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Biochemical aspects of almond microcuttings related to *in vitro* rooting ability

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Abstract

Microcuttings of seven genotypes of almond (*Prunus dulcis* Mill) used as peach rootstocks, showed different rooting ability *in vitro*. No direct relationship was found between peroxidase activity and total phenol content, determined in the whole microcuttings prior to the root inducing treatment, and the rooting ability of these genotypes. However, a positive relationship was found between free indole-3-acetic acid (IAA) level and IAA-aspartate and the rooting response. After transferring into the rooting medium, peroxidase activity of the easiest-to-root genotype increase up to a peak and then declined and the total phenol content showed an opposite trend. These variations did not occur in the most difficult-to-root genotype.

Additional key words: endogenous IAA, IAA-aspartate, peroxidase, phenols, *Prunus dulcis*, rhizogenesis.

Introduction

Rooting initiation involves cell division of induced cells followed by the formation of a root meristem. Cells competent for root formation are able to respond directly to an inducing stimulus by the formation of the root meristem. The shift in the new developmental fate of the cells involves complex interacting changes at biochemical and physiological levels.

There is substantial evidence that auxins play a crucial role in the formation of adventitious roots and positive correlations between endogenous IAA levels in cuttings and rooting ability have been reported (Weigel *et al.* 1984, Alvarez *et al.* 1989). However, auxins are not the sole determinant in adventitious rooting, peroxidases and phenols play also an important role. Peroxidases are reported to be

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involved in the adventitious root formation of some species where it appeared that rooting occurred after the cuttings had reached and passed a peak of activity (Gaspar *et al.* 1992). Changes of these enzymes have been found to correspond to parallel changes in IAA-oxidase activity (Mato and Vieitez 1986), thus, a major role of peroxidases in the IAA metabolism has been proposed. The stimulating action of phenolic compounds on rhizogenesis has been described by several authors (Jones and Hatfield 1976, Zimmermann and Broome 1981), but their effect is influenced by the natural phenolic content of the cuttings. It could be presumed that phenols would not act directly in rooting induction but by modulation of IAA-oxidase activity; in fact, an inverse variation of total peroxidase activity and phenolic compound has also been described during the rooting process (Moncousin 1986, Gaspar *et al.* 1992).

All these interacting changes of endogenous factors during the rooting process have been observed in several species, including woody species, but only few information is recently available on almond (Caboni 1994). In this work we studied relationships between *in vitro* rooting ability and peroxidase activity, total phenol content and IAA endogenous levels in some almond rootstocks.

Materials and methods

Plant material and shoot proliferation conditions: *In vitro* cultures of some genotypes of almond (*Prunus dulcis* Mill.), M49, M50, M51, M52, M53, M54, M55, under evaluation as peach rootstock in the "Istituto Sperimentale per la Frutticoltura" of Rome, have been used. The axillary shoots of all the genotypes, originated for all the genotypes from buds of twigs in the same physiological state, collected from 6-year-old mother-plant, were maintained in 2-week subculture cycle for 2 years on a multiplication medium, consisting of Murashige and Skoog (1962) (MS) salts, as previously described (Caboni and Damiano 1994). Shoots were subcultured every 15 d. Cultures were maintained at 16-h photoperiod, irradiance of 30 µmol m^{-2} s^{-1} (*Osram 140* white fluorescent tubes) and temperature of 21 °C.

Rooting conditions: Microcuttings, 1.5 to 2.0 cm in length, were excised from 15-d-old cultures and transferred into a rooting medium consisting of MS salts containing 30 g dm^{-3} saccharose, supplemented with indole-3-butyric acid (IBA) (5 µM) and solidified with 7 g dm^{-3} *Difco Bacto* agar. The pH was adjusted to 5.6, prior autoclaving. Microcuttings were maintained for 5 d in dark, then transferred to the light. Growth chamber conditions were the same as reported for the multiplication phase.

The rooting percentage and the average number of roots per rooted shoot were recorded after 30 d in the rooting medium. Triplicate treatments consisting of 10 plants each were used. The experiment was repeated twice.

Peroxidase activity: Whole shoots (250 mg of fresh mass) were quickly frozen in liquid nitrogen, ground in 1 cm^3 phosphate buffer (0.06 M, pH 6.1) with 100 mg of

polyvinylpyrrolidone and centrifuged for 10 min at 11 000 *g* at 4 °C to extract total soluble peroxidase. On the supernatant, the crude extract, were performed the peroxidase assays according to Moncousin and Gaspar (1983). Enzyme activity was expressed in units of enzyme (*U = the enzyme amount catalyzing the oxidation of* 1 μM of substrate min^{-1}) mg^{-1}(protein). Proteins were measured by the method of Bradford (1976). Duplicate treatments, consisting of 10 explants each, were used. Spectrophotometrical determinations were repeated twice, with similar results. Samples were taken from 15-d-old cultures of all the genotypes, just before applying the root inducing treatment, or each day till the 4th day after the transplanting in the rooting medium in one easy and one difficult to-root-genotype.

