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Peroxidase, acid phosphatase, RNase and DNase activity and isoform patterns during *in vitro* rooting of *Petunia* × *hybrida* microshoots

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Abstract

Specific activities and isoform patterns of peroxidases, acid phosphatases, DNases and RNases were studied in relation to *in vitro* rooting of *Petunia* × *hybrida* microshoots in the presence of 4 μM indole-3-butyric acid (IBA). Specific activities of the above enzymes increased in the course of rooting. Rhizogenesis could be related with an increased specific activity of peroxidases during the initiation phase, in parallel with increased lignin content. Twelve peroxidases, six anionic (A1 - A6) and six cationic (C1 - C6), seven acid phosphatases (ACP1 - ACP7), seven RNases (R1 - R7) and four DNases (D1 - D4) isoforms were detected following native PAGE. Variation in the number of the above isoforms and their quantity was observed during different stages of rooting. Particularly, A2, A3, C3, C4, C5, ACP2, R1, R2, R3, and D4 isoforms appeared after the induction phase and could be related to emergence of root primordia. Additionally, R3 and D4 could be associated with cell division and differentiation, since these are only expressed in rooted microshoots. Moreover, the higher number of roots in IBA-treated microshoots could be related to the higher expression of RNase and DNase isoforms during initiation and expression phases.

Additional key words: electrophoresis, IBA, lignin, microcuttings, nucleases, vegetative propagation.

Introduction

Petunia (*Petunia* × *hybrida*) is an important ornamental bedding plant and several studies are reported on the promotion of regeneration *in vitro* (Economou and Read 1984, Cabaleiro and Economou 1991, Dimasi-Theriou *et al.* 1993, Auer *et al.* 1999 and references therein). Adventitious root formation (ARF) in microshoots (microcuttings), a unique and complex process, is essential in vegetative propagation and the analysis of ARF on a number of plant species has led to the general recognition of three phases in the rooting process: induction is defined as the period in which no morphological events are clearly observed and comprises the early molecular and biochemical steps preceding morphological modifications; initiation in which cell divisions take place, root meristems are formed and root primordia are established; expression in which root emergence and growth occurs (Moncousin and Gaspar 1983, Syros *et al.* 2004, Hatzilazarou *et al.* 2006 and references therein).

It is well established that auxins play a central role in the determination of rooting capacity, while the most commercial propagation is done by rooting with indole-3-butyric acid (IBA). Auxin type efficacy depends on the affinity for the auxin receptor protein involved in rooting, on the concentration of free auxin that reaches target competent cells, on the amount of endogenous auxin, and on metabolic stability (De Klerk *et al.* 1997 and references therein, Kevers *et al.* 1997, Tereso *et al*. 2008).

Peroxidase (POD; EC 1.11.1.7) belongs to a large family of enzymes able to oxidize several different substrates in the presence of H_2O_2 (Siegel 1993). On the other hand, POD could participate in the protection of cells and subcellular systems from the effects of H_2O_2 (Molassiotis *et al*. 2006a,b, Niknam *et al*. 2006). Studies on adventitious root formation have suggested a fundamental role of peroxidases in controlling rooting, while peroxidase activity was proposed to define rooting phases in several species (Moncousin and Gaspar 1983, Gaspar *et al.* 1992, Calderón-Baltierra *et al.* 1998, Rout *et al.* 2000, Syros *et al.* 2004, Hatzilazarou *et al.* 2006 and

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Abbreviations: BA - 6-benzyladenine; DMAB - 3-(dimethylamino)benzoic acid; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid; MBTH - 3-methyl-2-benzothiazolinone hydrozone hydrochloride monohydrate; PAGE - polyacrylamide gel electrophoresis; PMSF - phenylmethylsulfonic fluoride; PVPP - polyvinylpolypyrolidone; Tris - tris(hydroxymethyl)aminomethane.
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references therein). Anionic PODs are involved in the process of lignification (Mäder and Füssl 1982, Christensen *et al.* 1998, Repka *et al.* 2000) and suberinization (Espelie and Kollattukudy 1985, Šimonovičová *et al.* 2004). On the other hand, changes in POD activity (mainly due to cationic PODs) influence rooting by indole-3-acetic acid (IAA) catabolism (Kevers *et al.* 1997 and references therein). Nevertheless, cationic PODs are also involved in the biosynthesis of lignin and suberin (Quiroga *et al.* 2000).

Acid phosphatases (E.C.3.1.3.2) are a group of enzymes, which non-specifically catalyzes the hydrolysis of a variety of phosphate esters (Duff *et al.* 1994 and references therein). The morpho-physiological events leading to emergence of roots through various tissues have been associated with hydrolysis of cells and tissues (De and Roy 1984) and a relationship between increasing acid phosphatase (ACP) activity and adventitious root formation has been reported (Bell and McCulley 1970, Bhattacharya and Nanda 1979, Kumar and Kakkar 1989). The specific role of ACP in this process remains to be elucidated.

In plants, DNA-degrading enzymes, better known as nucleases type I (EC 3.1.30.2) as well as RNA-degrading enzymes (RNases) have been studied in several species (Green 1994, Sugiyama *et al.* 2000, Desai and Shankar 2003). No plant specific DNA-degrading enzyme (DNase) has been identified. The biological role of these enzymes is not well elucidated. It is suggested that nuclease type I enzymes participate in the process of DNA replication, recombination and repair and mainly in catabolic processes of nucleic acids (Grafi and Larkins 1995, Yupsanis *et al.* 2004 and references therein), while the main cell functions of plant RNases are their general housekeeping roles in RNA processing and turnover (Green 1994). The above enzymes could participate in diverse physiological processes in higher plants, ranging from senescence and xylogenesis to seed germination and fruit development (Thelen and Northcote 1989, Green 1994 and references therein, Yupsanis *et al.* 2004 and references therein). Little is known regarding the signal pathways involved in the regulation of nucleolytic enzymes (DNase, RNase, type I nuclease) (LeBrasseur *et al.* 2002). In one case, barley aleurone synthesizes and secretes a nuclease in response to gibberellic acid (Brown and Ho 1987). Although it has been reported abundant DNA and RNA amplification occurred at the time of adventitious root formation (Oppenoorth 1979, Duhrssen *et al.* 1984) few information on the behaviour of nucleolytic enzymes in the rooting process is reported (Bhattacharya *et al.* 1976a,b).

