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MICROPROPAGATION

Involvement of polyamines in the adventitious rooting of micropropagated shoots of the apple rootstock MM106

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Abstract Apple rootstock MM106 shoots, raised *in vitro*, rooted at 96.7% after culture on a medium supplemented with an auxin for 5 d in darkness followed by culture on a second medium without growth regulators for 25 d in light. In control conditions (in absence of auxin in the first medium), these shoots did not root. Putrescine (PUT), spermidine (SPD), cyclohexylamine (CHA), and aminoguanidine (AG) enhanced rooting when applied during the first d of culture in the absence of IBA; on the contrary, α-difluoromethylornithine (DFMO) added to the first medium with IBA inhibited rooting. The endogenous levels of indole 3-acetic acid (IAA) and indole 3-acetylaspartic acid (IAAsp) increased up to a maximum concentration at days 2 and 3, respectively, in initial rooting conditions. PUT, when added with IBA, did not affect the typical IAA and IAAsp increase; when applied alone, it provoked an increase of their levels. Similar results were recorded with CHA. SPD, AG, and DFMO did not induce an increase of IAA and IAAsp in nonrooting conditions. The levels of endogenous PUT increased to a maximum at day 2 in rooting conditions; it was slightly affected by exogenous PUT and CHA application but reduced by SPD, AG, and DFMO. In rooting conditions, if the first medium was supplemented with SPD or AG, a small increase in peroxidase activity was observed, similar to that obtained with PUT treatment. The present work indicates an involvement of polyamines in the control of rooting and an interaction with auxins during the physiological phase of rooting. The consequence of this relationship was a different rooting expression, according especially to the content of these regulators in the culture medium.

Keywords Auxin · Inhibitors of polyamine metabolism · Micropropagation · Peroxidase · Physiological phases

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Introduction

Among tree species, rooting remains one of the most critical stages of micropropagation *in vitro* (Kevers et al.. 1997; De Klerk et al. 1999; Liew et al. 1999; De Klerk 2002; Arena et al. 2005). Many authors have demonstrated that the rooting process comprises a series of independent physiological phases, which are associated with changes in peroxidase activity and in endogenous auxin concentration (Berthon et al. 1990; Hausman 1993). If auxin plays a central role in the adventitious rooting process (Blakesley 1994), polyamines are also involved (Biondi et al. 1993; Altamura 1994; Hausman et al. 1994; Heloir et al. 1996).

Polyamines content and expression of polyamine biosynthetic genes were associated with cell divisions and active growth and metabolism (Kusanp et al. 2007, Liu and Moriguchi 2007). Also, the results from the application of exogenous polyamines suggest that endogenous concentration of these polyamines could regulate growth and

development in higher plants (Tang and Newton 2005; Martinez-Pastur et al. 2007). A correlation between polyamine accumulation and the initial stages of adventitious root formation has been observed by several authors (Jarvis et al. 1985; Biondi et al. 1990; Heloir et al. 1996; Neves et al. 2002), suggesting that polyamines could also be used as markers of the rooting process.

The levels of endogenous polyamines, particularly putrescine (PUT), increased when auxin stimulated rooting and meristemoids appeared (Geneve and Kester 1991); the activity of ornithine decarboxylase, a key enzyme in PUT biosynthesis, increased in explants treated with auxin (Torrigiani et al. 1988; Faivre-Rampant et al. 2000; Tassoni et al. 2000; Gemperlova et al. 2005), and the inhibition of polyamine synthesis by specific inhibitors such as DFMO and DFMA inhibits rooting (Hausman et al. 1994).

The present work has been designed to determine the involvement of polyamine on apple rootstock shoots during the induction of the rooting phase *in vitro*. The succession of media used for rooting and the determination of the different phases of the process were previously determined (Naija et al. 2008) on the basis of morphogenesis observations and peroxidase activity variations. Auxin level, peroxidase activity, and polyamine content were monitored in shoots grown in the presence of polyamines or inhibitors of enzymes involved on polyamine metabolism during the first d of the rooting process.

Material and Methods

Plant materials and culture conditions. *In vitro* proliferating cultures of the apple rootstock 'MM106' obtained from the research centre 'Mabrouka' in Mornag (Tunisia) were used as the source material. The proliferation was maintained through subcultures (every 3 wk) on Murashige and Skoog (1962) medium supplemented with 1.8 μM BA (6-benzylaminopurine); 0.6 μM GA_3 (gibberellic acid), and 0.5 μM IBA (indolebutyric acid). The shoot culture was maintained at 24±2°C in the light (16 h photoperiod, 40 μE m^{-2} s^{-1}).

