*New Zealand Journal of Botany, 2005, Vol. 43*: 61–70
0028–825X/05/4301–0061 


# Histological events during *in vitro* rooting of *Nothofagus nervosa* (Fagaceae)

GUILLERMO MARTÍNEZ PASTUR
MIRIAM ARENA
Centro Austral de Investigaciones Científicas (*CONICET*)
cc 92 (9410)
Ushuaia, Tierra del Fuego, Argentina

LUIS HERNANDEZ
Departamento de Agronomía
Universidad Nacional del Sur
Altos del Palihue (8000)
Bahía Blanca, Buenos Aires, Argentina
and
Comisión de Investigaciones Científicas (*CIC*)
Calle 526 e/ 10 y 11
(1900) La Plata, Buenos Aires, Argentina

NÉSTOR CURVETTO
Departamento de Agronomía
Universidad Nacional del Sur
Altos del Palihue
(8000) Bahía Blanca, Buenos Aires, Argentina
and
Centro de Recursos Naturales Renovables de la Zona Semiárida (*CERZOS – CONICET*)
Km 7, Camino a la Carrindanga
(8000) Bahía Blanca, Buenos Aires, Argentina

ELEONORA ELIASCO
Centro Austral de Investigaciones Científicas (*CONICET*)
cc 92 (9410)
Ushuaia, Tierra del Fuego, Argentina

**Abstract** *In vitro* rooting protocols were previously performed on *Nothofagus nervosa* to optimise the culture medium and environmental conditions. A qualitative and quantitative tissue differentiation study during adventitious rooting in *N. nervosa* under different light/dark conditions was carried out to increase knowledge of the rhizogenesis process in shoots. The histological events through the rooting period were consistent with the proposed biochemical markers (peroxidases, tissue nutrient contents, polyamines, flavonoids). Development of parenchymatic tissues, shown by the staining pattern of the cell contents, and cambium activity were the best tissues to define the rooting phases. Knowledge of the histological changes could be used to optimise a protocol for micro-propagation improving timing, quantity, and quality of roots, which would positively affect the survival rate during hardening.

**Keywords** rooting phases; cambium activity; root anatomy; histo-chemistry; peroxidase activity

**Abbreviations** BTM: Broadleaved Tree Medium; IBA: indol-3-butyric-acid; IAA: indol-3-acetic-acid.

*B04020; Online publication date 17 March 2005*
*Received 8 June 2004; accepted 31 August 2004*

## INTRODUCTION

Micropropagation rooting protocols have been developed in the past by testing different culture media and environmental conditions (Chalupa 1983). However, most of these trials were not done in parallel with biochemical or histological studies, which are a more adequate approach to better understanding of tissue differentiation underlying rhizogenesis in microshoots in woody species. A micropropagation protocol has been developed for *Nothofagus nervosa* (Phil.) Dim. et Mil. (raulí) (Martínez Pastur & Arena 1996), and the rhizogenesis process has been studied through biochemical markers (Calderón et al. 1998; Martínez Pastur et al. 2000, 2001, 2003). Consideration of the occurrence of rooting phases (induction and expression) (Gaspar 1981) is necessary when trying to improve the rooting media. These phases have already been established for *N. nervosa* based on the variation of peroxidase activity of *in vitro* shoots (Calderón et al. 1998).

It is also known that physical and chemical factors affect peroxidase activity during rooting phases (Druart et al. 1982; Berthon et al. 1993) affecting both the quantity and quality of differentiated roots.

Thus, histological and histochemical studies will allow an understanding of the tissue changes underlying rhizogenesis, and can be used to visualise the performance of the new roots (Filiti et al. 1987; Moreira et al. 2000). Other histological studies of *in vitro* rooting carried out in clones of woody species with different rhizogenesis performance (Ranjit et al. 1988; Zhou et al. 1992) or development phases of the tissues (Ballester et al. 1999) have increased the knowledge of the process. The aim of this study was to describe the histological events during the adventitious rooting of *N. nervosa* growing in the light (16:8, light:dark) or in the dark (0:24, light: dark). Evaluation of tissue differentiation during rhizogenesis included: (1) identification of the *in vitro* shoot base tissues before rooting, and observing the possible presence of preformed roots; (2) histological changes during the rooting process, from the induction stage and radicle primordium formation to root emergence; (3) quantification of histological changes during the rooting process; and (4) differences between the photoperiod conditions and their correlation with the morphogenetic processes.

## MATERIALS AND METHODS

### *In vitro* plant material

One clone of *in vitro* cultivated *N. nervosa* was selected for the healthy appearance of its shoots, low stem callus production, growth homogeneity, and its multiplication performance. Shoots were subcultured every 21 days to fresh BTM (Chalupa 1983) and divided every 63 days. The multiplication ratio, defined as the number of microshoots obtained from each original one, was 5:1 to 10:1, in agreement with previously reported ratios (Martínez Pastur & Arena 1996).

### Rooting explants, culture conditions and sampling

For rooting trials, 2.5 cm long shoots with 2–4 leaves and 4–6 buds were used. BTM including the modifications proposed by Martínez Pastur & Arena (1996) (half strength of macronutrient salts) was used. Fifty ml of medium were placed in 350 ml flasks and then sterilised for 20 min at 0.1 MPa and 121°C. Six shoots were placed in each flask and cultures were incubated at 24 ± 2°C. Two light conditions were used: light/dark treatment (16 h photoperiod: L) and dark treatment (total dark: D). Light was supplied with standard 20-watt cool white fluorescent tubes at 57 μmol $m^{-2}$ $s^{-1}$ PPD.

