Bulg. J. Plant Physiol., 2001, 27(1–2), 43–53

CHANGES IN ISOPEROXIDASE PATTERNS DURING THE *IN VITRO* ROOTING OF *NOTHOFAGUS ANTARCTICA*

G. Martinez Pastur[1]; D. Zappacosta[2]; M. Arena[1]; N. Curvetto[2]

[1]Centro Austral de Investigaciones Cientificas (CONICET). cc 92 (9410) Ushuaia, Provincia de Tierra del Fuego (Argentina)
[2]Laboratorio de Fisiologia Vegetal, Departamento de Agronomia, Universidad Nacional del Sur. Altos del Paligue (8000) Bahia Blanca, Provincia de Buenos Aires (Argentina)

Received April 25, 2001

Summary. Peroxidases play a major role in several physiological processes. Specifically, *in vitro* activity of this enzyme was used as a marker to identify different rooting phases of *Nothofagus*. The study of peroxidase profile patterns during rhizogenesis in this genus could give additional information about this topic, to justify the use of peroxidase isoforms as a more specific biochemical marker than total peroxidase activity. This work gives information on qualitative and quantitative changes in isoperoxidase expression (basic and acidic peroxidases) during *in vitro* rooting of *N. antarctica* microshoots through native PAGE. Time course analysis of isoperoxidase activity during rhizogenesis allowed positive correlation with specific morphogenic stages. Seventeen peroxidase bands were detected, 4 basic and 13 acidic. Four of the acidic peroxidases appeared during the induction stage of rooting. Variation in the number and relative activity of the isoperoxidases was found during different stages of adventitious rooting. The variation in the number and activity of isoperoxidases provides a good indicator of the processes involved in adventitious rooting of *N. antarctica*. Possible linkage of basic isoperoxidases with auxin metabolism and the role of certain isoperoxidases in specific rooting processes are also discussed.

Keywords: micropropagation, rhizogenesis, biochemical marker, basic and acidic peroxidases

* Corresponding author, Tel. +54-2901-422310/424142, Fax: +54-2091-430644, e-mail: cadicforestal@arnet.com.ar

Introduction

Nothofagus is the dominant component of the temperate and subantartic forests from 35° to 54° South latitude, being represented by six species. The forests Patagonian Andes are the result of geologic and climatic changes occurred through millennia and even millions of years; they are true relics of the past in very unstable balance. Regrettably, they are an example of the great reduction of the forest surface occurred during the past century (Dimitri, 1972). Hence, vegetative micropropagation is a good strategic approach in conservation and improvement projects of these species (Martínez Pastur et al., 1997).

Tree improvement requires successful propagation techniques to clone the best phenotypes. Rooting is the most difficult step in the process (Quoirin et al., 1974; Druart et al., 1982; Berthon et al., 1987). *In vitro* culture of *N. antarctica* (Martínez Pastur et al., 1997) and other *Nothofagus* species (Martínez Pastur and Arena, 1995; 1996; 1997) using meristems of young and mature plants has been successfully accomplished.

Peroxidase activity was proposed by Gaspar (1981) as a marker of inductive and initiative phases of root formation in *N. antarctica* and *N. nervosa* (Calderón et al., 1998). During the inductive phase there was a remarkable rise in peroxidase activity without any apparent histological and cytological modification (Berthon et al., 1987). At present, peroxidases have been claimed as the best rooting phase markers (Kevers et al., 1997); however, the reliability of this marker has been questioned (De Klerk, 1996). It is well known that peroxidases participate in several physiological processes (Gaspar et al., 1985). On rooting, *basic peroxidases* participate in auxin catabolism and *acidic peroxidases* are involved in the formation of the cell wall, contributing to its rigidity (Mader, 1992). Quoirin et al. (1974) have suggested that the study of isoperoxidases during rhizogenesis could give useful information, eventually providing a better biochemical tool than total peroxidase. To test this hypothesis, changes in qualitative and quantitative isoperoxidase patterns during *in vitro* rooting of *Nothofagus antarctica* were studied and related with the evolution of rooting and root apparition.

