Changes in peroxidases and catalase activity during *in vitro* rooting

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Abstract

Enzyme changes in non-rooted (treated with Fe-EDTA) and rooted (treated with Fe-EDDHA) stems of rootstock GF-677 (*Prunus amygdalus* × *P. persica*) during adventitious root formation *in vitro* have been recorded. The first roots appeared approximately after 12 d on the rooting medium. By contrast to non-rooted stems, rooted stems showed a maximum of soluble peroxidase activity on the 9th day, of ionically bound peroxidase to cell wall on the 6th and 12th day and of catalase on the 6th and the 15th day. A time course study of changes of soluble peroxidases isoenzymes showed that there was a band visible only in the rooted stems and also a new band appeared three days before the emergence of roots.

Additional key words: GF-677 (*Prunus amygdalus* × *P. persica*), iron.

Introduction

Peroxidases (H_2O_2 oxidoreductase; EC 1.11.1.7) are found both in the cell wall and in the cytoplasm and catalyse the oxidation of diverse hydrogen donors and peroxidase activity is implicated in many biological events in plants, depending upon the nature of the donor. According to some authors there was no relationship between peroxidase activity and rooting process (Pythoud and Buchala 1989, Dalet and Cornu 1989). However, other authors state that peroxidase plays an important role in the regulation of auxin content during the rooting of cuttings (Garcia-Gómez *et al.* 1995). Peroxidase activity and its isoenzymatic pattern has been studied in relation to IAA-oxidase catabolism, and also as a marker of the successive rooting phases (Gaspar *et al.* 1994, Saxena *et al.* 2000). Catalase (EC 1.11.1.6) is present in the peroxisomes of nearly all aerobic cells and facilitates the breaking down of H_2O_2 to water and O_2 and its role in the rooting process has not been fully studied, so far.

Adventitious root formation consists of three basic processes: cell division, cell enlargement, and cell differentiation. DNA synthesis is a prerequisite and protein synthesis is a common event before cell division (Molnar and LaCroix 1972).

The aim of this paper was to study the changes in catalase, soluble and ionically bound to cell wall peroxidase and their isoenzymes patterns, during the period of initiation and development of roots on stems of GF-677 (*Prunus amygdalus* × *P. persica)* as affected by iron forms and concentrations.

Materials and methods

The experiment was conducted twice using GF-677 (*Prunus amygdalus* × *P. persica* L.) rootstock explants and the reported data are the mean of two consecutive experiments. The explants were shoot tips (15 mm in length) preserved from previous *in vitro* cultures and maintained in a growth chamber. Each explant was transferred aseptically onto 10 cm^3 Murashige and Skoog (1962; MS) medium in 25 × 100 mm test tubes. The MS nutrient medium was supplemented with 30 g dm^{-3} sucrose, 7 g dm^{-3} agar, 0.6 mg dm^{-3} benzyladenine (BA), 0.2 mg dm^{-3} gibberellic acid (GA_3) and 0.05 mg dm^{-3} 3-indolebutyric acid (IBA) and contained three types of

Received 17 January 2003, *accepted* 8 August 2003.
Abbreviations: DMAB - 3-dimethylaminobenzoic acid; EDDHA - ethylenediamine-di(*o*-hydroxyphenyl)acetic acid; EDTA - ethylenediaminetetraacetic acid; MBTH - 3 methyl-2-benzothiazolinonhydrazon hydrochloride hydrate; MS medium - Murashige and Skoog medium; PAGE - polyacrylamide gel electrophoresis; PVPP - polyvinylpolypyrrolidone.
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iron compounds ($FeCl_3$, Fe-EDTA, Fe-EDDHA). Each form was supplied at three concentrations (0.05, 0.1 and 0.2 mM Fe), while the control was MS medium without iron (-Fe). Their pH was adjusted to 5.2 before autoclaving at 121 °C for 15 min. In each treatment, 25 replicates (tubes) were included, arranged randomly in the growth chamber, and maintained at temperature of 22 ± 1 °C and 16-h photoperiod with irradiance of 45 μmol m^{-2} s^{-1} (*Philips TLD 54/36W* fluorescence tubes), for a total period of 24 d.