### Microscopy studies

Six entire shoots with roots were collected at Days 1, 2, 3, 5, 7, 9, 11, 13, 15, and 20. Half of them were fixed in the fixative solution of Ruzin (1999) for 7 days and then maintained in 70% alcohol (method A); and the other half were fixed in 5% (m/v) glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.0) (method B). Ten cross sections 100 μm thick, taken from the microshoot bases, were fixed by method A. They were dehydrated in a graded ethanol series for 24 h in each one, then stained with Safranin-Fast Green and mounted for light microscope observation (Ruzin 1999). Shoots fixed by method B and embedded in Spurr epoxy resin (Spurr 1969) were used to obtain cross and longitudinal sections (5–40 μm) using crystal knives and an ultramicrotome. Sections fixed to the slide by heat were stained in toluidine blue for 5 min.

The stained sections were photographed using white light with a blue filter, polarised light, or ultraviolet light using a Nikon Labophot-2 light microscope. Photographs were digitised (12.2 pixels per $mm^2$) and analysed with the software Scion Image v1.0 (http://www.scioncorp.com). Stained cell contents, cell diameter, and density of tissues (pith, xylem, medullar rays, cambium, phloem, parenchyma, and cortex) were analysed comparing the rooting days and light conditions.

Fresh material was cut by hand to determine tissues exhibiting peroxidase activity. Shoots from Day 0 and Day 3 grown in light/dark treatment (L) conditions were tested. Ten 1.0 mm thick cross sections at the microshoot base were obtained. The sections were immediately submerged in 5 ml of 0.1 *M* acetate buffer, pH 4.6, and 1 ml of a mixture of guaiacol (8 m*M*) and hydrogen peroxide (8 m*M*) were added. After 1 minute of reaction, the sections were washed with distilled water, air dried, and photographed with the microscope as above.

### Statistical analysis

The results were evaluated by an analysis of the variance using the Fisher test, and media separation was done using the Tukey test. All tests were performed at a 5% significance level.

## RESULTS AND DISCUSSION

### Identification of the shoot tissues at Day 0

As in *Pyrus* (Baraldi et al. 1995), a secondary vascular structure with no differentiation of preformed roots was observed in the *in vitro* microshoots at Day

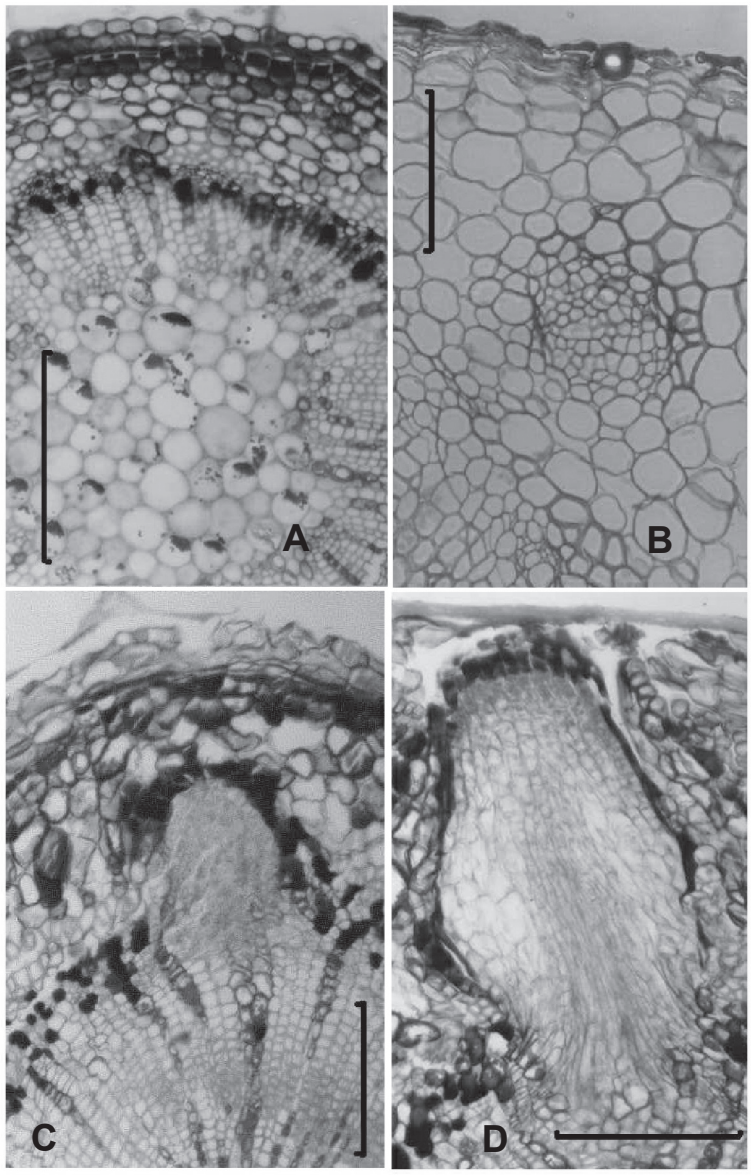


**Fig. 1** Transverse sections of development of a root in *N. nervosa*. **A**, Tissue of the microshoot at Day 1 (L treatment) (bar = 180 µm); **B**, radicle nodule at Day 9 (L treatment) (bar = 55 µm); **C**, root at Day 5 (D treatment) with its vascular connection (bar = 130 µm); **D**, root before the emergence at Day 7 (D treatment) (bar = 155 µm).