Phenolic compounds: Total phenolic compounds were extracted from 150 mg of whole shoots by Legrand's method (1977) and assayed with the Folin-Ciocalteu reagent following Bray and Thorpe (1954). Samples were taken at the times reported above for peroxidases. Triplicate treatments, consisting of 10 explant each, were used and spectrophotometrical determinations were repeated twice, with similar results. Total phenol content was expressed as μg mg^{-1} *of fresh mass (f.m.).*

IAA determination: Free indole-3-acetic acid (IAA) and IAA-aspartate were determined on samples of basal part of shoot stems (cut at the 3rd node from the base), *taken from* 15-d-old cultures, which were quickly frozen in liquid nitrogen and ground to a fine powder. The resultant powder was extracted in K-phosphate buffer (pH 6.5) and purified by solid-liquid extraction according to Nordstrom and Eliasson (1991).

Samples were analyzed on reversed-phase on HPLC (*Waters 600,* Milford, USA) coupled to a *Waters 470* fluorescence detector (excitation 280 nm, emission 360 nm) and to a *Waters 746* integrator. The column was a *Water Resolve C18*, 15 cm long and with 5 μm particle size. The mobile phase was 8 % acetonitrile, 1 % acetic acid and water with a flow rate of 0.5 cm^2 min^{-1}. The system was operated *isocratically. Identification of free IAA and IAA-aspartate peaks was confirmed* adding commercial IAA or IAA-aspartate (*Sigma,* St. Louis, USA) to a part of the sample. Duplicate treatments, consisting of 30 explants each, were used. IAA quantitative determinations at HPLC were repeated twice, with similar results.

Results

The seven almond genotypes showed very different rooting ability (Table 1): while 75 % of rooting (the highest percentage among the genotypes) was induced by IBA treatment in M51, only 5 % of the microcuttings of M50 produced adventitious roots. Furthermore, M51 produced also significantly more roots per rooted explant than the other genotypes which showed intermediate morphogenetic capacity (Table 1).

Total peroxidase activity as well as total phenolic compound contents were different in the samples of the seven clones collected just before the application of

the root inducing treatment: the highest peroxidase activity was showed in M51 and M55 and the lowest in M54; the highest phenol content was found in M52 and the lowest values were determined in M50 and M51 (Table 1).

Table 1. Peroxidase activity and total phenol content in almond genotypes at time of transferring into the rooting medium and rooting percentage and number of roots per rooted explant treated with 5 μM IBA for 5 d in dark. Values are the means of two independent experiments (each performed with 3 replicates) ± SE.

Genotype	Peroxidase [U mg^{-1}(protein)]	Phenols [mg g^{-1}(f.m.)]	Rooting [%]	Number of roots [$explant^{-1}$]
M49	3.04 ± 0.11	0.47 ± 0.01	35 ± 2.5	2.1 ± 0.2
M50	2.75 ± 0.19	0.33 ± 0.02	5 ± 0.3	1.2 ± 0.1
M51	3.20 ± 0.05	0.31 ± 0.01	75 ± 3.0	3.6 ± 0.2
M52	2.59 ± 0.11	0.54 ± 0.03	40 ± 2.1	2.0 ± 0.2
M53	2.42 ± 0.06	0.39 ± 0.02	65 ± 3.6	3.1 ± 0.1
M54	2.32 ± 0.10	0.40 ± 0.03	60 ± 3.7	3.0 ± 0.3
M55	3.19 ± 0.10	0.41 ± 0.03	30 ± 1.2	3.0 ± 0.2

Peroxidase activity, measured daily for the first 4 d during the IBA treatment, slightly decreased after one day, then steeply increased after 3 d, then decreased in the easy-to-root genotype (M51); in M50, the difficult-to-root one, only a very slight increase of activity was detected after 3 d in the rooting medium (Fig.1). Total phenol content increased after 1 d, then decreased till day 3 and afterwards again