The stems were harvested at 0, 3, 6, 9, 12, 15, 18, 21 and 24 d, after the establishment of the culture. The stems were homogenized in a cold pestle with a mortar in a sixth-fold volume of 10 mM Na-phosphate buffer (pH 6.5) containing 2.0 % (m/v) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 15 000 *g* for 30 min at 4 °C and the supernatant was used as crude enzyme extract for determination of catalase and soluble peroxidase activity. The pellet obtained was resuspended in the same buffer and centrifuged (15 000 *g*, 30 min, 4 °C). The new pellet obtained was resuspended in the extraction buffer containing 1 M NaCl and shaken overnight at 4 °C. After centrifugation (15 000 *g*, 30 min, 4 °C) the new supernatant contained the ionically bound to cell wall peroxidase.

Catalase activity was measured according to the floating disc method (Wang 1995). Aliquots of crude enzyme extract were applied on glass fibre discs, 1 cm in diameter (*Schleicher and Schuell*) and then placed at the bottom of a *LaMotte* test tube (*Code 0829*, USA) following the addition of 1.5 cm^3 of 2.25 % H_2O_2 and 18.5 cm^3 of deionised water, at 20 °C. The elapsed time for the disc to float was recorded. The activity of catalase in the crude extract was quantified based on the activity of bovine liver catalase (*Sigma*). One unit of CAT activity (U) was defined as the decomposition of 1 μmol H_2O_2 per min at pH 7.0 and 25 °C.

Peroxidase activity (soluble and ionically bound to cell wall) was determined according to Ngo and Lenhoff (1980). Adequate amounts of crude enzyme extract were applied in 3 cm^3 Na-phosphate buffer 0.1 M (pH 6.5) containing 0.1 M NaH_2PO_4, 0.2 mM 3 methyl-2-benzothiazolinonhydrazon hydrochloride hydrate (MBTH), 10 mM 3-dimethylamino benzoic acid (DMAB) and of 0.3 mM H_2O_2. Absorbance changes were recorded at 590 nm with a *Shimadzu UV-1601* spectrophotometer (*Shimadzu*, Kyoto, Japan) at room temperature (25 ± 2 °C). One unit of peroxidase activity (U) was defined as the increase of one unit of absorbance per minute, under the assay conditions and the enzymatic activity was referred to fresh mass (U g^{-1}(dm). Results were analysed by analysis of variance and the standard error of the means was also calculated.

Electrophoresis was performed using non-denaturing native polyacrylamide gel electrophoresis (PAGE) with a mini *Protean II cell* (*Bio-Rad*, Hercules, CA, USA) through a 7.5 % polyacrylamide gel according to Laemmli (1970). The electrode buffer was 0.025 M Tris/0.19 M glycine (pH 8.3). The same amount of peroxidase units was loaded in each lane. Electrophoresis was performed at 75 V until the samples entered the separating gel (about 20 min) and at 125 V for 1 h, thereafter. Peroxidase active bands were revealed by immersing the gels in a freshly prepared solution consisting of 100 mM Na-phosphate (pH 6.5), 5.55 mM H_2O_2 and 0.1 % *o*-dianisidine, until all the peroxidase bands were visible (20 - 30 min).

Results and discussion

Full rooting (100 %) was observed in stems in medium with Fe-EDDHA, while lower rooting percentages were obtained in the absence of iron or in the presence of $FeCl_3$. On the contrary, no root formation was observed in plantlets in medium with Fe-EDTA (data not presented). The first roots in rooted stems appeared approximately after 12 d. The rooted stems showed a maximum in soluble peroxidase activity 3 d before the appearance of roots (9^{th} day on the rooting medium) (Fig. 1). This maximum was not observed in non-rooted stems (Fig. 1). The rooted stems showed peaks in ionically bound to cell wall peroxidase activity on the 6^{th} and 12^{th} day on the rooting medium, with an intermediate decline on the 9^{th} day (Fig. 1). Stems of non-rooted plantlets did not show similar behavior in ionically bound to cell wall peroxidase activity (Fig. 1). However, catalase activity in rooted stems raised at the 6^{th} and the 15^{th} day, with an intermediate decline at the 9^{th} - 12^{th} day. Similar behaviour was not recorded in the non-rooted stems (Fig. 1).

Electrophoresis revealed two anionic isoenzymes (P_1 and P_2) in soluble fraction (Fig. 2) and one fade band in the ionically bound fractions (data not presented). A new band in soluble fraction (P_3) appeared on the 9^{th} day in plantlets treated with Fe-EDDHA. The activity of the anionic isoenozymes P_1 and P_2 was diminished at the 6^{th} and 9^{th} day and reappeared at the 12^{th} day (root appearance) in rooted plants. Furthermore, in rooted plantlets a new fade band appeared (Fig. 2).