0 (the beginning of the rooting period). In contrast, several species such as *Populus*, *Salix*, *Ribes nigrum*, and *Fagus sylvatica* (Haissig 1972; Fink 1982) have been shown to have preformed root primordia in the microshoots. These preformed roots could develop or not into roots depending on culture conditions (Lovell & White 1986; Altamura 1996).

At early stages of differentiation, cells of the microshoot, mainly in the pith, were large, with a conspicuous nucleus and small vacuoles. The pith occupied 20–40% of the transverse section and was consisted of parenchymatic lax cells with a low vacuole content. Nearly 16% of cell contents were stained and cells were large (20–30 µm diam.) (Fig. 1A). Around the pith were xylem strands, formed by isodiametric cells with thick walls, which gradually increased their size from the pith toward the cambium. The xylem occupied a variable area, 10% to 40% of the section, with many small cells (8 µm).

The xylem was regularly crossed by strands of 1–2 parenchyma cells which formed the medullary rays, leaving 5–10 intermediate rows of xylem cells.

The parenchyma of the rays was formed by small cells (9–10 μm) without stained contents (just 10% of the cells had small stained portions). Medullary rays presented a radial distribution, but in some slices the rays converged at one point, as is the case for many conifers (Esau 1965), although this feature could be due to the proximal bud. Around these tissues, the highly stained cambium with its large cells (13–15 μm) was observed. The cambium formed a more or less regular ring 2–3 cells thick, occupying 2–5% of the total section. Phloem appeared in variable groups of 6–12 small (8–9 μm) thickened cells. These tissues were better viewed with ultraviolet or polarised light and occupied 3–6% of the sections. Between phloem and cortex there was a parenchymatic tissue formed by cells 18–20 μm diameter of lax appearance with large spaces in the symplasm. None of the parenchymatic cells had stained contents. Loewe (1990) emphasised this particular root tissue condition (auriferous tissue) as favourable for root initiation in *Juglans regia*. The primary cortex was in the peripheral zone, formed by 4–5 layers of cells which were intensely stained. Also, green glands and red-stained modified cells (hairs) were observed. The overall density of the cells varied between 4000 and 8000 cells $mm^{-2}$, with a lower density (1000–1300 cells $mm^{-2}$) in the pith and higher density (14 000–15 000 cells $mm^{-2}$) in the xylem and the phloem.

**Recognition of rooting phases through the histological changes**

The rooting phases proposed by Gaspar (1981) include an induction stage in which morphological changes are not observed in the tissue, and an expression stage in which the root formation process can be observed. In this study, the first signs appeared as a change in the directions of cellular division in the medullary rays, which was observed in longitudinal ultra-thin sections. As stated by Filiti et al. (1987), these cells had a more isodiametric shape than that of their neighboring cells. From this point, different stages could be observed during root primordia formation (Fig. 1B–D). A change in the cell division pattern gave rise to small cell nodules with prominent nuclei which became intensely stained, evidence of high mitotic activity (Loewe 1990). Their location was variable, i.e., very close to the cambium in the xylem rays, or isolated in the parenchyma between the phloem groups (Fig. 1B). These results are in good agreement with others in the literature, where the parenchyma surrounding the phloem (*Eucalyptus ficifolia*, Gorst et al. 1983; *Prunus* sp., Filiti et al. 1987) or the xylem parenchyma (*Juglans regia*, Loewe 1990) are the most frequent initiation places. However, initiation of rooting can also appear in other tissues (Esau 1965; Lovell & White 1986; Apter et al. 1993; Ballester et al. 1999). Although root differentiation was observed to be concentrated in time at the beginning of the expression phase (Martínez Pastur et al. 2003), some root nodules appeared throughout the duration of the phase.

The formed nodules displaced the surrounding parenchymatic tissue, forming a ring between the xylem and the cortex which differentiated from the remaining tissues, originating a root primordium (Fig. 1C). This primordium had high cellular activity in the borders, where the enzymes digested the cells of the parenchyma between the primordium and the primary cortex (Lowell & White 1986). Subsequently the vascular tissues were differentiated, establishing a vascular connection with the xylem cells (Fig. 1D). By this time differentiated roots were well formed and ready to emerge.

It must be emphasised that during root formation no callus growth was detected. The absence of callus represented a clear advantage, since this undifferentiated tissue could interfere with the normal vascular connection in the formed roots or with the cicatricial tissues formed in the explant wound (Filiti et al. 1987; Loewe 1990; Pretová et al. 1993; Moreira et al. 2000). Moreover, no external abnormality was observed, e.g., swelling or presence of callus in the base of the explants, in the histological processes described above.

A change in division pattern was observed in the L treatment at Day 5 when the induction stage ended. Nodules appeared at Day 7 in L treatment and at Day 5 in D treatment (Fig. 1C). Thus, these first nodules must have formed earlier in the D treatment than in L. Root emergence was observed on Day 9 in the L treatment and on Day 7 in D. The timing of induction and expression of rooting stages for these morphogenetic events was coincident with those defined in *N. nervosa* by Calderón et al. (1998). These stages were defined using peroxidases as biochemical markers. The induction stage ended at Day 3 in D treatment and at Day 5 in L treatment, after which the expression stage began.