Shoots (40 mm long, six leaves) were isolated and used for rooting experiments as described previously (Naija et al. 2008). In rooting conditions (RC), rooting was induced by culturing the shoots for 5 d in darkness on 1/2-MS medium supplemented with 0.4 mgl^{-1} thiamine and 1 mgl^{-1} IBA before to be transferred in the light for 25 d on the same medium without auxin. The use of two successive media without growth regulator was considered as control and used for tests in non-rooting conditions (NRC). All media were supplemented with 30 g l^{-1} sucrose and adjusted to pH 5.7–5.8 with KOH or HCl prior to the addition of agar (5 g l^{-1}) and autoclaved for 30 min at 110°C. These cultures were done in 500-ml cylindrical glass jars "Le parfait" (Le Parfait, Reims, France; 15 shoots on 100 ml medium) closed with plastic film. Rooting percentage (six independent experiments of 15 shoots) was determined after 25 d on the second medium without auxin. The first roots appeared after 15 d of culture.

Chemical treatments. Shoots were grown 5 d on the first medium (with or without auxin) containing exogenous polyamines or inhibitors of polyamine metabolism. Three inhibitors of polyamine metabolism were used: cyclohexylamine (CHA) which inhibits spermidine (SPD) synthase, aminoguanidine (AG) which inhibits diamine oxidase and α-difluoromethylornithine (DFMO) which blocks PUT formation by inhibiting the activity of ornithine decarboxylase. These compounds were sterilized by filtration (0.22 μm pore size) and added to the first medium after autoclaving. PUT, SPD, CHA, AG, and DFMO were supplied at 10 μmol l^{-1} in all media for 5 d in dark conditions.

The biochemical analyses were performed to the first 6 d of the rooting process because Naija et al. (2008) have previously showed that minimum 5 d on auxin medium (RC) are needed to induce the rooting process.

Extraction and determination of polyamines. Extraction, separation, identification by high performance liquid chromatography (HPLC), and measurement of free polyamines were performed as described by Walter and Geuns (1987).

Whole shoots of frozen plant material (150 mg) stored at −80°C were homogenized in 1 ml of 4% $HClO_4$ containing 1,7-diaminoheptane-2HCl as internal standard. After 1 h in darkness at 4°C, the samples were centrifuged for 10 min at 11,000×*g*. To 100 μl of supernatant, 200 μl of carbonate buffer (1 M, pH 9) and 200 μl of dansyl chloride solution (7 mg ml^{-1} acetone) were added. After heating for 1 h at 60°C, the dansylated polyamines were extracted with 600 μl toluene. The extract was purified by passage through 0.05-g silica gel column and washed with 250 μl toluol and 250 μl toluol triethylamine (10:0.3). Polyamines were eluted with 2×200 μl ethyl acetate and the volume reduced under vacuum. The HPLC analysis was performed with a programmed acetonitrile: water solvent gradient, changing from 58% to 91% over 8 min. The solvent flow was 1 ml min^{-1}. Before injected, the sample was dissolved in methanol (1 ml). The eluate was monitored with a fluorescence detector at 340 nm for excitation and 510 nm for emission. The results are means of data from at least three separate experiments (three replicates each time).

Extraction and determination of auxins. Frozen whole shoots (150 mg) were homogenized in liquid nitrogen and extracted with 4 ml of phosphate buffer (pH 6.5). To the homogenate were added 50 μl butylated hydroxytoluene as antioxidant and 30 μl IAA^3H as internal standard. After 1 h in darkness, the samples were centrifuged (10 min at

Table 1. Effect of addition of polyamines or polyamine metabolism inhibitors in the first medium used in RC and NRC on the rooting percentage

Added compound	Condition	Rooting %
–	RC	96.67±4.46[a]
	NRC	0[e]
PUT	RC	94.44±4.01[a]
	NRC	42.22±6.41[c,d]
SPD	RC	84,44±12[a]
	NRC	26.66±13.49[d]
CHA	RC	92.22±6.23[a]
	NRC	53.33±13.49[b,c]
AG	RC	85.55±7.85[a]
	NRC	26.66±13.39[d]
DFMO	RC	63.33±13.30[b]
	NRC	0[e]

Values followed by different *letters* are significantly different (P=0.05)

14,000×*g* at 4°C) and the supernatants were filtered with the Whatman GF/C filter. The filter was washed with 4 ml of phosphate buffer (pH 6.5). The filtrates were loaded on Bond-Elut C18 columns conditioned to pH 6.5.