The present research was conducted to study the lignin content and the quantitative and qualitative changes of peroxidases, acid phosphatases, RNases and DNases during *in vitro* rooting of *Petunia* × *hybrida* microshoots in order to associate them with successful root formation and relate them with the interdependent phases of *in vitro* rooted microshoots.

Materials and methods

***In vitro* cultures:** New and fully expanded leaves from the top of the shoots of greenhouse grown petunia (*Petunia* × *hybrida*, cv. Ultra Blue) plants were properly disinfected (Economou and Read 1984), washed in sterile distilled water at least three times, cut into segments (1.0 × 1.0 cm; cross sections of the midrib of the leaf lamina) and subsequently transferred in a culture media consisted of Murashige and Skoog (1962; MS) basal salts supplemented with 2 μM 6-benzyladenine (BA) for adventive bud proliferation and microshoots multiplication (Dimasi-Theriou *et al.* 1993). The pH of the media was adjusted to 5.8 before gelling with 6.5 g dm^{-3} of agar (*Sigma-Aldrich*, St. Louis, USA). For root induction treatments, 6-week-old microshoots (1.5 - 2.0 cm) were separated from the mother cultures and transferred to MS basal salts supplemented with 4 μM IBA, while an MS medium without a growth regulator was used as control. One excised shoot was placed in culture tube (25 × 150 mm) having 20 cm^3 of the MS rooting medium. All the cultures were maintained at 22 ± 2 °C and 16-h photoperiod (irradiance of 40 μmol m^{-2} s^{-1}, cool white fluorescent lamps; *Osram*, München, Germany). Peroxidase, phosphatase, DNase and RNase activities and isoform patterns as well as lignin content were evaluated in the basal parts of the microshoots on day 0, and after 12 h, 1, 3, 7, 11, 15 and 19 d. In addition, on day 19, unrooted microshoots without the presence of callus in the basal part, were also used.

Enzyme extraction: Control and treated microshoots (50 mg of lyophilised tissue) were extracted with ice cold buffer (1:10), which consisted of 100 mM K-phosphate (pH 6.5), 1 mM phenylmethylsulfonic fluoride (PMSF) and 10 % (m/v) polyvinylpolypyrolidone (PVPP). The homogenate was clarified by centrifugation (20 min; 10 000 *g*). The supernatant was dialyzed against the extraction buffer (except of PVPP) and was used as soluble enzymes source (Syros *et al.* 2004). All preparations were performed at 0 - 4 °C.

The protein concentration was determined following the method by Bearden (1978) with bovine serum albumin as a standard.

Peroxidase activity and PAGE: Peroxidase (POD) activity was determined spectrophotometrically by monitoring the formation of an indamine dye from 3-(dimethylamino)benzoic acid (DMAB) and 3-methyl-2-benzothiazolinone hydrozone hydrochloride monohydrate (MBTH) at 590 nm in the presence of H_2O_2 (Ngo and Lenhoff 1980). Anionic and cationic soluble peroxidases were submitted to electrophoresis on a polyacrylamide

slab gel consisting of 10 % (m/v) separating gel (5.0 cm × 1.5 mm) and 5 % stacking gel (3.0 cm × 1.5 mm), according to the Laemmli (1970) and Reisfeld *et al.* (1962) for anionic and cationic peroxidase isoforms, respectively. The running buffer for the anionic peroxidase was 25 mM Tris-glycine (pH 8.8) and for the cationic peroxidase 350 mM β-alanine-acetic acid (pH 4.5). Peroxidase isoforms were detected by incubating the gels in 50 cm^3 100 mM sodium acetate buffer (pH 5.0), with 3-amino-9-ethyl-carbazole (dissolved in N,N-dimethylformamide) in the presence of 0.05 cm^3 H_2O_2 (30 %) (Syros *et al.* 2005a). Gel development was stopped with distilled water. Gels were photographed using a *Kodak T-Max 100* film. For quantifications of enzyme patterns, densitometric analysis of the enzyme bands was done using *Gel-Pro Analyser Software* (*Media Cybernetics*, Silver Spring, MD, USA).

Acid phosphatase activity and PAGE: Acid phosphatase (ACP) activities were assayed by measuring the amount of *p*-nitrophenol produced according to the method of Bessey *et al.* (1946). The enzyme unit was defined as the amount of phosphatase changing an increase in absorbance (A_{410}) of 0.01 min^{-1}. For acid phosphatase electrophoresis, 10 μg of protein was used (see peroxidase activity and PAGE; anionic peroxidase). ACP isoforms were visualized using Na-α-napthylacid phosphate as substrate (Syros *et al.* 2005b). Gel development stopped and the isoenzyme patterns were photographed and quantified as referred above.

DNase and RNase activity and PAGE: DNase and RNase were assayed according to Yupsanis *et al*. (2001) in the presence of 0.6 mg dm^{-3} substrate (ssDNA or RNA), 33 mM ammonium acetate (pH 5.5) and 1 mM Zn^{2+}. The reaction stopped by adding chilled $HClO_4$ (12.5 %); the non-degraded substrate was removed by centrifugation (15 min; 12 000 *g*) and the absorbance of the supernatant was measured at 260 nm. ssDNA was prepared by heating 2 mg cm^{-3} aqueous solution of DNA in a boiling-water bath for 10 min and immediately cooling in an ice bath. The enzyme unit was defined as the amount of nuclease changing an absorbance (A_{260}) of 0.01 min^{-1}. Enzyme extracts (4 μg protein) were subjected to native PAGE as above mentioned (see peroxidase activity and PAGE) according to Laemmli (1970) on active gel polymerized in the presence of RNA or ssDNA (90 mg dm^{-3}) according to Matoušek and Tupý (1987) as modified by Yupsanis *et al.* (2001). Following electrophoresis, the gel was stained with ethidium bromide (0.5 μg cm^{-3}) and the appeared DNase and RNase isoforms (dark bands) were identified on a UV-transilluminator (*TFX-20M*, *Gibco BRL*, Carlsruhe, Germany).

