotic nuclei marked by arrows and a narrow nucleus from the elongation zone by an arrowhead. C: BrdU-incorporation of the nuclei behind the apical meristem as seen with laser scanning confocal microscopy. Note the higher magnification in C than in A and B. IC = initial cell. Bar = 10 μm.

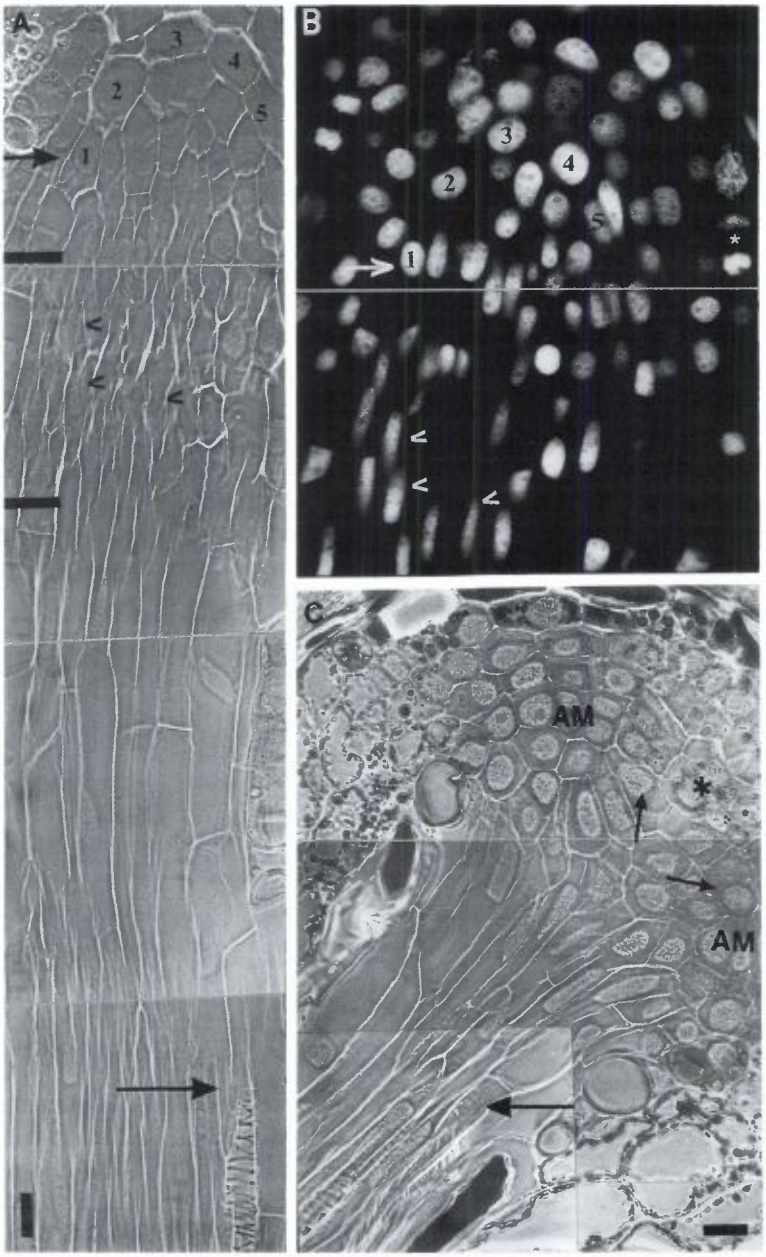

Figure 4. The structure of a truncated short root (A, B) and a mycorrhizal short root apex (C) of *Pinus sylvestris*. A: Phase contrast microscopy and B: DAPI-staining of the same longitudinal section of the truncated short root. The numbers in A and B mark the same cells, a black (A) and a white (B) arrow point to a cell layer behind the apical meristem in which vertical cell divisions occur (star in B).

big vacuoles. In short roots, the BrdU-labelled nuclei had a more restricted distribution than in lateral roots. The BrdU-label occurred in the nuclei of apical meristem and in the layer next to it.