The eluates were acidified to pH 2.5 with 2.8 M phosphoric acid and then applied to C18 columns conditioned to pH 2.5. The columns were washed with 2 ml distilled water and 2 ml of acetic ethanol (ethanol/acetic acid/water, 20:2:78). The auxins were eluted from the second column with 100 µl of methanol (100%) and 500 µl of methanol (80%). Fifty microliters were injected in a fully automated Merck-Hitachi HPLC system. The HPLC column was a Merck Lichrocart 100RP18, 12.5 cm long, 5 µm particle size; solvent and column temperatures were 30°C; and the mobile phase was acetonitrile/acetic acid/water (10:2:88). The eluate was monitored with a fluorescence detector (excitation at 292 nm; emission at 358 nm); the elution patterns were similar to those shown by Nordstrom and Eliasson (1991) and Nordstrom et al. (1991).

Peroxidase activity. Frozen whole shoots (150 mg) stored at −80°C were ground in 1 ml of phosphate buffer (pH 7) and centrifuged for 20 min at 12,000×*g* at 4°C. The supernatants were used as crude enzyme extracts. Peroxidase assays were performed as described by Moncousin and Gaspar (1983). Activity was expressed in ΔDO min^{-1} mg^{-1} Prot. Protein concentration was determined by the Coomassie blue method (Spector, 1978) with bovine serum albumin as standard.

Each sample analysis was performed in triplicate. All results presented are the means (±SE) of at least three independent experiments. Statistical analysis (analysis of variance with statistical significance level fixed at $P \leq 0.05$) was carried out using Microsoft Excel (Microsoft, Roselle, IL).

Results

Effect of polyamines and inhibitors of their metabolism on shoot rooting. In control conditions (without auxin treatment, NRC), shoots of apple rootstock MM106 did not root at all. In RC (5 d in presence of auxin), shoots rooted at 96.7% after 25 d on the second medium (Table 1).

Rooting percentage was not significantly modified when PUT, SPD, CHA, or AG were added in the presence of auxin (RC). Only the application of DFMO decreased rooting percentage when added in the presence of IBA. In contrast, PUT, SPD, CHA, and AG increased rooting percentage when applied in NRC during the first d (first medium without auxin) of the rooting process.

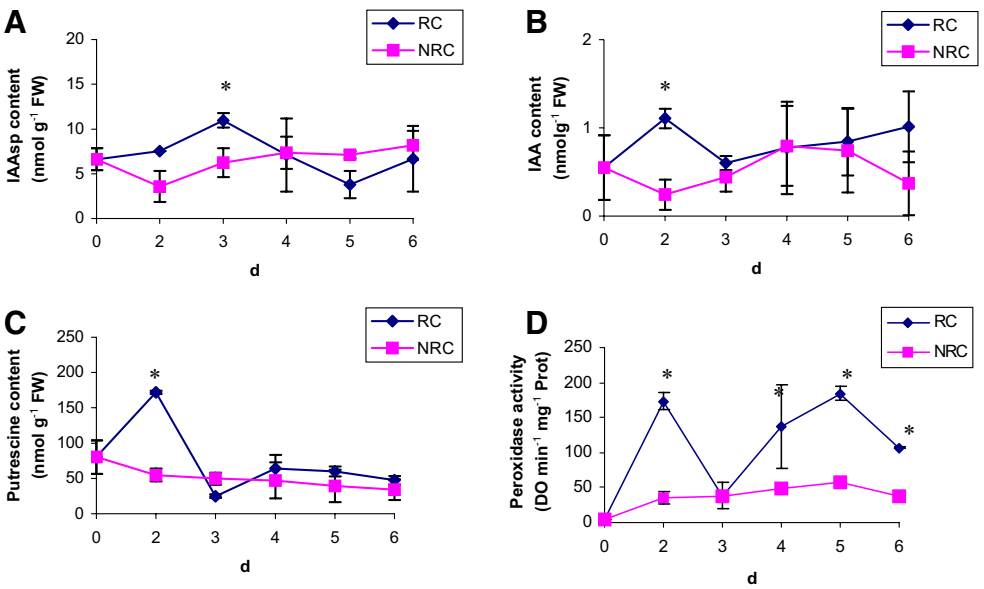

Figure 1. Changes in endogenous IAAsp (*A*), IAA (*B*), free PUT (*C*), and peroxidase activity (*D*) in RC (*filled diamond*) and NRC (*gray square*). Values significantly different from time 0 are indicated by an *asterisk* (P=0.05).