Lignin determination: Microshoots from the rooting experiment were assayed for lignin content by applying the thioglycolic acid procedure (Syros *et al.* 2005a) with some modifications. After extraction of peroxidases and centrifugation (see above) the pellets (10 mg) were resuspended twice in ethanol and centrifuged at 8 000 *g*. The pellets were then air dried at 60 °C. The insoluble cell wall material was resuspended in 1 cm^3 2 M HCl and 0.1 cm^3 thioglycolic acid in an Eppendorf tube. The mixture was heated for 4 h in a boiling water bath and centrifuged at 15 000 *g* for 15 min. The pellets were washed with water, resuspended in 1 cm^3 0.5 M NaOH and left overnight at room temperature. The insoluble residues were removed by centrifugation and lignin thioglycolate was precipitated by the addition of 0.4 cm^3 concentrated HCl (3 h, 4 °C). After centrifugation at 15 000 *g* for 20 min, the pellets were resuspended in 1 cm^3 0.5 M NaOH. The absorbance was recorded at 280 nm and lignin content was determined from calibration curve with commercial lignin alkali.

Rooting data and statistical analysis: The rooting percentage, the number and the length of the roots were determined on day 19 after microshoot planting. In each treatment, 110 microshoots were used; 30 and 80 for rooting evaluation and biochemical investigations, respectively. A microshoot was considered rooted if it had at least one root ≥ 0.1 cm. The experiments were conducted at two subsequent periods with similar results. Data on rooting and survival rate were subjected to arcsin transformation before statistical analysis. Mean separation in the rooting data was performed with the *z*-test, while data on enzyme activity and lignin content with Duncan's multiple range test at $P < 0.05$.

Results

Control and IBA-treated microshoots rooted satisfactorily and achieved high rooting percentages, while the greatest number of roots was counted with the use of IBA (Table 1). The length of roots was similar for both control and IBA-treated microshoots. The emergence of the adventitious roots took place on day 11 in control microshoots, while this was noticed two days earlier in the IBA-treated microshoots.

A sharp decrease of lignin content from day 0 to day 1 during adventitious root formation was noticed in control and IBA-treated microshoots (Fig. 1*A*). This content increased from day 1 to day 15 for control but less in IBA-treated microshoots. The lignin content was lower in un-rooted microshoots in comparison with rooted microshoots on day 19 (Table 2).

POD activity in control and IBA-treated microshoots decreased from day 0 to day 1, increased from day 2 to day 11 and slightly decreased thereafter (Fig. 1*B*). This activity was higher in IBA-treated than in control microshoots and in rooted microshoots in comparison

Table 1. Rooting rate, number and length of roots of *Petunia* × *hybrida* microshoots treated with 4 μM IBA on day 19 of rooting. Values are the mean of 3 replicates of 10 microshoots each. In each column, the values with the same letters are not significantly different at $P \leq 0.05$ according to the Duncan's test.

	Rooting [%]	Root number	Root length [cm]
Control	55.0ab	6.55b	0.36a
IBA	60.0a	12.44a	0.34a

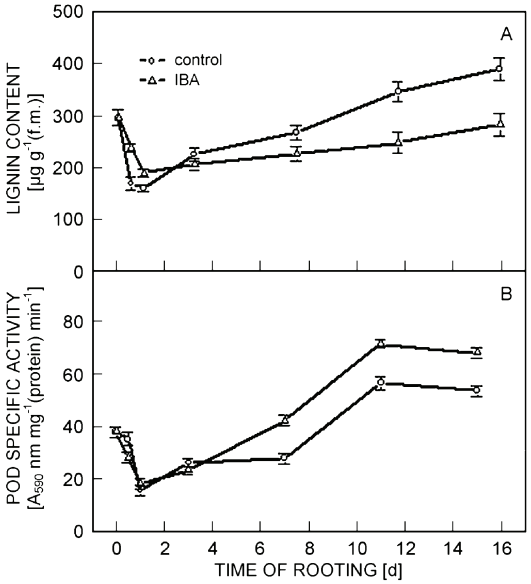

Fig. 1. Lignin content (*A*) and POD specific activity (*B*) of *Petunia* microshoots during adventitious rooting. The *vertical bars* indicate SE, $n = 4$.

with un-rooted microshoots on day 19 (Table 2).

PAGE analysis of control and IBA-treated microshoots revealed the appearance of six (A1 - A6) anionic PODs isoforms from day 0 up to day 15 (Fig. 2). In controls, A1, A4, A5 and A6 appeared from day 0 to day 1, while two slow migrating A_2 and A_3 POD isoforms were revealed from day 3 and thereafter (Fig. 2). On the other hand, the above-mentioned A1, A4, A5 and A6 were also noticed in IBA-treated microshoots from day 0 to day 3, while the A2 and A3 were revealed from day 7 and thereafter (Fig. 2). The amount of the above-mentioned A2 and A3 anionic POD isoforms slightly increased during adventitious root formation (Fig. 2). In un-rooted microshoots A2 and A3 (in IBA-treated) along with A6 (in both control and IBA-treated) were not noticed (Fig. 2).

Table 2. Lignin content [μg g^{-1}(f.m.)], POD specific activity [(A_{590} mg^{-1}(protein) min^{-1}], and ACP, RNase and DNase specific activities [U mg^{-1}(protein)] on day 19 of *in vitro* rooted and un-rooted *Petunia* microshoots. Means of 4 replicates. In each row, the values with the same letters are not significantly different according to the Duncan test at $P < 0.05$.