Materials and Methods

In vitro plant material

In vitro culture of *N. antarctica* was performed as described elsewhere (Martinez Pastur et al., 1997). Briefly, culture was initiated from seeds and maintained on multiplication medium until the beginning of the experiments. Explants were transferred to fresh medium every 21 days and subdivided every 9 weeks during the multiplication stage.

Rooting media and growing conditions

Micro-shoots of 2.5-3.0 cm long with two leaves and four buds were used as explants. Broad-leaved Tree Medium *(BTM)* (Chalupa, 1983) with half strength macronutrient salts containing 0.61 µM indol-butyric acid *(IBA)* was used as basal medium. Fifty ml of media were dispensed into 350 ml glass flasks. Cultures were maintained in a growth chamber at 22±2°C and 16 h photoperiod using fluorescent lamps (57 $\mu mol.m^{-2}.s^{-1}$ photosynthetically active radiation).

Samples and data collection

One-gram of fresh weight micro-shoots was sampled during the rooting period every one or two days. This material was kept frozen at –20 °C until enzyme analysis. Rooting percentage and root number (thirty micro-shoots in six flasks) were recorded every two days until day 28 when the experiment was ended.

Protein determination

Samples of 100 mg green tissue were homogenized with 5 ml 0.2 M citrate-phosphate buffer, pH 7.5, containing 0.1% Triton X-100. The homogenates were kept at 4 °C for 20 min and then were centrifuged at 10500×g during 20 min. Protein level was measured by Bradford method (Bradford, 1976). Briefly, 100 µl of the homogenate was mixed with 2 ml of Bradford protein stain. Reaction was spectrophotometrically measured at 595 nm. Calibration was done using bovine serum albumin (SIGMA). Each sample had three replications. The extracts were kept at –20 °C until use.

Gel electrophoresis and pattern determinations

The isoperoxidase patterns were obtained by native *PAGE* in bed gels of 16×10×0.3 cm using *Bio-Rad Protean II* system, essentially according to protocols for acidic (Davis, 1964) and basic proteins (Reisfeld et al., 1962). For acidic proteins, separating and stacking gels were made of 7.5% polyacrylamide at pH 8.9 and 3.1% polyacrylamide at pH 7.0, respectively. Both gels contained 30% acrylamide and 0.8% bisacryalmide. Electrolyte for electrode reservoirs was Tris-glycine, pH 8.3. Bromophenol blue (0.01%) was used as tracking dye. Electrophoresis was carried out at 4 °C applying 25 mA/gel during ca. 6 hours. In the case of basic proteins, the same polyacrylamide concentrations and acryalmide/bis-acrylamide ratios were used for separating and stacking gels, being pH 4.3 and 6.7, respectively. The electrolyte for electrode reservoirs was acetic acid–β-alanine, pH 4. The tracking dye was methylene blue (0.01%) and the etectrophoresis was run for 7 hours at 30 mA/gel and 4 °C.

Direct detection of isoperoxidase activity in gel was done by dark incubation in 200 ml of 50 mM acetate buffer solution (pH 5), containing 2 mM $CaCl_2$, 0.1 ml H_2O_2

(35%) and 2.4 mM 3-amino-9-ethylcarbazole (Medina et al. 1985). Aliquots of seventy µg protein in extraction buffer containing (10% sucrose) were added to the gel wells. Assays were carried out twice. Protein bands were identified on a transilluminator with white fluorescent light and their relative activity was recorded using a relative scale (0–5), the higher stained protein band having the higher number. To obtain the time course evolution of total acidic or basic isoperoxidases, corresponding relative activities were summed.

Results and Discussion

Band Identification

17 bands were detected (Fig. 1), 4 of them (B1-B4) were basic peroxidases located in the lowest R_f range (3 to 27%) (Fig. 2) and 13 (A1–A13) were acidic peroxidases in a more extended R_f range (4 to 85%) (Fig. 3) than the basic peroxidases. In this work was not determined whether the bands represent true isozymes or compounds derived of the protein-phenolic interaction as a result of altered charging of the protein.