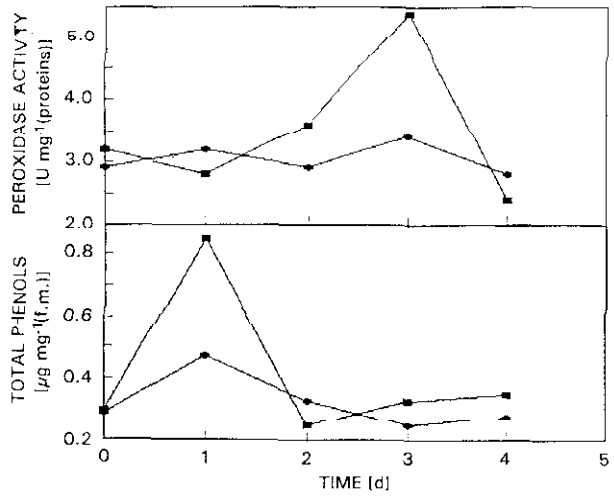

Fig. 1. Peroxidase activity (*above*) and total phenol content (*below*) in microcuttings of M51 (*rectangles*) and M50 (*rhombs*) during the first 4 d in the rooting medium. SE was lower than 10 % for all the means.

increased slightly at day 4 in M51; in M50, only poorly increased after one day (Fig. 1).

Contents of endogenous free IAA and IAA aspartate were significantly higher in the genotype M51, which showed the highest rooting ability, than in M50 which rooted very poorly (Table 2).

Table 2. Content of endogenous free IAA and IAA-aspartate in M51 and M50 almond genotypes, measured in basal part of stem of 15-d-old cultures before the rooting treatment. SE was lower than 10 % for all the means.

Genotype	Free IAA [ng g^{-1}(f.m.)]	IAA-aspartate [ng g^{-1}(f.m.)]
M50	19.3	82.0
M51	107.7	438.5

Discussion

Previous works showed that different genotypes of almond need different inducing treatments to show a satisfactory rooting response (Rugini and Verma 1983, Caboni and Damiano 1988). In our study we found very different rooting percentage in the seven almond genotypes applying the same treatment (IBA, 5 μM and 5 d of dark). These data confirmed that rooting is strictly genotype related in almond.

Peroxidases have been shown to be involved in the IAA catabolism and in the rooting process. In some cases they have been used as predictor of the rooting performance of cuttings for propagation (Quoirin *et al.* 1974). Peroxidase activity of microcuttings of many species, when transferred to a rooting inducing medium, underwent a typical curve with an early transient minimum followed by an increase up to a peak and then a decline (Gaspar *et al.* 1992). This variation normally corresponds to an inverse trend of the level of total phenol compounds which, presumably modulate peroxidase activity (Gaspar *et al.* 1992). In our studies on almond microcuttings no direct relationship was found between rooting ability of each genotype and peroxidase activity or total phenol contents, measured on *in vitro* grown explants just before the transferring to the rooting medium. Otherwise, measuring the enzyme activity during the first 4 d of inducing treatment in the easiest-to-root genotype (M51) and the most difficult-to-root one (M50) it was possible to show that in M51 the peroxidase activity variations follow the curve above mentioned. It could be presumed that this variation correspond to opposite changes in free IAA endogenous level and this aspect is now being investigated. Furthermore, in this genotype it was also found that the total phenol content changed inversely to the peroxidase activity which suggests that phenols act modulating activity of the enzyme also in the root induction phase of almond as reported for other species (Moncousin 1986). Thus, our data show that interacting changes of total phenols and peroxidases during the first day of the rooting process

are involved in the rooting process in almond and that these variations are critical for obtaining a satisfactory rooting response.

Previous researchers have investigated the rooting performance of genotypes in some woody species in relation with the constitutive IAA endogenous levels (Le 1985, Foret *et al.* 1986). Findings of Alvarez *et al.* (1989) suggested that differences in *vitro* rooting ability of 2 apple rootstocks are related to differences in free IAA levels in basal section: the higher the free endogenous level, the higher the rooting response. An inverse relationship was found by the same authors for IAA-aspartate endogenous levels of basal part of microcuttings. There is no available information on IAA levels in tissue cultured almond. We measured the free IAA and IAA-aspartate endogenous level in the basal part of one difficult-root almond genotype (M50) and in a very easy-to-root one (M51). The level of free IAA was significantly higher in M51 than in M50. These data support Alvarez *et al.* (1989) findings on the direct relationship of free endogenous levels of basal part of microcuttings and rooting ability. However, we found also higher IAA-aspartate endogenous level in M51 then in M50. Thus, we can hypothesize that in almond root formation is related to the level of the free IAA but that also constitutive higher level of the IAA-aspartate is present in the easy-to-root genotypes perhaps due to a higher capacity of these genotypes to synthesize IAA.

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