	Control rooted	un-rooted	IBA rooted	un-rooted
Lignin	356.13a	187.67b	248.16a	129.17b
POD	168.52a	77.87b	153.53a	53.70b
ACP	2.05a	0.72b	1.43a	0.79b
RNase	3.08a	2.10b	2.45a	0.88b
DNase	2.34a	0.56b	1.84a	0.42b

Electrophoretic analysis revealed the similar appearance of six (C1 - C6) cationic PODs isoforms (Fig. 3 and results not shown) in control and IBA-treated microshoots from day 0 up to day 15. The C3 and C4 POD isoforms were noticed especially on 12 h and day 1, while a fast migrating C5 isoform was revealed from day 11 and thereafter. Un-rooted microshoots revealed similar patterns of C1- C6 isoforms.

In control and IBA-treated microshoots, ACP specific activity was minimum from day 0 to day 1 and maximum from day 3 to day 11. This activity was higher in the case of IBA-treated microshoots from day 7 and thereafter (Fig. 4*A*). ACP activity was lower in un-rooted microshoots (Table 2). Electrophoretic analysis revealed the appearance of seven (ACP1 - ACP7) ACP isoforms from

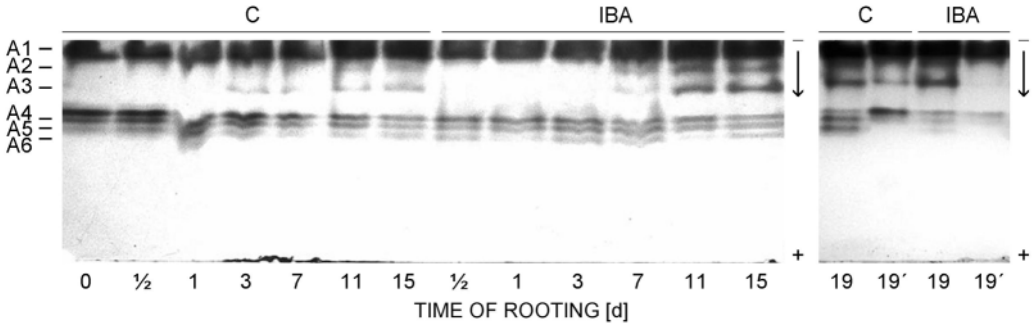

Fig. 2. PAGE on native gels (10 %) of anionic POD isoforms of *Petunia* control (C) and IBA-treated (IBA) microshoots during adventitious rooting. The indication ½ corresponds to the 12 h treatment; the indication 19 and 19′corresponds on day 19 of rooting, of rooted and un-rooted microshoots, respectively).

day 0 upto day 15. In detail, in the control and IBA-treated microshoots ACP1, ACP3, ACP4, ACP6 and ACP7 were displayed from day 0 to day 3 and ACP2 and ACP5 from day 3 and thereafter (Fig. 5 and results not shown). ACP2 and ACP5 were not presented in un-rooted microshoots (results not shown).

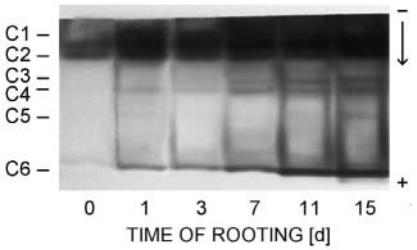

Fig. 3. PAGE on native gels (10 %) of cationic POD isoforms of control *Petunia* microshoots during adventitious rooting.

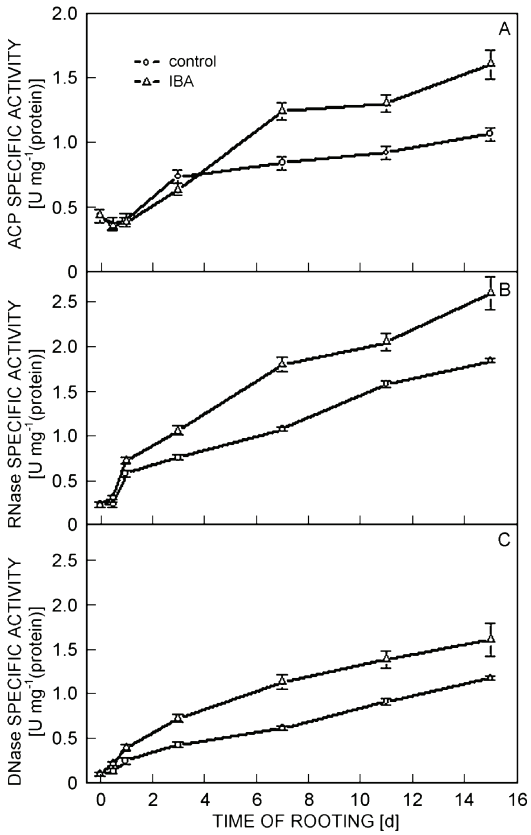

Fig. 4. ACP (*A*), RNase (*B*) and DNase (*C*) specific activitiesy of *Petunia* microshoots during adventitious rooting. The *vertical bars* indicate SE, $n = 4$.

RNase and DNase activity increased from day 0 to day 15 for both control and IBA-treated microshoots and was higher in IBA-treated microshoots at the respective days (Fig. 4*B*,*C*). RNase and DNase activities were lower in un-rooted microshoots (Table 2). Electrophoretic analysis of control and IBA-treated microshoots revealed the appearance of seven RNase (R1 - R7) isoforms from day 0 to day 15 (Fig. 6). R5, R6 and R7 were displayed during the whole period, while R1, R2 and R3 were revealed from day 11 to day 15 and from day 7 to day 15 in control and IBA-treated microshoots, respectively. In control and IBA-treated rooted microshoots R1, R2, R3, R5, R6 and R7 appeared on day 19, on the other hand, in the un-rooted microshoots only slight the appearance of R1, R5, R6 and R7 but additional R4 was observed (Fig. 7).

Electrophoretic analysis of control and IBA-treated microshoots, revealed the appearance of D1, D2, D3 and D4 DNase isoforms from day 0 to day 15 (Fig. 6). D1, D2 and D3 were observed from the beginning of the rooting, while the fast migrating D4 appeared from day 7 and day 3 and thereafter for control and IBA-treated microshoots, respectively (Fig. 6). The above-mentioned DNase isoforms strongly appeared in the IBA-treated microshoots (Fig. 6) but very slightly in un-rooted microshoots (Fig. 7) where the fast migrating D4 isoform did not appear.