In mycorrhizal short roots, the elongation of the cells appeared to take place even more abruptly than in non-mycorrhizal short roots. No cell layers with vertically divisions or cells with pointed ends, as in the root tips of laterals and short roots, could be distinguished. The elongated cells with large vacuoles and elongated nuclei appeared to develop almost directly from the apical meristem (Fig. 4C). In the mycorrhizal short root apex, the number of horizontal cell layers and the number of cells in each layer was higher than in a non-mycorrhizal short root apex. On the other hand, the size of individual cells was smaller. In dichotomous roots cortical cells with vacuoles and amyloplasts occurred between the two meristems (Fig. 4C). Dividing or BrdU-labelled nuclei were detected only in the apex of mycorrhizal short roots.

The central cylinder of the roots develops into vascular tissues, in which the mature xylem elements are easy to recognize due to their wall thickenings. In the lateral roots, the first mature xylem elements occurred 749 ± 121 μm from the meristem (n=6), while in the short roots the measured distance between the meristem and the first mature xylem element was 222 ± 26 μm (n=6). In the mycorrhizal short roots, the distance between the first mature xylem elements and meristem was the same as in non-mycorrhizal short roots, although in Fig. 4C the first differentiated xylem element appears to be much closer, about 80 μm apart from the upper border of the meristem.

The investigation of thick sections of the central tissues of non-mycorrhizal and mycorrhizal short roots labelled with tubulin antiboby and observed with laser scanning confocal microscopy showed the typical orientation of microtubules in different parts of the root tip. In elongated cells above the apex the orientation of the cortical microtubules were horizontal (Figs. 5A and B) while vertically orientated microtubules were detected in the horizontally elongated cells of the apical meristem (Figs. 5A and C). Both in mycorrhizal and non-mycorrhizal short roots, the cells with the different orientation of cortical microtubules were separated by a layer of round cells with loosely orientated microtubules. These cells were probably the initial cells of the

Figure 4. Continuation. Black bars in A define the region of the short root with elongated, spindle shaped cells and arrowheads in A and B points to the elongated nuclei of these cells. A differentiated tracheid (black arrow, A) is seen 216 μm behind the meristem initial cells. C: An apex of a dichotomously branching mycorrhizal root. Small black arrows points to the two apical meristems (AM) with differentiated cortical cells in the middle (star). Long arrow points to the first differentiated tracheid 83 μm from the border of the meristem. Bar = 10 μm.