The rooting in grapevine cuttings as described by the model of Gaspar *et al.* (1992) includes three phases: *1*) an induction phase, characterized by a sharp decline in peroxidase activity, *2*) a phase of root initiation with corresponding increase in peroxidase activity, and *3*) an expression phase, characterized by a gradual decline in peroxidase activity, which is followed by the first histologically visible signs of root primordia (Rival *et al.* 1997). The results of our work partly agree with this model, as the rooted plantlets showed an increase in the ionically bound to cell wall peroxidase activity on the 6^{th}

day, reduction on the 9th day and an increase on the 12th day on the rooting medium. However, our data did not show the initial passage through a minimum peroxidase activity as reported by Gaspar *et al.* (1992). One explanation of this discrepancy may stem from the fact that peroxidase activity was measured in crude extracts where (poly)phenols apparently modulated the enzyme activity (Gaspar *et al.* 1990). The period of time between the peroxidase minimum and the peroxidase maximum might be considered as the initiative phase of rooting, itself preceding an expressive one (Barthon *et al.* 1990). According to Chao *et al.* (2001) the decreased peroxidase activity after its peak is due to the inhibition of the de novo synthesis of the peroxidase isoenzymes during the adventitious root formation. Those changes in activity of cell wall bound peroxidase during root induction indicate a key role of cell wall peroxidase in rooting of microshoots of GF-677 *in vitro*. The decrease of cell wall peroxidase activity leads to a less rigid cell wall and thus promotes cell expansion and plant growth (Brownleader *et al.* 2000).

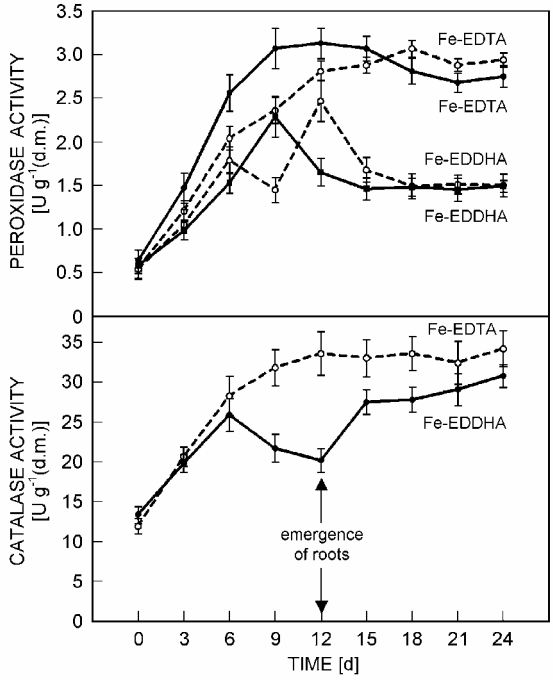

Fig. 1. Changes in catalase and soluble (*squares*) and ionically bound to cell wall (*circles*) peroxidase activity of GF-677 (*Prunus amygdalus* × *P. persica* L.) stems treated with 0.2 mM Fe as Fe-EDTA or 0.2 mM Fe as Fe-EDDHA *in vitro* on MS rooting medium. *Vertical bars* represent SE for three determinations, each one from three different extracts. *Arrow* indicates the time of root emergence (only on medium with Fe-EDDHA).

Additionally to the model of the ionically-bound peroxidase isoenzymes involvement, this work revealed the influence of soluble peroxidase isoenzyme on rooting process. Because in the cytoplasm more than one of peroxidase isoenzymes exist (Siegel and Galston 1967), the increase of soluble peroxidase activity could be due to a general increase in all isoenzymes, to an increase in certain already existing isoenzymes, or to the appearance of new isoenzymes (Gardiner and Cleland 1974). Thus, the increase of soluble peroxidase activity at the 9th day was probably due to the appearance of the new band P_3. The observed decline of soluble peroxidase activity at the 12th day is correlated to the disappearance of the band P_3 during the adventitious root formation. Also, the peroxidase activity of the Fe-EDTA samples is very high, suggesting that the difference is not at the level of total activity, but isoenzymes.