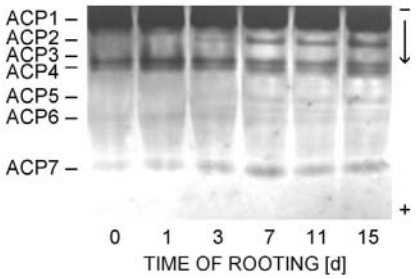

Fig. 5. PAGE on native gels (10 %) of ACP isoforms of IBA-treated *Petunia* microshoots during adventitious rooting. Numbers indicate the relevant days of rooting.

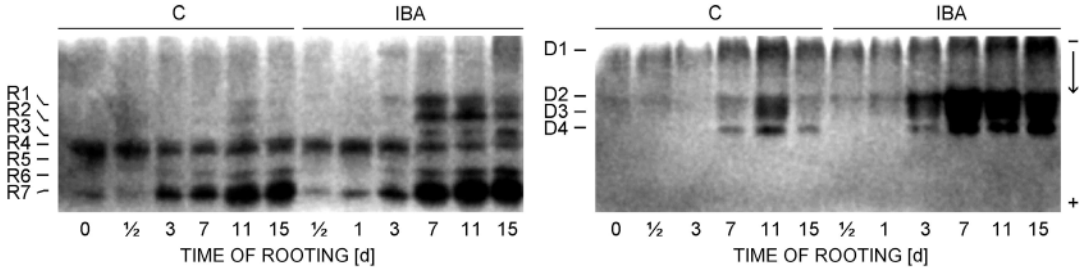

Fig. 6. PAGE of RNases and DNases isoforms on active (10 %) RNA and DNA gel, respectively, of *Petunia* microshoots during adventitious rooting in control and IBA-treated microshoots. The isoforms appeared after staining the gels with ethidium bromide. Numbers indicate the relevant days of rooting (the indication ½ corresponds to the 12 h treatment).

Discussion

Petunia microshoots revealed similar rooting percentages as well as length of roots for control and IBA-treated microshoots while microshoots exposed to IBA had higher number of roots (Table 1). Adventitious rooting is promoted by treatment with auxins, particularly with IBA (*e.g.* Ludwig-Muller 2000). Additionally, the appearance of the roots was noticed earlier in the IBA-treated microshoots. This may be related to the shorter required rooting time in IBA-treated cuttings (Hassanein *et al.* 1999 and references therein). The rooting response with IBA could also be influenced by its capacity of being acropetally transported (Ludwig-Muller 2000) along with transpiration stream in the xylem. This feature may be facilitated by auxin conjugation and/or metabolism.

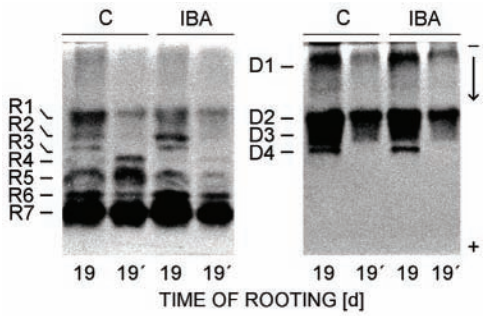

Fig.7. PAGE of RNases (*on the left*) and DNases (*on the right*) isoforms on (10 %) RNA and DNA gel, respectively, of *Petunia* on day 19 of rooting in control and IBA-treated microshoots. The isoforms appeared after staining the gels with ethidium bromide. Numbers 19 and 19′corresponds on day 19 of rooting of rooted and un-rooted microshoots, respectively.

The rooting process, in successive interdependent physiological phases, involves changes in interrelated biochemical factors (Bhattacharya 1988). Peroxidases are the most traditional biochemical markers used in rooting studies (Moncousin and Gaspar 1983, Hatzilazarou *et al.* 2006). According to the Gaspar *et al.* (1992) the induction period is characterized by a sharp reduction of peroxidase activity, the initiation phase by an increase and the expression phase by a reduction in peroxidase activity. Consequently, it is necessary to detect minimum and maximum peroxidase activity, in order to define the rooting phases (Gaspar *et al.* 1992). In the course of adventitious root formation of *Petunia* × *hybrida* control, as well as on IBA-treated microshoots, the soluble peroxidase activity showed the typical change (Fig. 1*B*) described for other species (Moncousin and Gaspar 1983, Rout *et al.* 2000, Metaxas *et al.* 2004, Syros *et al.* 2004, Hatzilazarou *et al.* 2006) and showed a minimum on day 1 (in induction phase) and a peak on day 11 (in initiation phase). The maximum activity and the time taken to reach it could be related to the rooting capacity of the species (Berthon *et al.* 1987, Calderón-Baltierra *et al.* 1998), while the duration of the phases (induction, initiation and expression) varies depending on the plant species and the culture conditions (Moncousin and Gaspar 1983, Metaxas *et al.* 2004 and references therein). Several authors have observed a positive correlation between peroxidase activity and rooting (Moncousin and Gaspar 1983, Kevers *et al.* 1997, Metaxas *et al.* 2004, Syros *et al.* 2004). According to them, root formation occurred after the cuttings reached and passed a peak of maximum enzyme activity.

With regard to peroxidase isoforms, petunia microshoots were characterized by a generally increased expression of both anionic and cationic peroxidase isoforms from day 1 to day 15 (Figs. 2, 3), as well as with the appearance of additional anionic (A2 and A3) isoforms from day 7 to day 15 and cationic isoforms (C3, C4 and C5) from day 3 to day 15. Microshoots treated with IBA showed the most expressive changes in these isoforms. Changes in anionic and cationic peroxidase isoform patterns could be positively correlated. Similar correlations were reported in a number of plant species (Gaspar *et al.* 1992, Faivre-Rampant *et al.* 1998, Metaxas *et al.* 2004, Syros *et al.* 2004). Anionic peroxidase isoforms are involved mainly in growth and development (Gaspar *et al.* 1985), associated with lignin polymerization (Mäder and Füssl 1982) and suberinization (Espelie and Kallattukudy 1985). For example, the fast-migrating anionic peroxidase isoforms of tobacco (Mäder and Füssl 1982) and poplar (Christensen *et al.* 1998) have been considered to be involved in the polymerization of lignin monomers. On the other hand, changes in cationic peroxidase influence rooting by IAA catabolism (Kevers *et al.* 1997).