Total relative isoperoxidase activity variation

With respect to the overall behaviour of the activity variation of the main peroxidases groups, the total basic isoperoxidase activity increased quickly during the first days until day 11, decreased at day 13, and declined to a minimum at day 15 (Figs. 1 and 4A). A second maximum of lower relative activity was measured at day 17, which could be related to the apparition of new roots (Fig. 4A and 4C). This quite probably reflects the growth promoting effect of recently synthesized auxins, as was previously suggested after correlation studies between total peroxidases and rhizogenesis (Caspar et al., 1985; 1991; 1997; Kevers et al., 1997; Calderon et al., 1998). These basic isoenzymes showed a similar pattern of temporal variability. These enzymes probably have a role in auxin catabolism, and could be used as specific markers of rooting during the induction stage (Van der Berg et al., 1983; Gaspar et al., 1985; 1991; Faivre-Rampant et al., 2000).

The total acidic isoperoxidase activity increased rapidly from the first days of rooting (Figs. 1 and 4A). An initial peak of isoperoxidase activity was observed at day 2–3, followed by a minimum at day 3–4 and an increase again toward day 11. The marked decrease in total activity observed later reached a minimum until day 20 and a subsequent increase started from day 24.

The results on the activity of both kinds of isoperoxidases in *N. antarctica* showed that the rooting induction stage would be until day 4 (when the minimum appeared) (Fig. 4A) instead of 7 days as reported by Calderón et al. (1998), who used identical