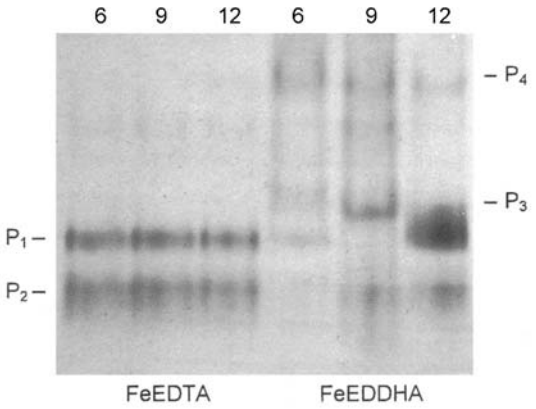

Fig. 2. Peroxidase isoenzymes profiles of soluble extracts from stems of plantlets nourished with 0.2 mM Fe in the form of Fe-EDTA (non-rooted) and Fe-EDDHA (rooted), obtained by anionic electrophoresis (PAGE) at different days of the rooting process. From left to right: days 6, 9 and 12. Fe-EDTA: *lane 1* (6 d), *lane 2* (9 d), *lane 3* (12 d), Fe-EDDHA: *lane 4* (6 d), *lane 5* (9 d), *lane 6* (12 d).

According to the literature peroxidases are involved in cell wall formation, because this enzyme catalyses the formation of diferulate (Fry 1987). Cell wall peroxidases are probably involved not only in the oxidative polymerization of hydroxylated cinnamyl alcohols, but also in the synthesis of the hydrogen peroxide necessary to the final step of lignification (Goldberg *et al.* 1985). Since the final phase of *in vitro* root differentiation involves the deposition of lignin (Miller *et al.* 1985), the observed increase in peroxidase activity could be related to the lignification of root primordia cells (Gonzáles *et al.* 1991). Also, during the root formation peroxidase activity was closely associated with cambial cell division and differentiation (Garcia-Gómez *et al.* 1995).

In non-rooted plantlets the activity of soluble and ionically bound to cell wall peroxidase was significantly higher than the one found in rooted plantlets. According to Zheng and Huystee (1992) higher peroxidase activities are closely associated with reduced growth of plants.

Catalase activity followed different pattern than peroxidase. Hence, it was low initially and after 6 d in culture was maximum. The smallest value recorded after 12 d. However, after this decrease its activity again increased. Recently, Racchi *et al.* (2001) found a strong activity of CAT-2 isoform in rooted microshoots of oak

(*Quercus robur* L.) suggesting that this isoform is a protein specifically related to rooting.

Catalase and peroxidases are involved in oxidative metabolism and also in the subsequent production of H_2O_2 (Scandalios 1993), which is involved in IAA oxidation (Nag *et al.* 2001). Weiser and Blaney (1967) showed that an increase in O_2 tension or supplementation of the medium with H_2O_2 enhanced root initiation, suggesting that oxidative metabolism plays an important role in root initiation. Furthermore, cells in the quiescent state produce hydrogen peroxide endogenously, which act as one of the mediators inhibiting DNA synthesis (Shibanuma *et al.* 1995).

An interesting point of this work is that Fe forms and concentrations can directly affect the rooting ability of GF-677 *in vitro* probably due to their effect on peroxidase and catalase activity. Based upon these results we propose, at least for the peach rootstock GF-677, the substitution of Fe-EDTA by Fe-EDDHA in the MS medium (Molassiotis *et al.* 2003/4).

More than five isoenzymes of peroxidase were identified in the extracts of the IBA-treated and the control tissues in soybean hypocotyls during adventitious root formation (Racchi *et al.* 2001). In our experiment, two isoenzymes of peroxidase (P_1 and P_2) were detected in non-rooted stems (treated with Fe-EDTA) of GF-677 and their induction was completed from the 6th day on the rooting medium. On the contrary, in rooted stems (treated with Fe-EDDHA) of GF-677 four isoenzymes of peroxidase were revealed. Two of them were also present in the non-rooted stems (P_1 and P_2) and two new ones (P_3 and P_4). However, the induction of P_1 and P_2 with Fe-EDDHA started from the 6th day and was completed on the 12th day on the rooting medium. Although we landed the same amount of peroxidase units in each lane, however, in Fig. 2 *lanes* 4 and 5 appear to have less peroxidase activity, probably due to the effect of Fe-EDDHA in the half life and stability of peroxidase isoenzymes.

Peroxidases are widespread in higher plants and there is a number of peroxidase isoenzymes, while each one is thought to serve a different function during plant cell growth and development. However, their actual roles in plant physiology are still obscure. Since many peroxidase isoenzymes exist in plants, it is important to isolate the genes that encode each isoenzyme and to characterize the expression and properties of the different genes (Kim *et al.* 2000). In our experiment both P_3 and P_4 bands should be considered as reliable molecular markers inducing root formation of GF-677 *in vitro* and their specific role should be further clarified. The expression of the gene(s), which controls the activity of P_3 and P_4, should be tested in each case of root formation, suggesting the possibility of using it as a universal marker.

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