The lignin content of petunia microshoots increased during adventitious root formation and was lower in the case of IBA-treated microshoots (Fig. 1*A*, from day 7 to day 11). This suggests an inhibition of the lignification in the IBA-treated microshoots in agreement with Aeschbacher *et al.* (1994), Ballester *et al.* (1999) and Syros *et al.* (2004).

Hydrolytic enzymes (including phosphatases) facilitate root primordia emergence from cuttings following digestion of middle lamella of cortical tissues (Bell and McCulley 1970). Phosphatases have also been reported to be involved in root emergence from hypocotyl cuttings of *Phaseolus mungo* (Bhattacharya and Nanda 1979). The specific activity of acid phosphatase of both control and IBA-treated microshoots slightly increased from day 0 to day 15, and ACP activity was higher in IBA-treated microshoots (Fig. 4*A*). Moreover, a new slow migrating ACP2 isoform (in both control and IBA-treated microshoots) was expressed after the induction phase (from day 7 and thereafter) (Fig. 5). These findings indicated that phosphatases were involved in the root emergence and could be associated with increased energy requirements as rooting progressed. Kumar and Kakkar (1989) reported that increased activities of acid phosphatase by auxin treatments might facilitate emergence of greater number of root primordia through biochemical

degradation of cortical tissues and assist in the absorption of material derived from these tissues. Additionally, phosphatases are referred to be involved in cell-to-cell communication, and in response to hormones (Smith and Walker 1996).

Quantitative and qualitative changes in DNA and RNA prior to rooting have had been determined in many plant species (Haissig 1982 and references therein). Furthermore, exogenous application of DNA and RNA stimulated rooting of *Phaseolus mungo* and *Impatiens balsamina* cuttings (Bhattacharya *et al.* 1976a,b). An increase of DNase and RNase activities in cuttings of the above-mentioned species was also observed during early stages of rooting. These results supported the hypothesis that applied DNA and RNA could be used, after partial catabolism by nucleases, for the synthesis of new DNA and RNA needed for rooting. The specific activities of both DNase and RNase in the basal parts of the petunia microshoots increased during rooting (Fig. 4*B*,*C*). Treatment with IBA further enhanced these activities and their increase appeared earlier in comparison with control microshoots. Bhattacharya *et al.* (1976a,b) suggested that RNase activity may be related to rooting, but the mechanism of involvement is unclear. In any case, according to our results, enhanced DNase and RNase activity and appearance of four DNase (D1 - D4) and seven RNase (R1 - R7) isoforms (Fig. 6) accompanied root formation. Undoubtedly, the R3 and D4 isoforms are associated with the process of root formation (cell division and differentiation), since these are not expressed in un-rooted microshoots. D4 DNase could be characterized as a nuclease type I since it displayed similar relative mobility (Rf = 2.5, Fig. 7). The higher quantity and the earlier appearance of DNase and RNase isoforms during adventitious rooting in the IBA-treated microshoots could be related to the higher number of roots observed in these microshoots (Table 1).

According to our results, the changes of specific activities and isoform patterns of peroxidases, acid phosphatases, RNase and DNase provide useful indicators of the processes involved in the adventitious rooting of *Petunia* × *hybrida* microshoots and could be associated with successful root formation. Hence, these enzymes might be used as potent biochemical markers.

References

Aeschbacher, R.A., Schiefelbein, J.W., Benfey, P.N.: The genetic and molecular basis of root development. - Annu. Rev. Plant Physiol. Plant mol. Biol. **45**: 25-45, 1994.

Auer, C.A., Motyka, V., Březinová, A., Kamínek, M.: Endogenous cytokinin accumulation and cytokinin oxidase activity during shoot organogenesis of *Petunia hybrida*. - Physiol. Plant. **105**: 141-147, 1999.

Ballester, A., San-Jose, M.C., Vidal, N., Fernandez-Lorenzo, J.L., Vieitez, A.M.: Anatomical and biochemical events during *in vitro* rooting of microcuttings from juvenile and mature phases of chestnut. - Ann. Bot. **83**: 619-629, 1999.

Bearden, J.C.: Quantitation of submicrogram quantities of protein by an improved protein-dye binding assay. - Biochim. biophys. Acta **533**: 525-529, 1978.

Bell, J.K., McCully, M.E.: A histological study of lateral root initiation and development of *Zea mays*. - Protoplasma **70**: 179-205, 1970.

Berthon, J.Y., Boyer, N., Gaspar, T.: Sequential rooting media and rooting of *Sequoiadendron giganteum in vitro*. Peroxidase activity as a marker. - Plant Cell Rep. **6**: 341-344, 1987.

Bessey, O.A., Lowry, O.H., Brock, M.J.: A method for the rapid determination of alkaline phosphatase with five cubic millimetres of serum. - J. biol. Chem. **164**: 321-329, 1946.

Bhattacharya, N.C.: Enzyme activities during adventitious rooting. - In: Davis, T.D., Haissig, B.E., Sankhla, N. (ed.): Adventitious Root Formations in Cuttings. Vol. 2. Pp. 88-101. Dioscorides Press, Portland - Oregon 1988.

Bhattacharya, N.C., Bhattacharya, S., Malik, C.P.: Transients in the activities of some hydrolases and their relationship with adventitious root formation in hypocotyls cuttings of *Phaseolus mungo*. - Plant Sci. **8**: 38-44, 1976a.

Bhattacharya, S., Bhattacharya, N.C., Nanda, K.K.: Effect of exogenous application of nucleic acids and auxin on the rooting of hypocotyls cuttings of *Impatiens balsamina*. Evidence for the uptake of information molecules. - Experientia **32**: 1301-1303, 1976b.