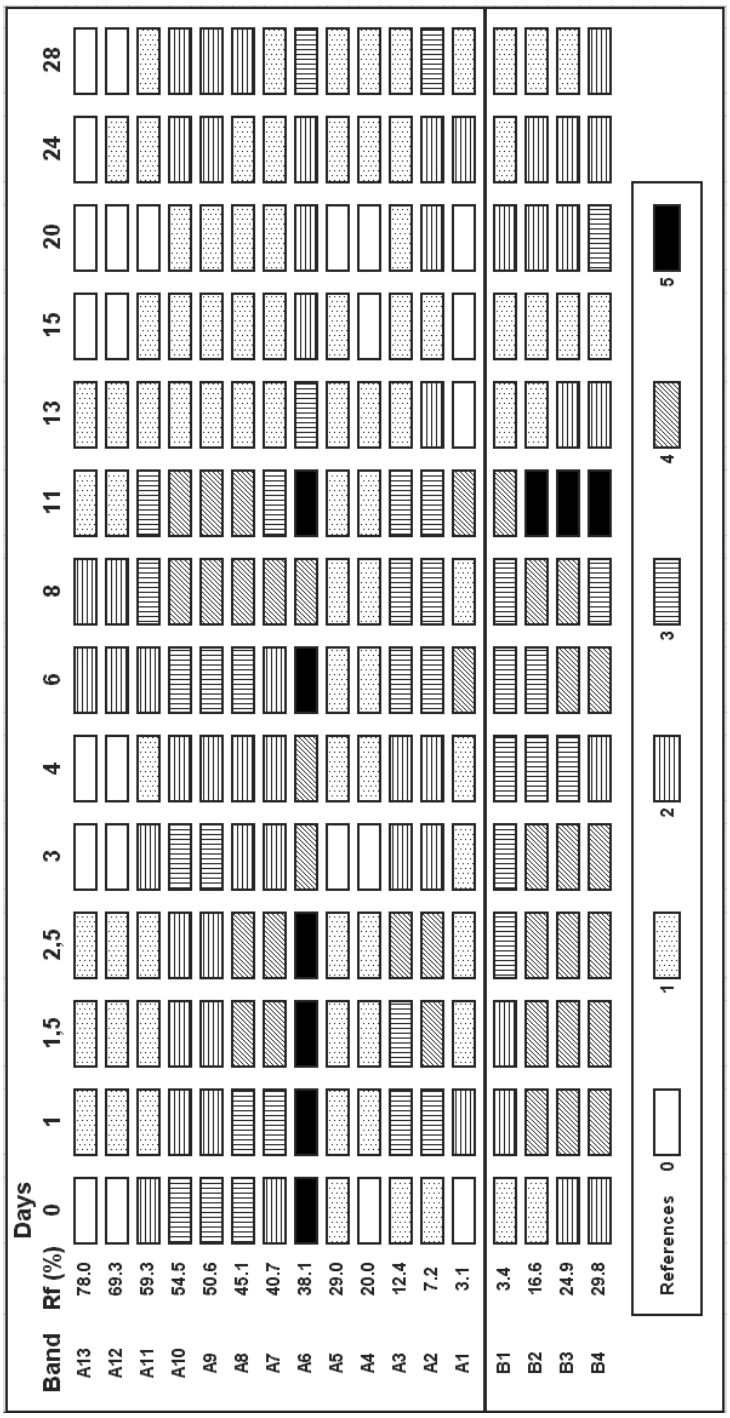

Fig. 1. Bands detected over the time course of relative isoperoxidase activity (References 0 to 5) during rhizogenesis in *Nothofagus antarctica*.

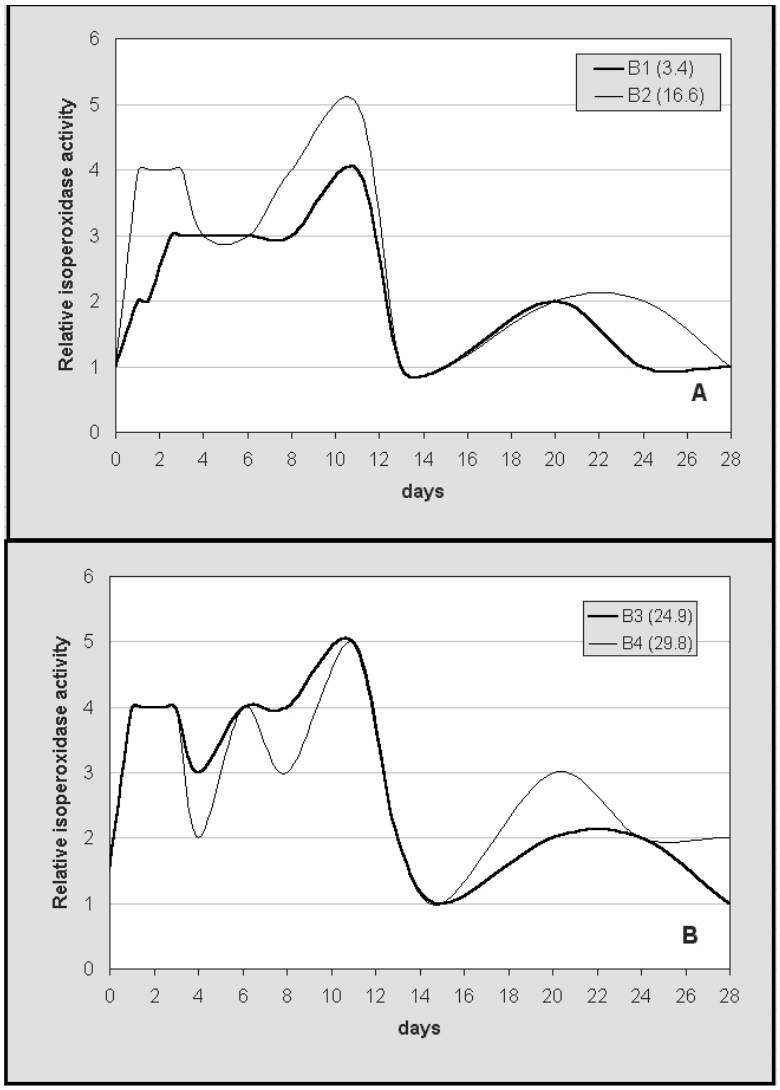

Fig. 2. Time course of relative basic isoperoxidase activity (0 to 5) (isoforms B1 and B2 in graph **A**, B3 and B4 in graph **B**) during rhizogenesis in *Nothofagus antarctica*. R_f values are given between brackets.

plant material and culture conditions. These differences indicate that the induction period could vary between 4 to 7 days.