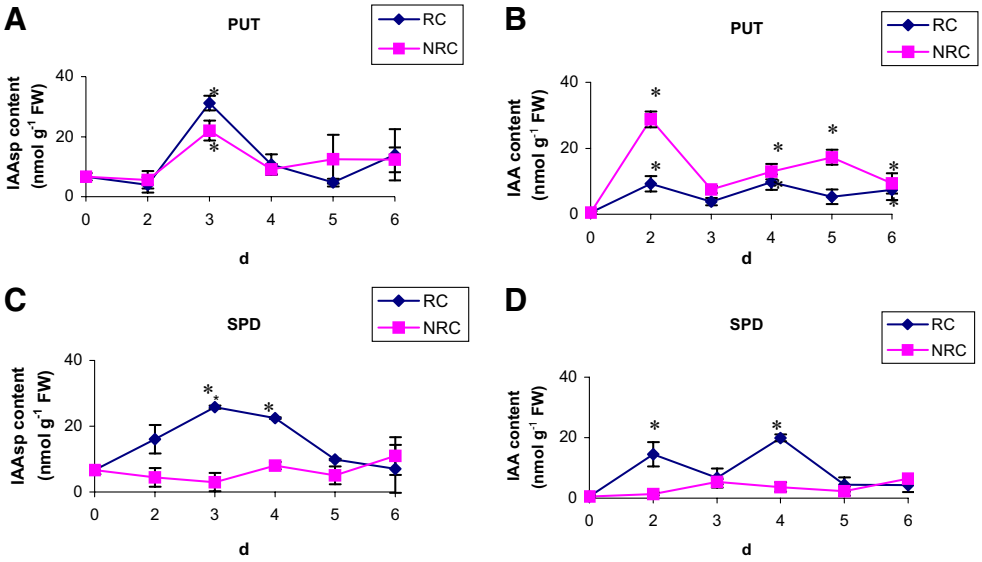

Figure 2. Changes in endogenous concentrations of AIAsp (*A*, *C*) and AIA (*B*, *D*) in RC (*filled diamond*) and NRC (*gray square*) when the first medium was supplemented with PUT (*A*, *B*) or SPD (*C*, *D*). Values significantly different from time 0 are indicated by an *asterisk* (P=0.05).

Changes in endogenous auxins, polyamines, and peroxidase activity during the first d of rooting process. No significant variations of auxin and PUT level and of peroxidase activity were observed in shoots grown in control conditions (NRC) without auxin (Fig. 1).

The IAAsp (indole 3-acetylaspartic acid) level of the shoots kept for 5 d in the presence of auxin in RC increased up to day 3 and then decreased (Fig. 1*A*). The IAA (indole 3-acetic acid) level showed a similar profile, but the maximum level was on day 2 (Fig. 1*B*). The shoot grown in RC showed an increase in PUT on day 2 followed by a minimum on day 3 (Fig. 1*C*). In these RC, after an increase on day 2, peroxidase activity decreased to day 3 and then increased rapidly to day 5 (Fig. 1*D*).

Effects of exogenous polyamines on endogenous changes in auxins, polyamines, and peroxidase activity. PUT incorporated in the first medium of RC did not affect the IAAsp content which reached its maximum at day 3 (Fig. 2*A*). Such a peak at day 3 was observed in shoots kept in NRC when the first medium was supplemented with PUT. The peaks of IAA were observed at day 2 in RC and NRC (Fig. 2*B*), higher on NRC.

In RC, when the first medium was supplemented with SPD, the IAAsp level was highest on day 3. In the absence of auxin (NRC), no significant variation of IAAsp level was detected (Fig. 2*C*). The IAA level of the shoots showed a peak at days 2 and 4 in RC while in NRC, no significant variation was shown (Fig. 2*D*).

PUT content in shoots grown in RC with the first medium supplemented with PUT increased during the first 2 d, then decreased and increased again at day 4. In shoots grown on the first medium of the NRC supplemented with PUT, an increase in this compound was also observed on day 2 (Fig. 3*A*).