Bhattacharya, S., Nanda, K.K.: Promotive effect of purine and pyrimidine bases and their role in the mediation of auxin action through the regulation of oxidases and phosphatases in rooting cuttings of *Phaseolus mungo*. - Indian J. exp. Biol. **17**: 40-45, 1979.

Brown, H.B., Ho, T.D.: Biochemical properties and hormonal regulation of barley nuclease. - Eur. J. Biochem. **168**: 357-364, 1987.

Cabaleiro, C., Economou, A.S.: Effect of light on rooting *in vitro* of petunia microshoots. - Acta Hort. **300**: 189-196, 1991.

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

Christensen, J., Bauw, G., Welinder, K., Montagu, M., Boerjan, W.: Purification and characterization of peroxidases correlated with lignification in poplar xylem. - Plant Physiol. **118**: 125-135, 1998.

De, K.K., Roy, S.C.: Role of an acid phosphatase isoenzyme in callus tissue during cytodifferentiation. - Theor. appl. Genet. **68**: 285-287, 1984.

De Klerk, G.-J., Brugge, J.T., Marinova, S.: Effectiveness of indoleacetic acid, indolebutyric acid and naphthaleneacetic acid during adventitious root formation *in vitro* in *Malus* Jork 9. - Plant Cell Tissue Organ Cult. **49**: 39-44, 1997.

Desai, N.A., Shankar, V.: Single-stand-specific nucleases. - FEMS Microbiol. **26**: 457-491, 2003.

Dimasi-Theriou, K., Economou, A.S., Sfakiotakis, E.M.: Promotion of petunia (*Petunia hybrida* L.) regeneration *in vitro* by ethylene. - Plant Cell Tissue Organ Cult. **32**: 219-225, 1993.

Duff, S.M.G., Sarath, G., Plaxton, W.C.: The role of acid phosphatase in plant phosphorus metabolism. - Physiol. Plant. **90**: 791-800, 1994.

Duhrssen, E., Lanzendorfer, M., Neumann, K.H.: Comparative investigation on DNA organization of some varietes of *Daucus carota* L. - Z. Pflanzenphysiol. **113**: 223-229, 1984.

Economou, A.S., Read, P.E.: *In vitro* shoot proliferation of Minnesota deciduous azaleas. - HortScience **19**: 60-61, 1984.

Espelie, K.E., Kollattukudy, P.E.: Purification and characterization of an abscisic acid-inducible anionic peroxidase associated with suberization in potato (*Solanum tuberosum*). - Arch. Biochem. Biophys. **240**: 539-545, 1985.

Faivre-Rampant, O., Kevers, C., Bellini, C., Gaspar, T. : Peroxidase activity, ethylene production, lignification and growth limitation in shoots of a non-rooting mutant of tobacco. - Plant Physiol. Biochem. **36**: 873-877, 1998.

Gaspar, T., Kevers, C., Hausman, J.F., Berthon, J.Y., Ripetti, V.: Practical use of peroxidase activity as a predictive marker of rooting performance of micropropagated shoots. - Agronomie **12**: 757-765, 1992.

Gaspar, T., Penel, C., Castillo, F., Greppin, H.: A two step control of basic and acidic peroxidases and its significance for growth and development. - Physiol. Plant. **64**: 418-423, 1985.

Grafi, G., Larkins, B.A.: Activity of single-stranded DNA endonucleases in mung bean is associated with cell division. - Plant mol. Biol. **29**: 703-710, 1995.

Green, P.J.: The ribonucleases of higher plants. - Annu. Rev. Plant. Physiol. Plant. mol. Biol. **45**: 421-445, 1994.

Haissig, B.E.: Activity of some glycolytic and pentose-phosphate pathway enzymes during the development of adventitious roots. - Physiol Plant. **55**: 261-272, 1982.

Hassanein, A.M., Ahmed, A.M., Abed-el-Hafez, A.I., Soltan, D.M.: Isozyme expression during root and shoot formation in *Solanum nigrum*. - Biol. Plant. **42**: 341-347, 1999.

Hatzilazarou, S.P., Syros, T.D., Yupsanis, T.A., Bosabalidis, A.M., Economou, A.S.: Peroxidases, lignin and anatomy during *in vitro* and *ex vitro* rooting of gardenia (*Gardenia jasminoides* Ellis) microshoots. - J. Plant Physiol. **163**: 827-836, 2006.

Kevers, C., Hausman, J.F., Faivre-Rampant, O., Evers, D., Gaspar, T.: Hormonal control of adventitious rooting: progress and questions. - J. appl. Bot. **71**: 71-79, 1997.

Kumar, K., Kakkar, R.K.: Changes in activities of some hydrolases and oxidoreductases in IAA-treated hypocotyl cuttings of *Phaseolus vulgaris* L. during adventitious root initiation and development. - Curr. Sci. **58**: 694-696, 1989.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. - Nature **227**: 680-685, 1970.

LeBrasseur, N.D., MacIntosh, G.C,, Perez-Amador, M.A., Saitoh, M., Green, P.J.: Local and systemic wound-induction of RNase and nuclease activities in *Arabidopsis*: RNS1 as a marker for a JA-independent systemic signalling pathway. - Plant J. **29**: 393-403, 2002.

Ludwig-Müller, J.: Indole-3-butyric acid in plant growth and development. - Plant Growth Regul. **32**: 219-230, 2000.

Mäder, M., Füssl, R.: Role of peroxidase in lignification of tobacco cells. II. Regulation by phenolic compounds. - Plant Physiol. **70**: 1132-1134, 1982.

Matoušek, J., Tupý, J.: Developmental changes in nuclease and other phosphohydrolase activities in anthers of *Nicotiana tabacum* L. - J. Plant Physiol. **129**: 351-362, 1987.