Relative acidic isoperoxidase activity variation

Acidic peroxidase patterns at day 0 compared to those obtained during the rhizogenesis process, clearly showed new bands (A1, A4, A12 and A13), in the latter. The

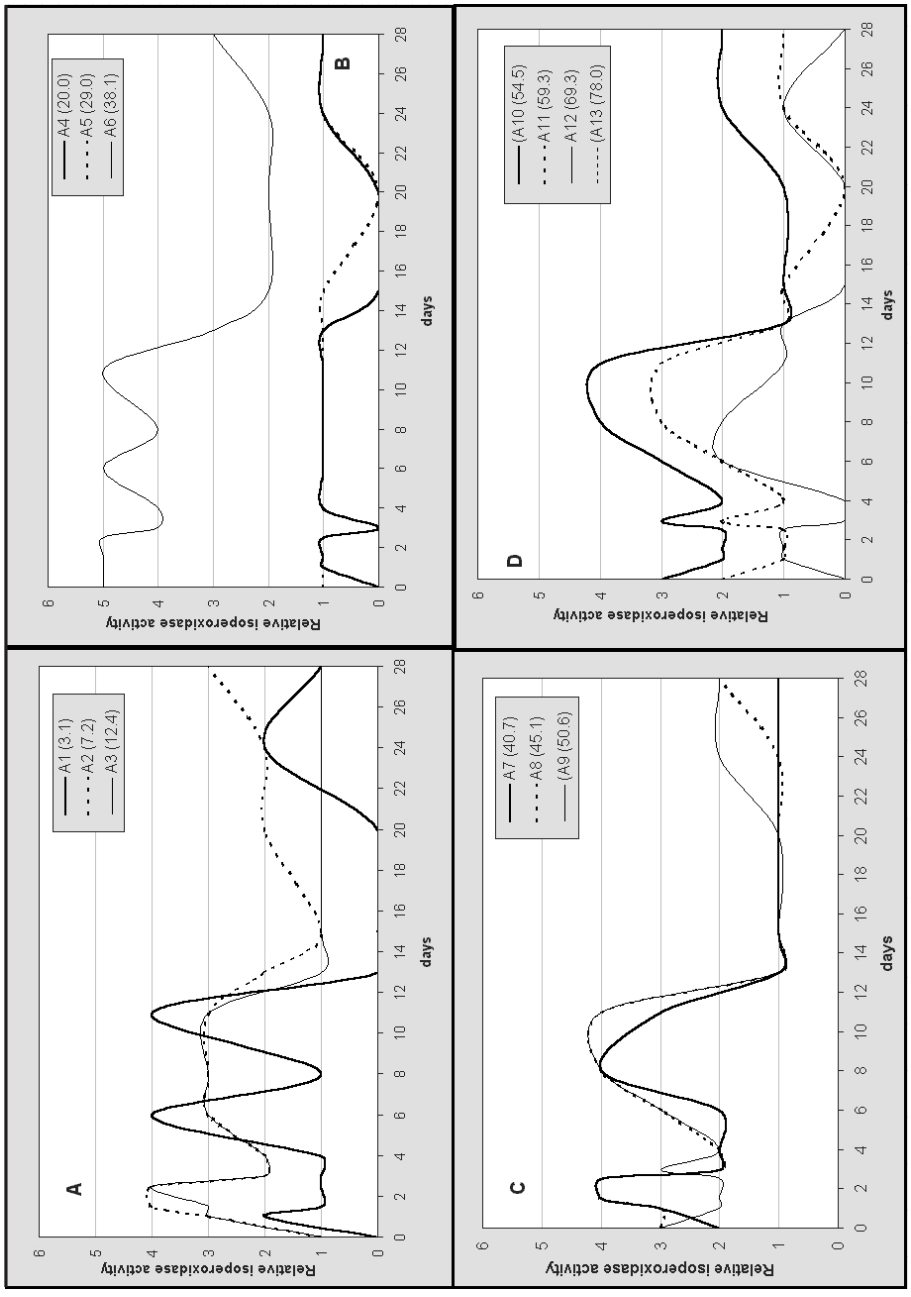

Fig. 3. Time course of relative acidic isoperoxidase activity (0 a 5) (isoforms A1 to A3 in graph **A**, A4 to A6 in graph **B**, A7 to A9 in graph **C**, A10 to A13 in graph **D**) during rhizogenesis in *Nothofagus antarctica*. R_f values are given between brackets.