Since tissue peroxidase enzyme activity may also reflect the level of free IAA in a particular tissue, which in turn may show the influence of this auxin in root growth elicitation (Kevers et al. 1997), an alternative way to track histologically the above morphological events is through histochemical studies localising the enzyme activity of the peroxidases.

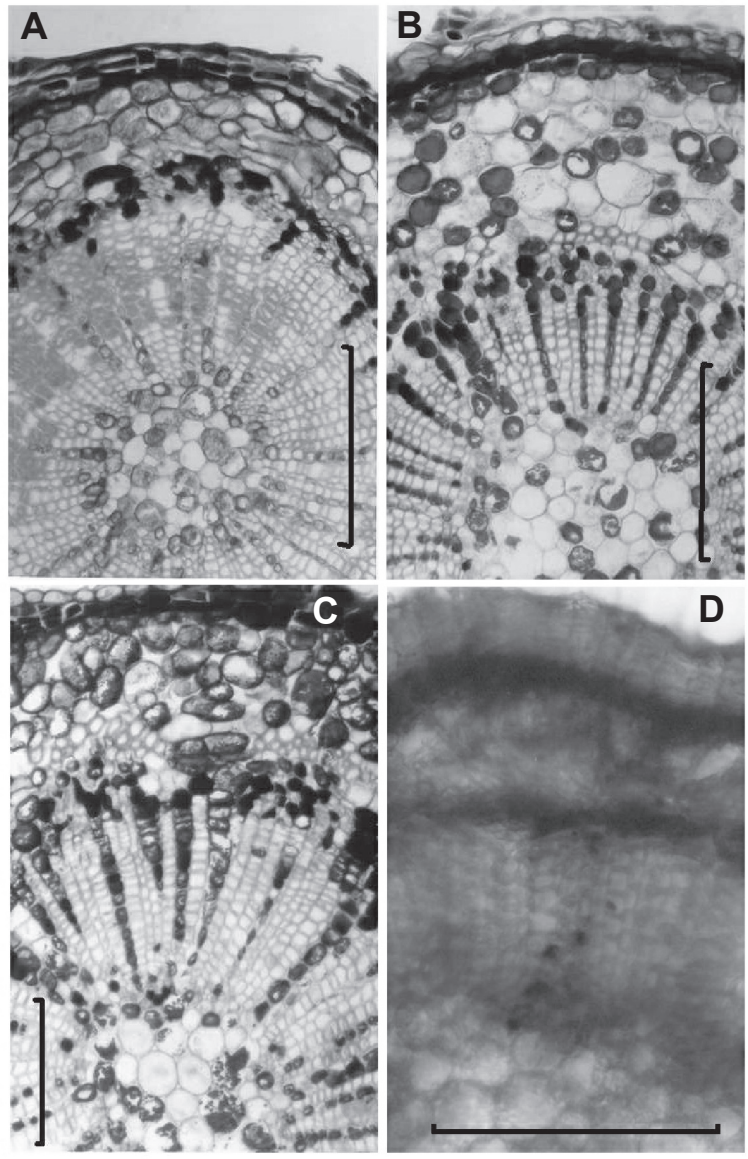


**Fig. 2** Transverse sections of the development of tissues in *N. nervosa* during the rooting process in the L treatment. **A**, Day 3 (bar = 180 μm); **B**, Day 9 (bar = 170 μm); **C**, Day 13 (bar = 130 μm); **D**, total peroxidase activity at Day 3 (bar = 192 μm).

When this was done, low peroxidase activity and an expected higher free IAA content were observed in all shoot tissues at both the beginning of the rooting period and at the preceding induction stage. However, a higher peroxidase enzyme activity, i.e., where the endogenous free IAA content (Gaspar et al. 1997) should be lower, was observed in the meristematic tissues such as the cambium and the cork cambium. In these meristems some spots of more stained cells were observed dispersed among the tissues, where the guaiacol reacted. During the induction stage peroxidase activity increased significantly. The stained tissues no longer appeared as dispersed spots, and two well-marked rings could be seen in the meristems (Fig. 2D). In the first internal ring, the roots were initiated more frequently at the point where the cambium was observed and medullary rays ended. This higher peroxidase activity is consistent with other findings which have allowed the definition of the rooting stages using

other biochemical markers (tissue nutrient contents, polyamines, flavonoids) (Martínez Pastur et al. 2000, 2003; Arena et al. 2003) and also with those defined in this study through the histological observations.

### Quantification of the histological changes along the rooting process

All morphometric values of the explants varied during the rooting period (Fig. 1A, 2) under the influence of both photoperiods (Tables 1 and 2). As expected, the percentage of each tissue in the sections varied significantly. Some tissues showed important changes, since the explants grew vigorously at the beginning of the rooting period and then compressed the tissues, e.g., the pith (from 45% to 15% in L treatment).

Cell diameters varied significantly depending on the tissue type and stage of development (Table 1). The pith cells reduced their average size in the L treatment compared with the following days, while in the D treatment the behaviour was the opposite. In the medullary rays formed in L, there was a progressive increase of cell size during the rooting period, while in D only small changes were observed. The xylem cells in L increased their size until Day 11 and then diminished significantly, whereas no significant changes were observed in D. Cambium cells varied markedly in size during the rooting period. In L there was a significant maximum at Day 7 with a variable average size in the following days. The same was also observed in D at Day 7 but to a lesser extent. The phloem exhibited similar behaviour in L with a maximum at Day 7, but in D the maximum cell size occurred on Day 11. The parenchymatic cells around the phloem showed variable cell sizes with maxima at Day 3 and Day 9 in the L treatment, but there were no significant differences in the D treatment.