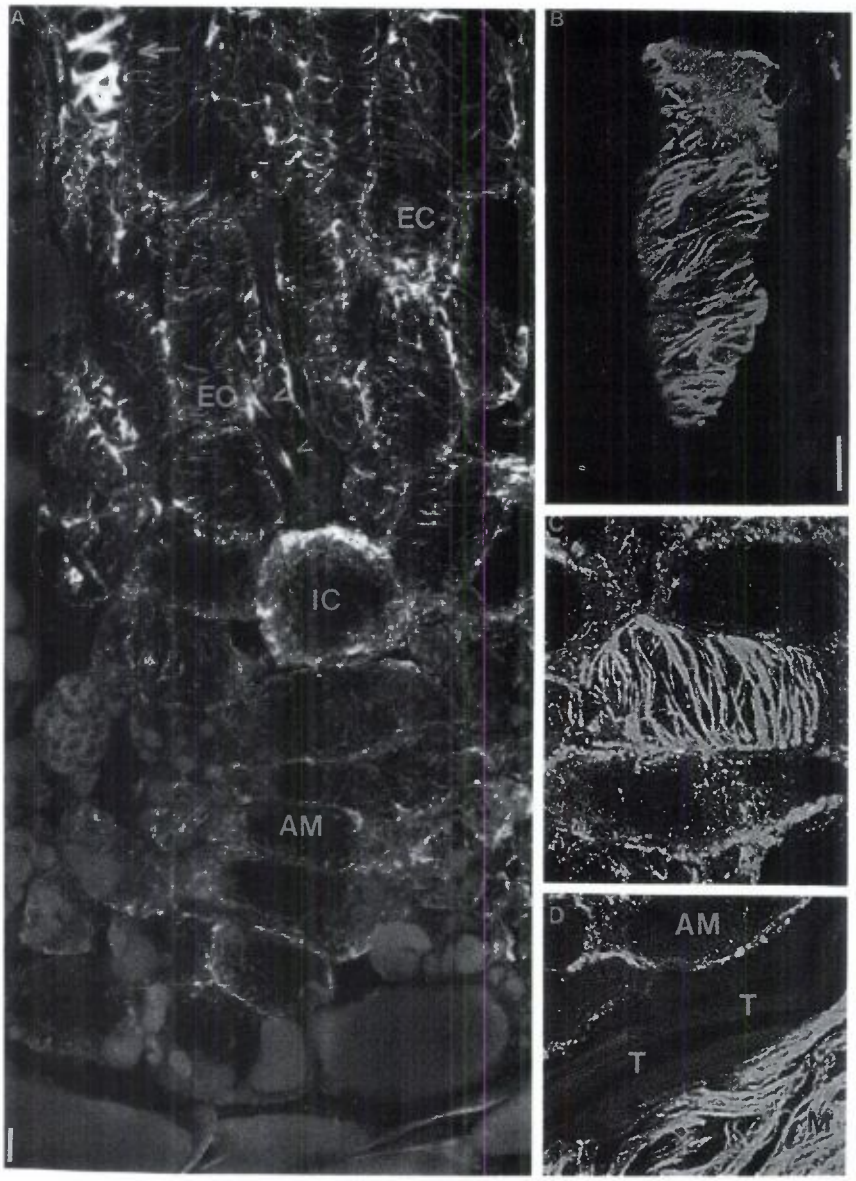

Figure 5. Cortical microtubules in the apical cells of the short roots as revealed by α-tubulin antibody in laser scanning confocal microscope. A–C: Non-mycorrhizal truncated short root. D: Mycorrhizal short root. A: In the horizontally elongating meristem cells of the apex (AM) the microtubules are orientated vertically as shown in the cell in C, in which the sectioning has left the cortical microtubules intact. In the round meristem initial cells (IC) the microtubule organization is flexible. The elongated cells (EC) behind the intial cells have mainly horizontally arranged cortical microtubules as shown clearly in B.

meristem (Esau, 1977), behind which the layer of vertically dividing cells occurred in mycorrhizal and non-mycorrhizal (Fig. 5A) short roots. In the mycorrhizal short roots the microtubule cytoskeleton of the fungal hyphae surrounding the root apex were clearly distinguished (Fig. 5D).

4. Discussion

In pine the early development of ectomycorrhizal short roots and the formation and structure of the mantle and Hartig net in short roots have been thoroughly investigated (Piche et al., 1982, 1983; Harley and Smith, 1983; Kottke and Oberwinkler, 1986, 1987; Smith and Read, 1997). However, the differences in the growth pattern between laterals and short roots and the reasons for the formation of ectomycorrhiza only in short roots have achieved less attention. During root development the elongation is determined by (1) the rate of cell production in the meristem and (2) the rate of elongation of the cells after leaving the meristem (Barlow et al., 1991). In *Pinus sylvestris* the labelling of the replicating DNA with BrdU and DAPI staining of the dividing nuclei drew attention to the differences in the rate of cell production for the elongation zone between lateral roots and truncated short roots. The distribution of the BrdU-labelled and mitotic nuclei correlated well in lateral and short roots which confirmed that the BrdU visualized nuclei in cells with an actively progressing cell cycle and led thus to a more comprehensive view of the process than DAPI-staining of the nuclei. In plants the BrdU-labelling has previously been successfully used for investigation of the DNA replication in pea (Levi et al., 1987) and maize (Kerk and Feldman, 1995) root meristems, soybean protoplasts (Wang et al., 1989 a, b) and tobacco leaves (Stroobants et al., 1990).