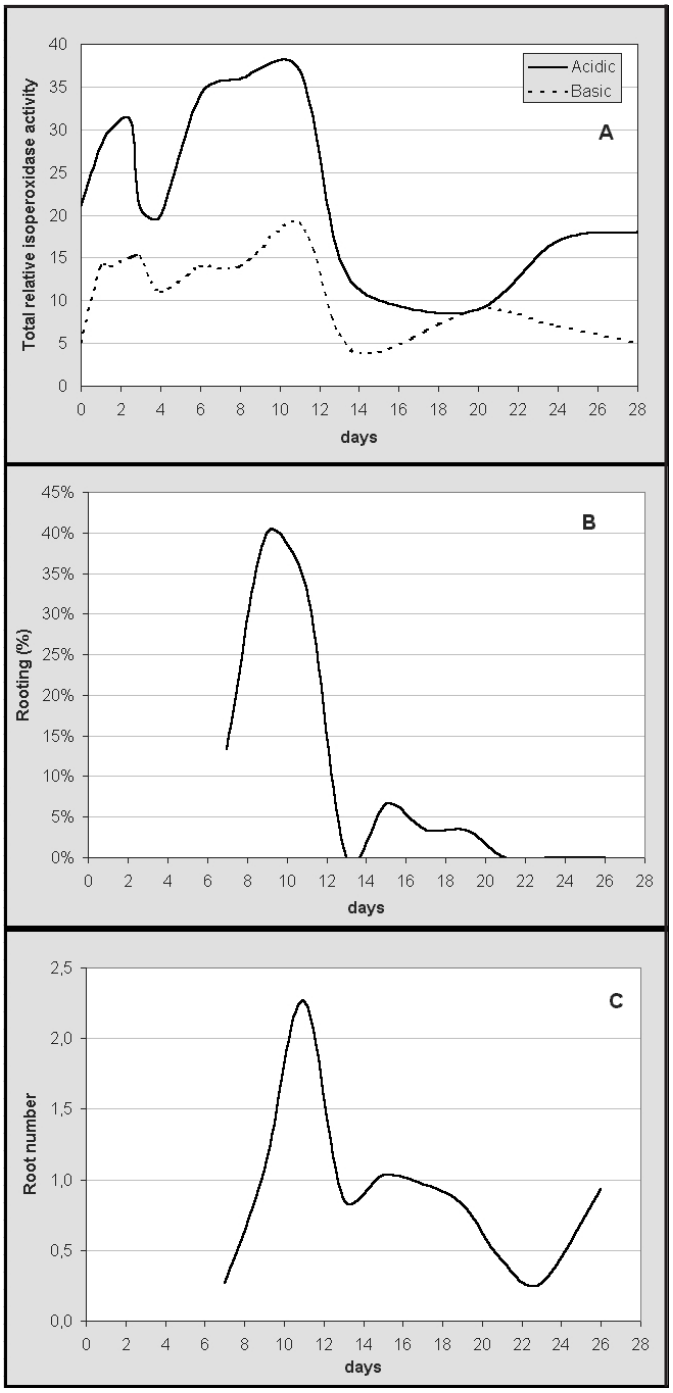

Fig. 4. Time course variation of total relative isoperoxidase activity (*acidic* and *basic*) (*A*), non-cumulative rooting (*B*) and non-cumulative apparition of new roots (*C*) during the *Nothofagus antarctica* rooting process. The time course evolution of total acidic and basic isoperoxidases was obtained by summing up the corresponding relative activities.

enzyme activity of A1 band exhibited a maximum at day 6 and 11, and A12 and A13 bands at days 6–8 (Fig. 3). Qualitative and quantitative analysis of the relative activity of the isoenzyme bands A1, A4 and A12 before and during the rooting process, may make them useful indicators of the induction phase of rhizogenesis.