Cell density (Table 2) varied significantly depending on the tissue type and stage of development, being inversely related to the quadratic cell diameter. The cell density of the pith in the L treatment was greatest at Days 3 and 11, while in D it decreased during the rooting process. In the medullary rays the density decreased to Day 7, then increased in L. In

**Table 1** Average cell diameter of tissues during rhizogenesis in microshoots of *N. nervosa*. *F* values and significance: Light/Dark treatment: Pith, 3.29(0.0016); Medullary rays, 3.91(0.0003); Xylem, 4.51(0.0001); Cambium, 8.75(0.0000); Phloem, 4.62(0.0001); Parenchyma, 9.74(0.0000). Dark treatment: Pith, 4.26(0.0001); Medullary rays, 2.31(0.0222); Xylem, 1.17(0.3229); Cambium, 3.79(0.0004); Phloem, 8.65(0.0000); Parenchyma, 2.51(0.0135). For each column and treatment, values with different letters are significantly different at $P < 0.05$. $n = 30$ cells (cuts from 3 microshoots) of each treatment.

| Day | Pith (µm) | Medullary rays (µm) | Xylem (µm) | Cambium (µm) | Phloem (µm) | Parenchyma (µm) |
|---|---|---|---|---|---|---|
| **Light/Dark treatment** | | | | | | |
| 1 | 28.2b | 9.8 ab | 8.0 ab | 13.0 ab | 8.2 ab | 18.4 ab |
| 2 | 25.5ab | 12.7 abc | 8.4 ab | 15.2 ab | 10.2 abc | 19.8 abc |
| 3 | 20.4ab | 11.8 abc | 10.5 ab | 13.8 ab | 9.0 abc | 28.4 cd |
| 5 | 24.7ab | 11.2 ab | 11.2 b | 14.4 ab | 9.4 abc | 17.2 a |
| 7 | 26.4ab | 12.8 abc | 10.3 ab | 26.1 c | 11.4 c | 23.5 abc |
| 9 | 25.8ab | 10.8 ab | 10.4 ab | 18.0 b | 10.4 bc | 36.0 d |
| 11 | 20.1a | 14.6 bc | 11.1 b | 16.7 b | 10.0 abc | 26.1 bc |
| 13 | 27.3ab | 8.8 a | 7.2 a | 9.7 a | 7.7 a | 21.2 abc |
| 15 | 21.5ab | 13.6 abc | 7.7 a | 13.4 ab | 9.4 abc | 18.8 ab |
| 20 | 19.9a | 17.3 c | 7.9 ab | 19.4 bc | 11.6 c | 18.7 ab |
| **Dark treatment** | | | | | | |
| 1 | 21.5 ab | 9.4 ab | 7.4 a | 11.0 a | 5.9 a | 22.4 a |
| 2 | 20.3 a | 11.3 ab | 8.3 a | 10.9 a | 7.9 abc | 20.6 a |
| 3 | 22.8 abc | 11.3 ab | 9.1 a | 10.5 a | 7.4 ab | 20.0 a |
| 5 | 22.9 abc | 12.0 ab | 8.8 a | 11.7 a | 9.2 abc | 19.9 a |
| 7 | 24.4 abc | 12.1 ab | 10.8 a | 16.2 ab | 10.0 bcd | 26.1 a |
| 9 | 23.0 abc | 8.8 a | 8.6 a | 12.3 a | 11.0 cd | 26.6 a |
| 11 | 29.5 bc | 12.0 ab | 8.2 a | 14.4 ab | 13.2 d | 24.7 a |
| 13 | 29.1 bc | 11.9 ab | 8.5 a | 14.0 ab | 11.3 cd | 20.5 a |
| 15 | 28.9 bc | 12.9b | 9.7 a | 14.5 ab | 11.3 cd | 24.7 a |
| 20 | 30.2 c | 9.8 ab | 10.2 a | 19.4 b | 8.4 abc | 26.9 a |

D there was a delay in reduction of the medullary ray density, where the minimum only appeared at Day 13. The area occupied by the parenchyma of the medullary rays in D was lower than in the L treatment (2–8% and 7–15%, respectively). In L the microshoots could photosynthesise their organic compounds or obtain them from the culture medium, to be subsequently transported, utilised, or deposited in different tissues. The microshoots changed their morphogenesis, achieving higher efficiency of their development, trying to maximize the use of their own available resources (Salisbury & Ross 1992). It is possible that the higher number of parenchymatic cells in the medullary rays has been generated due to a huge requirement of photo-assimilates in the shoot.

In the xylem, there was a continuous decrease in cell density in the L treatment. In D the density was highest between Days 3 and 5 then diminished until the end of the assay. The proportion of xylem in the shoots varied according to the treatment. Those that had been grown in the D treatment had a higher proportion than those grown in L, which had smaller xylem in terms of cellular density until the end of rhizogenesis. Although the development of this tissue was related to the number of formed cells, independently of the photoperiod, it probably affected the development of other tissues, modifying their final ratio.

The cambium density increased to Day 5 in the L treatment, then diminished until the assays ended. In D the cambium density diminished through the experiments. The percentage of the area occupied by the cambium was higher in L than in D. In D, the area occupied by the cambium varied from 2% to 6% at Day 7 and then fell to less than 1%. In L it reached 8% at Day 7, and then decreased to 3%.