In lateral roots the BrdU-signal and nuclear divisions occurred regularly in the apical meristem and in the four to five layers of cells behind the apical meristem, which proved that the cell cycle was in progress in these cells. In the fourth or fifth layer of cells the BrdU-labelling and the number of dividing nuclei decreased and the nuclei and cells became longer suggesting that cell proliferation gradually changed to cell elongation (Baluska et al., 1990, 1996,

Figure 5. Continuation. In the cell above the round initial cell the nucleus is dividing and the fluorescence in the cell is due to spindle microtubules (arrowheads). This was confirmed by DAPI-staining of the nuclei of the same section (not shown). A differentiating xylem element marked by an arrow. D: Mantle (M) formed by *Suillus bovinus* is distinguished due to the strong fluorescence of microtubule tracks in the hyphae. Between the mantle hyphae and meristem cells (AM) the outer root cells are flattened and filled with tannins (T). Bar = 10 μm.

1997; Ishikawa and Evans, 1995). In the truncated short roots, the BrdU-labelled and dividing nuclei were detected only in the apex and in the cell layer behind the apical meristem. Behind this layer, nuclear division or BrdU-labelling of nuclei were rare. Instead, the cells close to the apical meristem were elongated and had long narrow nuclei, which are features typical to the cells of root elongation zone.

These observations suggest that the different growth pattern in the laterals and short roots is related to the difference in the rate of cell production in the meristem. In lateral roots, the cell divisions in four to five layers of cells produce initials for the cell elongation zone while in the truncated short roots, studied here, cell divisions in only one cell layer seem to provide initials for the elongation zone. This must affect the growth rate of the roots and could explain why the elongation of the short roots is retarded and tissue maturation occurs closer to the meristem in short roots than in laterals. Whether the rate of cell elongation after leaving the meristem (Barlow et al., 1991) is also affected in the short roots remains to be clarified. The limited growth of the short roots is probably the prerequisite for the establishment of the ectomycorrhiza in these roots. In truncated short roots the slow-growing ectomycorrhizal fungi (Niini and Raudaskoski, 1993) are able to cope with the growth rate of the plant tissue and to form the mantle around the root and the Hartig net between the cortical cells (Gianinazzi-Pearson, 1984).

In mycorrhizal short roots the BrdU-labelling and the dividing nuclei occurred only in the very apex and were absent behind the apex, as in the non-mycorrhizal short roots. In the presence of the fungus the apical cell divisions led to the formation of a broader and flatter meristem than in the non-mycorrhizal short roots, which indicates that the fungal invasion affects the rate and direction of divisions in the meristem. The differentiation of non-dividing, vacuolated cortical cells into the middle of the broad meristem splits it in two parts and initiates the development of the dichotomous short root as described previously (Faye et al., 1980; Piche et al., 1983). In the mycorrhizal short roots the elongation of the cells appeared to start next to the apical meristem and was followed by rapid differentiation of root tissues, including xylem elements, which could be advantageous for transport of metabolites between the symbiotic partners.

The laser scanning confocal microscopical investigations of the microtubule cytoskeleton in the apical region of non-mycorrhizal and mycorrhizal short roots showed that in both root types the horizontally elongated cells with vertically orientated cortical microtubules in the root apex changed abruptly to vertically elongated cells with transversely or obliquely arranged cortical microtubules. Between these cells only one cell layer with randomly orientated microtubules was located. This is in agreement with the present anatomical

and cytological observations on short roots which suggested that between meristematic and elongating cells in short roots only one intervening cell layer may be present. It also showed that the orientation of cortical microtubules may change profoundly from one cell layer to another. The laser scanning confocal microscopical results agree with the previous IIF-microscopical studies on microtubule cytoskeleton in mycorrhizal short roots, in which spindles and vertically orientated cortical microtubules were detected in the root apex and transversely orientated cortical microtubules in the root centre in cells differentiating to vascular tissue (Timonen et al., 1993). In *P. sylvestris* non-mycorrhizal and mycorrhizal short roots, a short root-specific α-tubulin isotype pattern was recently identified (Niini et al., 1996) which could be significant for the specific growth pattern of the short roots described here.

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