On media supplemented with PUT, the SPD content of the shoots did not change significantly (data not shown).

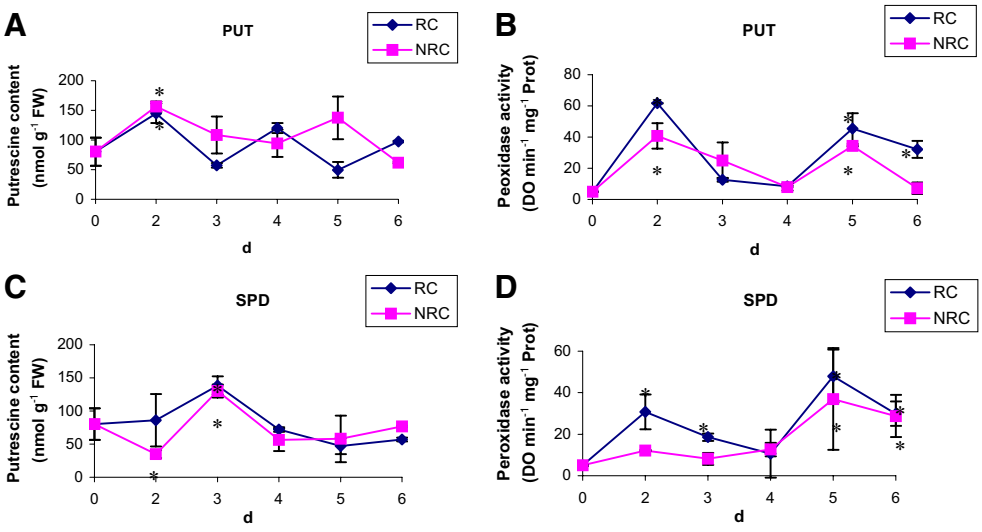

Figure 3. Changes in endogenous concentrations of PUT (*A*, *C*) and peroxidase activity (*B*, *D*) of shoots grown in RC (*filled diamond*) and NRC (*gray square*) when the first medium was supplemented with PUT (*A*, *B*) or SPD (*C*, *D*). Values significantly different from time 0 are indicated by an *asterisk* (P=0.05).

In the RC and NRC, when the first medium was supplemented with SPD, endogenous PUT level increased to a maximum on day 3 and decreased (Fig. 3*C*). No variation of the SPD content in shoots grown on media supplemented with SPD was observed (data not shown).

PUT incorporated in RC induced a first increase of peroxidase activity on day 2 followed by a decrease up to day 4. A similar profile was observed in shoots grown in NRC supplemented with PUT (Fig. 3*B*).

A small increase in peroxidase activity was measured on day 2 in shoots in RC with the first medium supplemented with SPD. The activity decreased to reach a minimum on day 4 and another maximum on day 5. In the NRC with the first medium supplemented with SPD, no variation in peroxidase activity was observed (Fig. 3*D*) during the first 4 d, but it increased later with a maximum on day 5.

Effects of inhibitors of polyamine metabolism on endogenous changes in auxins, polyamines, and peroxidase activity. When both of the first media used in RC and NRC were supplemented with CHA, maxima of IAAsp content were observed, respectively, on day 3 (as in RC without CHA) and day 2 (Fig. 4*A*). On the same media, maxima of IAA levels were observed on day 2 (Fig. 4*B*), as observed for IAA without CHA. In RC and NRC supplemented with AG, no significant variation of IAAsp was recorded (Fig. 4*C*), while a maximum of IAA was measured on day 2 (Fig. 4*D*) as in RC without inhibitors. In the RC and NRC supplemented with DFMO, no variation of IAAsp and IAA levels was detected (Fig. 4*E*, *F*) during the first 5 d.

PUT content in shoots grown in RC when the first medium was supplemented with CHA showed an increase with a maximum on day 2 before a decrease, as without inhibitors. In NRC, supplemented with CHA, only a decrease of PUT content was observed after day 2 (Fig. 5*A*). In the two conditions, PUT content increased again on day 6.

When both of the first media used in RC and NRC were supplemented with AG, PUT level in shoots showed no significant variation (Fig. 5*C*). The SPD content in the shoots in the shoots in RC, with AG showed an increase on day 2 and a decrease for the next 2 d (data not shown).

In RC with DFMO, no variation of PUT and SPD (data not shown) levels in shoots was observed (Fig. 5*E*).