The size of the cells and the proportion of cambial tissue reflected the meristem activity during the rooting process. The density of the phloem fell from the beginning until Day 11 in both treatments, being most remarkable in D. The phloem was a

**Table 2** Average cell density of tissues during rhizogenesis in microshoots of *N. nervosa*. *F* values and significance: Light/Dark treatment: Pith, 11.49(0.0000); Medullary rays, 5.71(0.0000); Xylem, 3.62(0.0007); Cambium, 6.70(0.0000); Phloem, 8.95(0.0000); Parenchyma, 18.93(0.0000). Dark treatment: Pith, 11.67(0.0000); Medullary rays, 22.78(0.0000); Xylem, 2.80(0.0061); Cambium, 41.67(0.0000); Phloem, 88.34(0.0000); Parenchyma, 36.33(0.0000). For each column and treatment, values with different letters are significantly different at $P < 0.05$. $n = 30$ sections (cuts from 3 microshoots) of each treatment.

| Day | Pith (cell mm$^{-2}$ ×10$^3$) | Medullary rays (cell mm$^{-2}$ ×10$^3$) | Xylem (cell mm$^{-2}$ ×10$^3$) | Cambium (cell mm$^{-2}$ ×10$^3$) | Phloem (cell mm$^{-2}$ ×10$^3$) | Parenchyma (cell mm$^{-2}$ ×10$^3$) |
|---|---|---|---|---|---|---|
| **Light/Dark treatment** | | | | | | |
| 1 | 1.35 a | 13.82 cd | 14.52 c | 8.03 bc | 14.94 d | 3.63 b |
| 2 | 2.46 bcd | 9.66 abc | 13.60 bc | 6.21 ab | 14.40 d | 4.14 b |
| 3 | 3.31 de | 10.91 abcd | 10.64 abc | 6.11 ab | 8.37 abc | 2.11 a |
| 5 | 2.52 bcd | 11.16 abcd | 12.46 abc | 9.33 c | 11.17 abcd | 3.53 b |
| 7 | 1.85 ab | 7.60 a | 10.32 abc | 6.79 abc | 11.62 bcd | 1.81 a |
| 9 | 1.82 ab | 14.31 d | 11.92 abc | 4.35 a | 7.49 abc | 1.25 a |
| 11 | 3.12 de | 11.93 bcd | 8.00 a | 5.13 ab | 7.28 ab | 1.83 a |
| 13 | 1.95 abc | 13.04 cd | 13.79 bc | 7.79 bc | 11.86 cd | 3.46 b |
| 15 | 2.89 cde | 10.98 abcd | 11.48 abc | 6.00 ab | 11.14 abcd | 3.32 b |
| 20 | 3.57 e | 8.61 ab | 9.28 ab | 3.98 a | 6.85 a | 3.16 b |
| **Dark treatment** | | | | | | |
| 1 | 3.26 d | 16.47 e | 11.96 ab | 11.65 g | 23.59 e | 3.88 ef |
| 2 | 3.45 d | 12.97 de | 9.62 ab | 10.52 fg | 9.56 cd | 4.66 f |
| 3 | 2.01 abc | 13.74 de | 13.53 b | 9.44 ef | 11.33 d | 3.61 de |
| 5 | 2.56 bcd | 12.52 cd | 13.48 b | 7.54 d | 10.07 cd | 2.87 cd |
| 7 | 2.70 cd | 10.67 bcd | 12.27 ab | 5.51 bc | 8.60 bc | 2.26 bc |
| 9 | 2.30 abc | 9.02 bc | 10.81 ab | 7.76 de | 9.65 cd | 1.97 ab |
| 11 | 1.75 ab | 6.96 ab | 10.79 ab | 7.18 cd | 5.91 a | 1.34 a |
| 13 | 1.65 ab | 4.62 a | 10.41 ab | 4.64 ab | 6.72 ab | 2.26 bc |
| 15 | 1.51 a | 10.15 bcd | 9.76 ab | 6.77 cd | 9.80 cd | 2.00 ab |
| 20 | 1.77 ab | 5.07 a | 8.73 a | 3.28 a | 9.51 cd | 1.93 ab |

higher proportion of tissue in L than in D, as in the medullary rays (average of 3.7% in D and 5.4% in L). This could be explained by a higher requirement of organic compounds in the shoots grown under the light. Similarly, the parenchyma around the phloem was least in the L treatment between Days 7 and 11, and between Days 9 and 11 in D.

Some of the vacuoles of the parenchymatic cells had stained cell contents at the beginning of the rooting period (16% of the cells of the pith, 8–20% of medullary rays, and no tinted contents in the parenchyma around the phloem) (Fig. 1A), which were strongly related to the morphogenic activity of the explants. In the L treatment the stained content of the pith changed dramatically during the rooting period (Fig. 2A–C) (Table 3). The content of the vacuoles in the parenchymatic cells (source cells) could be related to the radicle primordium (sink cells). The changes described were more noticeable in the L treatment, since the microshoots have the facility to photosynthesise for themselves a percentage of the compounds needed, while the microshoots in D could only extract them from the culture medium. Loewe (1990) described variations of the reserve substances in the neighbouring cells, where the morphogenic processes develops.

The main source of energy storage in *N. nervosa* is through the aleuronic granules, which can be seen using the xylol and safranine test. In other trees starch has been cited as the main storage substance (*Juglans regia*, Loewe 1990; *Malus communis*, Jasik & De Klerk 1997), but it could not be detected in *N. nervosa* tissues observed with polarised light.