During the induction stage, the acidic isoenzymes bands A2, A3, A7 and A8 presented activity peaks between days 1–2 and 2–3 (Fig. 3). At the beginning of the expression stage a second maximum between days 6–11 was in good agreement with an increase in total peroxidases by day 9 reported by Calderón et al. (1998). This maximum represented the activity of the isoenzyme bands A1, A2, A3, A7, A8, A9, A10, A11, A12 and A13. A decrease occured in the activity previously described that reverted at the end of the period for some isoenzymes. Among them, isoenzymes bands A1 and A2 exhibited a significant increase in activity, while bands A6, A8, A9 and A10 showed less marked increases. These isoenzymes would be related to cell digestion during the radical emergence and the rigidity of the cell walls of the new roots (Caspar et al., 1985; 1991).

Relationship of isoperoxidase patterns with rooting, apparition of new roots and total peroxidase activity

Comparative graphs of total isoperoxidase activities (*acidic* and *basic*) with the evolution of rooting and the apparition of new roots during the assays are presented in Fig. 4. Rooting was maximum at days 9–11 (73% of microshoots had rooted) and markedly decreased at day 13 (Fig. 4B). The average root number (2.3 per explant) was maximal at day 9, representing 25% of the total formed roots in the micro-shoots (8.9 roots per explant at day 28), another peak occurred between days 15 and 19 (Fig. 4C). The overall time course for rooting and root apparition closely matched the time course observed in the total isoperoxidases pattern (Fig. 4A) and consistently matched the amount of total peroxidases (Calderón et al., 1998). In fact, the first maximum recorded for root apparition (days 9–11) was in parallel with the second peak exhibited by acidic isoperoxidases. When the roots emerge through the tissues, digestion of cells found in their path occurs. Isoperoxidases could be part of this process which is evidenced by the methodology proposed in this study.

The maximum total isoperoxidase activities observed in the earliest stage of rhizogenesis could be responsible for the formation of the radical nodes, while the smaller rooting initiation recorded at the end of the assayed period could be related to a minimal activity increase presented by these isoenzymes (Fig. 4A).

The behavior observed in the evolution of rooting and the peaks of root apparition can also be correlated with total peroxidases. The total peroxidase activities determined in *N. antarctica* showed a maximum at day 3, followed by a minimum at day 7, another maximum at day 9 and a continuous decrease until the end of rooting (Calderón et al., 1998). These maxima are due to both acidic and basic peroxidases, and con-

sistently matched with the maxima and minima of total isoperoxidase activities detected in the present work. However, the studies carried out with total peroxidases do not permit evaluating the possible role of different isoenzyme types (*acidic* or *basic*), nor do they permit differentiating those isoforms having a relevant role during rhizogenesis.

We conclude that auxin induction in *N. antarctica* must occur early between days 0 and 4, and later, basic peroxidases are built up and would catabolise auxins, as was previously suggested (Van der Berg et al., 1983; Gaspar et al., 1985; 1991; Faivre-Rampant et al., 2000). The significant increase of acidic peroxidases is consistent with the formation and growth of the radical system, emerging through the cortex at day 7. The variation in the number and activity of isoperoxidases represents a good indicator of the processes involved in adventitious rooting stages of *N. antarctica*.

Monitoring the activity of specific isoperoxidase bands which label particular phases of tissue differentiation in the formation of roots in *N. nervosa* should be of particular utility in the evaluation of the effectiveness of putative rooting enhancer compounds which in turn would allow the generation of an improved protocol for micropropagation of this forest species.

Acknowledgements. The authors wish to thank Ricardo Devalis *(CERZOS)* and Gustavo Vater *(CADIC)* for helpful technical assistance.

References

Berthon, J., N. Boyer, Th. Caspar, 1987. Sequential rooting media and rooting capacity of *Sequoiadendron giganteum in vitro.* Peroxidase activity as a marker. Plant Cell Rep., 6, 341–344.