Metaxas, D., Syros, T., Yupsanis, T., Economou, A.S.: Peroxidases during adventitious rooting in cuttings of *Arbutus unedo* and *Taxus baccata* as affected by plant genotype and growth regulator treatment. - Plant Growth Regul. **44**: 257-266, 2004.

Molassiotis, A., Sotiropoulos, T., Tanou, G., Diamantidis, G., Therios I.: Boron-induced oxidative damage and antioxidant and nucleolytic responses in shoot tips culture of the apple rootstock EM 9 (*Malus domestica* Borkh). - Environ. exp. Bot. **56**: 54-62, 2006a.

Molassiotis, A.N., Sotiropoulos, T., Tanou, G., Kofidis, G., Diamantidis, G., Therios I.: Antioxidant and anatomical responces in shoot culture of the apple rootstock MM 106 treated with NaCl, KCl, mannitol or sorbitol. - Biol. Plant. **50**: 61-68, 2006b.

Moncousin, C., Gaspar, T.: Peroxidase as a marker of rooting improvement of *Cynara scolymus* L. cultured *in vitro*. - Biochem. Physiol. Pflanzen **178**: 263-271, 1983.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-479, 1962.

Niknam, V., Razavi, N., Ebrahimzadeh, H., Sharifizadeh, B.: Effect of NaCl on biomass, protein and proline contents, and antioxidant enzymes in seedlings and calli of two *Trigonella* species. - Biol. Plant. **50**: 591-596, 2006.

Ngo, T., Lenhoff, M.: A sensitive and versatile chromogenic assay for peroxidase and peroxidase-coupled reactions. - Anal. Biochem. **105**: 389-397, 1980.

Oppenoorth, J.M.: Influence of cycloheximide and actinomycin D on initiation and early development of adventitious roots. - Physiol. Plant. **47**: 134-138, 1979.

Quiroga, M., Guerrero, C., Botella, M.A., Barceló, A., Amaya, I., Medina, M.I., Alonso, F.J., Forchetti, S.M., Tigier, H., Valpuesta, V.: A tomato peroxidase involved in the synthesis of lignin and suberin. - Plant Physiol. **122**: 1119-1127, 2000.

Reisfeld, R.A., Lewis, U.J., Williams, D.E.: Disk electrophoresis of basic proteins and peptides on polyacryl-amide gels. - Nature **195**: 281-283, 1962.

Repka, V., Štetková, D., Fischerová, I.: The substrate preference and histochemical localization argue against the direct role of cucumber stress-related anionic peroxidase in lignification. - Biol. Plant. **43**: 549-558, 2000.

Rout, G.R., Samantaray, S., Das, P.: *In vitro* rooting of *Psoralea corylifolia* Linn: peroxidase activity as a marker. - Plant Growth Regul. **305**: 215-219, 2000.

Siegel, B.Z.: Plant peroxidases - an organismic perspective. - Plant Growth Regul. **12**: 303-312, 1993.

Šimonovičová, M., Huttová, J., Mistrík, I., Široká, B., Tamás, L.: Peroxidase mediated hydrogen peroxide production in barley roots grown under salt stress conditions. - Plant Growth Regul. **44**: 267-275, 2004.

Smith, R.D., Walker, J.C.: Plant protein phosphatases. - Annu. Rev. Plant Physiol. Plant mol. Biol. **47**: 101-125, 1996.

Sugiyama, M., Ito, J., Aoyagi, S., Fukuda, H.: Endonucleases. - Plant mol. Biol. **44**: 387-397, 2000.

Syros, T., Yupsanis, T., Zafiriadis, H., Economou, A.S.: Activity and isoforms of peroxidases, lignin and anatomy, during adventitious rooting in cuttings of *Ebenus cretica* L. - J. Plant Physiol. **161**: 69-77, 2004.

Syros, T.D., Yupsanis, T.A., Economou, A.S.: Expression of peroxidases during seedling growth in *Ebenus cretica* L. as affected by light and temperature treatments. - Plant Growth Regul. **46**: 143-151, 2005a.

Syros, T., Yupsanis, T., Petkou, D., Economou, A.S.: Protein, leucine aminopeptidase, esterase, acid phosphatase and photosynthetic responses of oleander (*Nerium oleander* L.) during cold acclimation and freezing treatments. - J. Plant Physiol. **162**: 886-894, 2005b.

Tereso, S., Miguel, C.M., Mascarenhas, M., Roque, A., Trindade, H., Maroco, J.: Improved *in vitro* rooting of *Prunus dulcis* Mill. cultivars. - Biol. Plant. **52**: 437-444, 2008.

Thelen, M.P., Northcote, D.H.: Identification and purification of a nuclease from *Zinnia elegans* L. A potential molecular marker for xylogenesis. - Planta **179**: 181-195, 1989.

Yupsanis, T., Kefalas, P.S., Eleftheriou, P., Kotinis, K.: RNase and DNase activities in the alfalfa and lentil grown in iso-osmotic solutions of NaCl and mannitol. - J. Plant Physiol. **158**: 921-927, 2001.

Yupsanis, T., Symeonidis, L., Kalemi, T., Moustaka, H., Yupsani, A.: Purification, properties and specificity of an endonuclease from *Agropyron elongatum* seedlings. - Plant Physiol. Biochem. **42**: 767-776, 2004.

Hell, R, Dahl, C., Knaff, D., Leustek, T. (ed.): **Sulfur Metabolism in Phototrophic Organisms**. - Springer, Dordrecht 2008. 516 pp. EUR 229.00. ISBN 978-1-4020-6862-1.

The new volume of the Advances in Photosynthesis and Respiration acquaints us with the metabolism of sulfur in photosynthetic systems.

Sulfur is one of the elements which are essential for metabolic processes of living organisms. Biologically important sulfur compounds – these include sulfur amino acids cysteine and methionine, glutathione, as well as numerous cofactors, e.g. coenzyme A, thiamin-diphosphate, lipoic acids or proteins that transfer acyls in lipid biosynthesis – contain sulfur chiefly in sulfide form. However, the source of sulfur for biochemical compounds is the sulfate anion. It is reduced and incorporated into organic substances by plants, bacteria and fungi only.

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