After the addition of CHA in the first medium in RC, peroxidase activity rose on day 2, dropped on day 3, and rose again on day 4. In NRC with medium supplemented with CHA (Fig. 5*B*), a maximum was observed on day 2 followed by a slow decrease.

In RC, with a first medium supplemented with AG, a small increase in peroxidase activity was observed on day 2 followed by a decrease on day 3; the variations were smaller than in without this inhibitor. In NRC supplemented with AG, the peroxidase activity increase was more

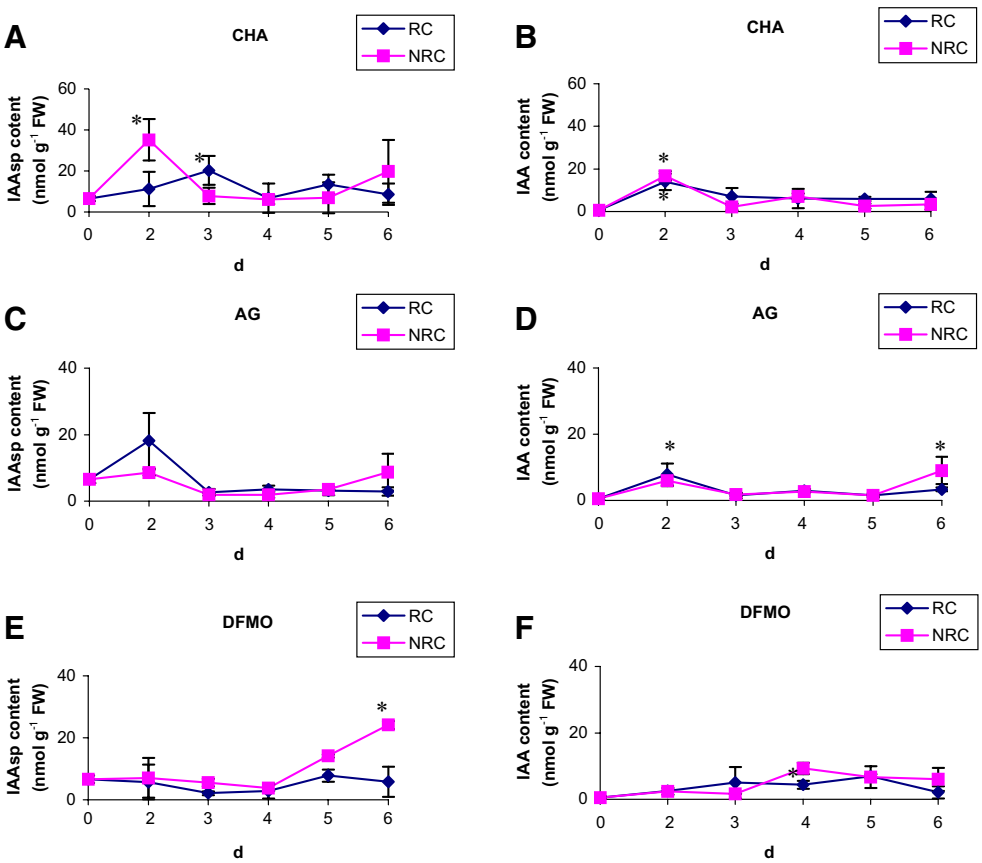

Figure 4. Changes in endogenous concentrations of IAAsp (*A*, *C*, *E*) and IAA (*B*, *D*, *F*) of shoots grown in RC (*filled diamond*) and NRC (*gray square*) when the first medium was supplemented with CHA (*A*, *B*), AG (*C*, *D*), or DFMO (*E*, *F*). Values significantly different from time 0 are indicated by an *asterisk* (P=0.05).