All the variations described in the tissues could be related to the changes observed in the micronutrient contents of the total tissues and in the peroxidase activity associated with the rooting morphogenic processes (Calderón et al. 1998; Martínez Pastur et al. 2000, 2003). During the induction stage, a maximum followed by a minimum is produced in the total peroxidase activity (Calderón et al. 1998), which is associated mainly with the action and degradation

**Table 3** Percentage of cells stained with stained cellular content of tissues during rhizogenesis in microshoots of *N. nervosa*. *F* values and significance: Light/Dark treatment: Pith, 17.53(0.0000); Medullary rays, 33.12(0.0000); Parenchyma, 17.44(0.0000). Dark treatment: Pith, 7.30(0.0000); Medullary rays, 33.04(0.0000); Parenchyma, 41.30(0.0000). For each column and treatment, values with different letters are significantly different at $P < 0.05$. $n = 30$ sections (cuts from 3 microshoots) of each treatment.

| Day | Pith (%) | Medullary rays (%) | Parenchyma (%) |
|---|---|---|---|
| **Light/Dark treatment** | | | |
| 1 | 16 ab | 8 a | 0 a |
| 2 | 66 e | 42 bc | 6 a |
| 3 | 47 cde | 40 bc | 2 a |
| 5 | 4 a | 20 abc | 16 ab |
| 7 | 43 cd | 88 d | 4 a |
| 9 | 30 bc | 96 d | 46 cd |
| 11 | 56 de | 82 d | 28 bc |
| 13 | 28 bc | 16 ab | 8 ab |
| 15 | 35 bc | 45 c | 27 bc |
| 20 | 36 c | 82 d | 50 d |
| **Dark treatment** | | | |
| 1 | 16 ab | 20 ab | 0 a |
| 2 | 6 a | 28 ab | 2 a |
| 3 | 24 abc | 6 a | 16 ab |
| 5 | 27 abc | 36 b | 14 ab |
| 7 | 30 abc | 64 c | 12 ab |
| 9 | 10 a | 68 c | 20 ab |
| 11 | 40 bc | 80 c | 60 c |
| 13 | 50 c | 80 c | 72 c |
| 15 | 50 c | 80 c | 74 c |
| 20 | 40 bc | 70 c | 24 b |

of the free IAA. In addition, some nutrients (calcium and boron) increased during the induction stage and decreased in the expression stage (Martínez Pastur et al. 2000), which was related to the root formation. Additional cofactors such as flavonoids have also shown changes associated with these phenomena (Martínez Pastur et al. 2003). These processes have been correlated with root emergence (macro observations), and with the quantity and quality of roots formed during the rooting process.

The results of this work complement the processes previously described and the timing of rooting phases (Calderón et al. 1998). The changes in the cambial activity and in the stained cell contents are related to increased meristematic activity in the formation of new radicle nodules. The increase in stained cells and in tissue activity precedes the massive formation of roots. The stained cells could act as source cells providing the energy requirements of the sink cells surrounding the radicle primordium, which could be acquired from the reserve cells. Also, stained cells are related to the formation, emergence, and differentiation of the roots during rooting. The changes detected in the tissues due to the photoperiod are similar to those cited in the literature, where the events in the D treatment are early in time when maxima and minima are compared (Martínez Pastur & Arena 1996; Martínez Pastur et al. 2003).

## CONCLUSIONS

The development of histological events in microshoots of *N. nervosa* subjected to rooting conditions as previously described were concurrent with the formation and emergence of roots, and consistent with biochemical markers. The cambium activity and the staining pattern development of the parenchymatic tissues appeared as the best histological characteristics for the definition of the rooting stages. The tracking of histological changes provides information together with the use of biochemical markers (peroxidases, tissue nutrient contents, polyamines, flavonoids), and are of great help in the development of new rooting successive media for *N. nervosa* and for other woody species. Moreover, this strategy of study may also be valuable when trying to detect the possible cause for the absence of root formation ability in some recalcitrant forest species. As here shown, histological studies may demonstrate the functional ability of the roots formed by showing the absence of tissue abnormalities such as callus and a true vascular connection. Additionally, knowledge of histological changes could be used to optimise a protocol for micro-propagation of *N. nervosa*, by improving the timing, quantity, and quality of roots, which would positively affect the survival rate during hardening.

## ACKNOWLEDGMENTS

In memory of Osvaldo Caso, for his scientific support and discussions during the course of the experiments. We also wish to thank to Cristina Dizeo (CEVEG), Ivonne Lindström (UNSur-CIC), and Cecilia Pellegrini (UNSur-CIC) for their helpful technical assistance.

## REFERENCES

Altamura MM 1996. Root histogenesis in herbaceous and woody explants cultured *in vitro*. A critical review. Agronomie 16: 589–602.

Apter RC, McWilliams EL, Davies FT 1993. *In vitro* and *ex vitro* adventitious root formation in Asian jasmine (*Trachelospermum asiaticum*) I. Comparative morphology. Journal of the American Society for Horticultural Science 118: 902–905.

Arena M, Martínez Pastur G, Benavides P, Zappacosta D, Eliasco E, Curvetto N 2003. Peroxidase and polyamine activity variation during the *in vitro* rooting of *Berberis buxifolia*. New Zealand Journal of Botany 41: 475–785.