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins using the principle of protein-dye binding. Anal. Biochem., 72, 248–254.

Calderon Baltierra, X., G. Martinez Pastur, M. Jofre, M. Arena, 1998. Activity variation of peroxidase during *in vitro* rooting of *Nothofagus nervosa* and *N. antarctica.* Phyton, 62(1/2), 137–144.

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

Davies, B. J., 1964. Disc electrophoresis II. Methods and application to human serum proteins. Ann. NY Acad. Sci., 121, 404–427.

Dimitri, M. J., 1972. La región de los bosques andino-patagonicos. Sinopsis general. Tomo X. Colección Cientifica del Institute Nacional de Tecnologia Agropecuaria, 381.

Druart, Ph., Cl. Kevers, Ph. Boxus, Th. Caspar, 1982; *In vitro* promotion of root formation by apple shoots through darkness effect on endogenous phenols and peroxidases. Z. Pflanzenphysiol., Bd. 108, 429–436.

De Klerk, G., 1996. Markers of adventitious root formation. Agronomie, 16, 609–616.

Faivre-Rampant, O., C. Kevers, Th. Caspar, 2000. IAA-oxidase activity and auxin protectors in nonrooting, *rac,* mutant shoots of tobacco *in vitro.* Plant Sci., 153, 73–80.

Caspar, Th., 1981. Rooting and flowering, two antagonistic phenomena from a hormonal point of view. In: Aspects and prospects of plant growth regulators (Ed. B. Jeffcoat). British Plant Growth Regulator Group, Wantage, 39–49.

Caspar, Th., C. Penel, F. Castillo, H. Greppin, 1985. A two-step control of basic and acidic peroxidases and its significance for growth and development. Physiol. plant., 64, 418–423.

Caspar, Th., C. Penel, D. Hagege, H. Greppin, 1991. Peroxidases in plant growth, differentiation and development processes. In: Biochemical Molecular and Physiological Aspects of Plant Peroxidases (Eds., J. Lobarzewski, H. Greppin, C. Penel, Th. Caspar). University of M. Curie-Sklodowska, 249–280.

Gaspar, Th., C. Kevers, J. F. Hausman, 1997. Indissociable chief factors in the inductive phase on adventitious rooting. In: Biology of root formation and development (Eds., A. Altman, Y. Waisel). Chapter 7. New York, Plenum Press, 55–63.

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

Mader, M., 1992. Compartmentation of peroxidase isoenzymes in plant cells. In: Plant Peroxidases 1980–1990 (Eds., C. Penel, Th. Gaspar, H. Greppin). University of Geneva, 37–46.

Martinez Pastur, G., M. Arena, 1995. *In vitro* propagation of *Nothofagus obliqua (Fagaceae).* Aust. J. Bot., 43, 601–607.

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

Martinez Pastur, G., M. Arena, O. Caso, 1997. *In vitro* propagation of *Nothofagus antarctica* from sphaeroblasts and saplings. Biocell, 21(3), 231–236.

Martinez Pastur, G., M. Arena, 1997. Micropropagación de *Nothofagus pumilio* (Poepp et Endl) Krasser. Revista Bosque, 18(2), 43–50.

Medina, Ml., M. A. Di Renzo, I. N. Tiranti, 1985. Identificatión de cultivares de pasto llorón *Eragrostis curvula* (Schrad.) Nees por medio de esterasas, fosfatasas y peroxidasas. Rev. Invest. Agrop. Argentina, 20, 1–9.

Quoirin, M., P. Boxus, Th. Gaspar, 1974. Root initiation and isoperoxidases of stem tip cuttings from mature *Prunus* plants. Physiol. Veg., 12(2), 165–174.

Reisfeld, R. A., U.J. Lewis, D. E. Williams, 1962. Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature, 195, 281–283.

Van der Berg, B. M., R. N. Chibbar, R. B. Van Huystee, 1983. A comparative study of a cationic peroxidase from peanut and an anionic peroxidase from *Petunia.* Plant Cell Rep., 2, 304–307.