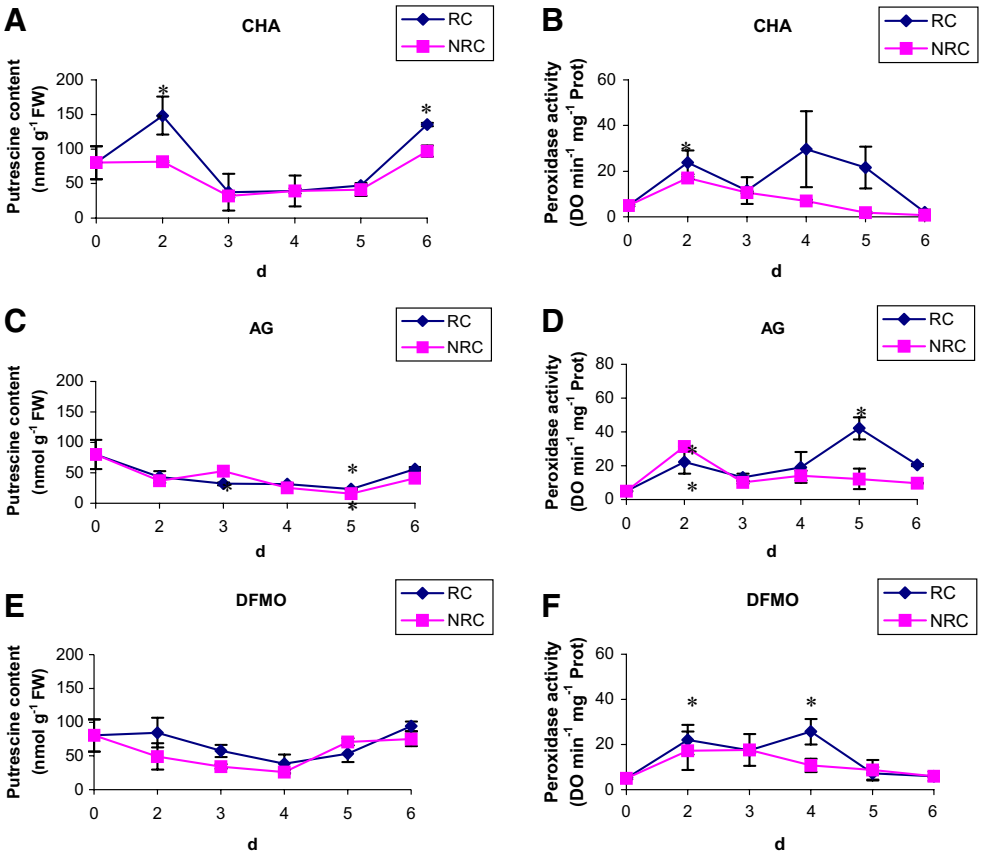

Figure 5. Changes in endogenous concentrations of PUT (*A*, *C*, *E*) and peroxidase activity (*B*, *D*, *F*) of shoots grown in RC (*filled diamond*) and NRC (*gray square*) when the first medium was supplemented with CHA (*A*, *B*), AG (*C*, *D*), or DFMO (*E*, *F*). Values significantly different from time 0 are indicated by an *asterisk* (P=0.05).

important on day 2 followed by a decrease and a further stabilization of this activity (Fig. 5*D*).Without this inhibitor, no variation was observed.

In RC and NRC, when the first medium was supplemented with DFMO, no significant variation of peroxidase activity was detected (Fig. 5*F*), as in control conditions NRC without inhibitors.

Discussion

The results indicated a typical increase–decrease variation in free IAA concentration during the first 5 d that correspond to the induction of rooting; this variation is similar to that observed in other rooting materials (Maldiney et al. 1986; Blakesley et al. 1991; Gaspar et al. 1992, 1994; Blakesley 1994). Moncousin et al. (1988), Gaspar et al. (1990), and Noiton et al. (1992) have shown that this transitory IAA peak corresponded to the end of the rooting inductive phase.

The application of exogenous IBA affected the concentration of endogenous IAA. Exogenous IBA was probably not directly converted to IAA but more probably enhanced IAA biosynthesis (Epstein and Lavee 1984; Wiesman et al. 1988, 1989).

The decreased IAA level might be interpreted through a conversion of IAA into IAAsp. This hypothesis agreed with the ideas that IAAsp formation was the first step of IAA storage (Hausman et al. 1995a). For some other authors, the transient peak of IAA might also be a result of conversion of IAAsp to IAA (Bandurski et al. 1995; Heloir et al. 1996; Stefancic et al. 2007).

A decrease in peroxidase activity characterized the rooting induction phase of some plant tissue (Gaspar et al. 1992, 1994; Hausman et al. 1995b; Metaxas et al. 2004). In our material, the decrease in peroxidase activity corresponded with the transient peak in IAA concentration as it was already observed in other materials (Gaspar et al. 1994; Ripetti et al. 1994). The determination of polyamine content in the shoots during the rooting process in RC showed a peak in the concentration of PUT followed by a decrease. Similar findings have been reported for other plant materials (Friedman et al. 1982; Jarvis et al. 1985; Tiburcio et al. 1989, Biondi et al. 1990; Altamura et al. 1991; Hausman et al. 1994; Tonon et al. 2001) in the early inductive phase of the rooting process.