Ballester A, San José MC, Vidal N, Fernández-Lorenzo J, Vieitez AM 1999. Anatomical and biochemical events during *in vitro* rooting of microcuttings from juvenile and mature phases of chestnut. Annals of Botany 83: 619–629.

Baraldi R, Bertazza G, Bregoli AM, Fasolo F, Rotondi A, Predieri S, Serafini-Fracassini D, Slovin JP, Cohen JD 1995. Auxin and polyamines in relation to differential *in vitro* root induction on microcuttings of two pear cultivars. Journal of Plant Growth Regulation 14: 49–59.

Berthon J, Battraw M, Gaspar T, Boyer N 1993. Early test using phenolic compounds and peroxidase activity to improve *in vitro* rooting of *Sequoiadendron giganteum*. Saussurea 24: 7–13.

Calderón X, Martínez Pastur G, Jofre MP, Arena M 1998. Activity variation of peroxidase during *in vitro* rooting of *Nothofagus nervosa* and *Nothofagus antarctica*. Phyton 62: 137–144.

Chalupa V 1983. Micropropagation of conifer and broadleaved forest trees. Communicationes Instituti Forestalis Cechosloveniae 13: 7–39.

Druart P, Kevers C, Boxus P, Gaspar T 1982. *In vitro* promotion of root formation by apple shoots through darkness effect on endogenous phenols and peroxidases. Zeitschrift fur Pflanzenphysiologie Bd 108: 429–436.

Esau K 1965. Plant anatomy. 2nd ed. New York, John Wiley and Sons. Pp. 481–538.

Filiti N, Montuschi N, Rosati P 1987. *In vitro* rhizogenesis: Histo-anatomical aspects on *Prunus* rootstock. Advances in Horticultural Science 1: 34–38.

Fink S 1982. Adventitous root primordia – the cause of abnormally broad xylem rays in hard and softwoods. International Association of Wood Anatomists 3: 31–38.

Gaspar T 1981. Rooting and flowering to antagonistic phenomena from a hormonal point of view. In: Jeeffcoat B ed. Aspects and prospects of plant growth regulators. British Plant Growth Regulator Group Mongraph 6: 39–49.

Gaspar Th, Kevers C, Hausman JF 1997. Indissociable chief factors in the inductive phase adventitious rooting. In: Altman A, Waisel Y ed. Biology of root formation and development. New York, Plenum Press. Pp. 55–63.

Gorst J, Slaytor M, De Fossard RA 1983. The effect of indole-3-butyric acid and riboflavin on the morphogenesis of adventitious roots of *Eucalyptus ficifolia* grown *in vitro*. Journal of Experimental Botany 34: 1503–1515.

Haissig B 1972. Meristematic activity during adventitious root primordium development. Plant Physiology 49: 886–892.

Jasik J, De Klerk G 1997. Anatomical and ultrastructural examination of adventitious root formation in stem slices of apple. Biologia Plantarum 39: 79–90.

Kevers C, Hausman J, Faivre-Rampant O, Evers D, Gaspar T 1997. Hormonal control of adventitious rooting: Progress and questions. Angewandte Botanik 71: 71–79.

Loewe V 1990. Analisi isto-anatomica sulla radicazione del noce (*Juglans regia*) *in vitro*. Rivista di Frutticoltura 12: 57–61.

Lovell PH, White J 1986. Anatomical changes during adventitious root formation. In: Jackson MB ed. New root formation in plants and cuttings. Dordrecht, Martinus Nijhoff Publ. Pp. 111–140.

Martínez Pastur G, Arena M 1996. *In vitro* propagation of *Nothofagus nervosa*. Phyton 58: 1–7.

Martínez Pastur G, Arena M, Curvetto N 2000. Calcium and boron for *in vitro* rooting of *Nothofagus nervosa*. Biocell 24: 65–71.

Martínez Pastur G, Zappacosta D, Arena M, Curvetto N 2001. Changes in isoperoxidase patterns during the *in vitro* rooting of *Nothofagus antarctica*. Bulgarian Journal of Plant Physiology 27: 43–53.

Martínez Pastur G, Arena M, Curvetto N, Zappacosta D, Eliasco E 2003. Successive media to improve *in vitro* rhizogenesis of *Nothofagus nervosa* (Phil.) Dim. et Mil. New Forests 26: 201–215.

Moreira MF, Appezzato-da-Glória B, Zaidan L 2000. Anatomical aspects of *IBA*-treated microcuttings of *Gomphrena macrocephala* St.-Hil. Brazilian Archives of Biology and Technology 43: 221–227.

Pretová A, Ostrolucká M, Samrov I, Borisovna Batygina T 1993. Morpho-histological examination of calli, somatic embryos and other structures derived *in vitro* in *Quercus* sp. Biológia 48: 451–456.

Ranjit M, Kester D, Polito V 1988. Micropropagation of cherry rootstocks: III. Correlations between anatomical and physiological parameters and root initiation. Journal of American Science 113: 155–159.

Ruzin SE 1999. Plant microtechnique and microscopy. New York, Oxford University Press. 322 p.

Salisbury FB, Ross CW 1992. Plant physiology. 4th ed. Belmont, CA, Wadsworth, Inc. 682 p.

Spurr AR 1969. A low-viscosity epoxy resin medium for electron microscopy. Journal of Ultrastructure Research 26: 31–43.

Zhou J, Wu H, Collet G 1992. Histological study of initiation and development *in vitro* of adventitious roots of minicuttings of apple rootstocks of M26 and EMLA9. Physiologia Plantarum 84: 433–440.