Our results also showed that PUT promoted rooting in the absence of auxin to 42% (Table 1). These results confirmed those of Altamura et al. (1991), Hausman et al. (1994, 1995a), and Kevers et al. (1997) who showed that exogenous application of PUT can stimulate root organogenesis.

Different inhibitors of PUT biosynthesis inhibited rooting in the presence of auxin (Jarvis et al. 1983; Biondi et al. 1990;

Rugini 1992). Addition of CHA in NRC (without IBA) during the period of rooting induction increased the rooting percentages (Table 1); this result can be explained by the specific inhibition of SPD synthase which converts PUT into SPD and so promoted the accumulation of PUT as shown by Hausman et al. (1994), Kevers et al. (1997), Faivre-Rampant et al. (2000), and Tonon et al. (2001). In RC, application of CHA did not modify the rooting percentage.

AG, an inhibitor of diamine oxidase which converts PUT into Δ^1-pyrroline, induced rooting in NRC, thus, in absence of auxin. In fact, it caused accumulation of SPD that can be converted to γ-aminobutyric acid through a polyamine oxidase. The activity of this enzyme is as essential as that diamine oxidase for root vascularization (Aribaud et al. 1998).

DFMO treatment affected rooting percentage in the presence of auxin, suggesting that polyamines were involved in the rooting process as observed by Kevers et al. (1997), Martin-Tanguy et al. (1997), and Tonon et al. (2001).

The application of PUT during the root induction phase resulted in an increase of endogenous PUT, endogenous auxins, and peroxidase activity. These observations provide evidence for the hypothesis that PUT played a role in the rooting induction phase (Friedman et al. 1982; Tiburcio et al. 1989; Altamura et al. 1991; Hausman et al. 1995a, b).

This polyamine was able to induce the endogenous variations of auxins, PUT, and peroxidase activity as observed in the presence of auxin. This was a proof that auxin and polyamine were active factors in the rooting process. As in poplar cuttings (Hausman et al. 1994), rooting depended on a concomitant variation in the levels of both regulators (Faivre-Rampant et al. 2000). The main difference consisted in the timing: some h in poplar (Hausman et al. 1994) to some d in our material.

A marked effect of CHA was an increase in concentration of endogenous auxins, PUT, and peroxidase activity during the induction of rooting. The accumulation of PUT resulted from the inhibition of its conversion to SPD by this inhibitor of SPD synthase, as described for other species (Hausman et al. 1995a, b; Neves et al. 2002). Moreover, the stimulation of *in vitro* rooting of poplar shoots in the presence of CHA was associated with the accumulation of γ-aminobutyric acid generated by polyamine catabolism (Hausman et al. 1997). Unlike cork oak and *Fraxinus angustifolia*, apple rootstock MM106 treated with CHA accumulated PUT but with a decrease in rooting rates (Tonon et al. 2001; Neves et al. 2002). By contrast, poplar and grapevine shoots treated with CHA showed a positive relation between PUT accumulation and adventitious rooting (Hausman et al. 1994).

An inverse relationship between peroxidase activity and concentration of endogenous PUT was observed when AG was added during rooting induction; these results might be associated to the hydrogen peroxide formation during the action of the PUT catabolism (Hausman et al. 1995a; Tonon et al. 2001; Guoxing et al. 2005).

All these results confirm the stimulatory effect of the application of exogenous PUT or CHA on rooting, allowing the accumulation of free PUT and the inhibitory effect of AG, preventing this accumulation of endogenous PUT.

In *Virginia pine*, Tang and Newton (2005) demonstrated that diamine activities inhibit root formation. Faivre-Rampant et al. (2000) and Couée et al. (2004) showed an increased level of ornithine and arginine decarboxilase activities and indicated that arginine decarboxylase was the major one in increasing PUT synthesis in roots. The involvement of genes related to polyamine synthesis and the role of polyamines in root development have been established (Couée et al. 2004).

The present work confirmed the implication of polyamines in the control of rooting of apple rootstock shoots. There is an interaction between auxins and polyamines during the physiological phase of rooting induction and the consequence of this relationship was a different rooting expression, according to the content of these regulators in the culture medium.

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