BASIC PEROXIDASES AND ROOTING IN MICROCUTTINGS OF *MALUS*

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Abstract

We have examined the relation between the activity of basic peroxidases (basPox) and rooting in microcuttings of *Malus*. (1) A positive correlation ($p < 0.001$) occurred between the activity of basPox in stems of shoots at the time of transfer to rooting medium and the number of roots 21 days after the transfer. This correlation, though, only occurred if the nonrooted shoots had been omitted from the calculation. (2) BasPox increased during the first stages of the rooting process (5- to 10-fold), but did not show a subsequent decrease. The increase was larger in an easy-to-root than in a hard-to-root subclone. The increase, though, also occurred in nonrooted shoots on rooting medium and in shoots cultured on propagation medium or hormone-free medium. We suggest that the increase of basPox is related to cell divisions, which may result in roots and/or callus on rooting medium, or in callus on propagation medium.

In hard-to-root shoots, the outgrowth of root primordia was not inhibited as compared with easy-to-root shoots. Auxins inhibited outgrowth up to 50%. During the first days of the rooting period, 0.7% of applied radioactive auxin (indole-3-acetic acid) was taken up per day. Approx. 50% of the label taken up accumulated in the basal 3-mm portion of the stem.

1. Introduction

Success of rooting of microcuttings depends on the genetical and physiological condition of the microcutting, and on the medium composition and physical conditions during the rooting treatment. In many cases, microcuttings are hard to root. We have initiated a study on rooting of microcuttings of *Malus*. As both the level and the course of basic peroxidase (basPox) are thought to be closely related with the successive stages of the rooting process (Gaspar *et al.*, 1985), we determined basPox in easy- and in hard-to-root subclones of *Malus*. Furthermore, we studied the effect of subclone type and auxins on the outgrowth of root primordia.

2. Materials and Methods

Shoot production of *Malus domestica* Borkh. cv. Elstar (in one experiment 'M 9' was used) was maintained by subculture cycles of 4 weeks. The propagation medium consisted of MS macro- and microelements (Murashige and Skoog, 1962), vitamins of the N6 medium of Chu *et al.* (1975) and 100 $mg.l^{-1}$ myo-inositol, 0.5 μM indolebutyric acid (IBA), 4 μM benzyladenine (BA), 3% (w/v) sucrose and 0.6% (w/v) agar (Difco Bacto). The pH was adjusted to 5.5 before adding the agar. The shoots were cultured on 15 ml medium in 22-mm culture tubes at 20°C and 30 $\mu E.m^{-2}.s^{-1}$ (Philips TLD 36W/33; 16 h per day). Two

subclones were available: E1 was subcultured at least 30 times and was easy to root; E2 was subcultured 6-10 times and was relatively hard to root.

Shoots of 1-2 cm were taken for rooting. The rooting medium was as the propagation medium, except for the macro- and microelements (the macroelements at half strength and the microelements at full strength Lepoivre; Quoirin *et al.*, 1977) and the hormones (IBA at 1 uM, no BA). In some experiments, riboflavin (1 $mg.l^{-1}$) was added. After 7 days in the dark, the shoots were transferred to the light. Root primordia were counted microscopically after 7-10 days. Roots were scored after 21 days.

Total peroxidase (Pox) was determined in crude extracts with a guaiacol/H_2O_2 mixture and protein with the Bradford-method. BasPox was determined by separating peroxidases by isoelectric focussing on Servalyt Precotes, pH range 3-10. The gels were stained with 3,3-diaminobenzidine/H_2O_2. The intensity of the bands was measured with a Zeineh 2-D/1-D Video densitometer. Uptake of [5(N)-^{3}H]-indole-3-acetic acid (925GBq.$mmol^{-1}$, Amersham) was determined as described previously (Smulders *et al.*, 1988).

3. Results

3.1. Relation between basPox in shoots at the time of transfer to rooting medium and the number of roots formed

At the time of transfer to rooting medium, the basal 3-mm portion of stems of individual shoots (the portion of the shoots in which the adventitious roots are formed) had very different activities of basPox, the highest being 12 times the lowest (Fig. 1a). The activities of acidic peroxidases, though, were similar. The activities of basPox in the basal 3 mm and in the adjacent 3-mm portion were very similar ($r = 0.78$; $p < 0.001$; $n = 15$).

Shoots from which the basal 3 mm had been taken to determine basPox, were transferred to rooting medium. The number of roots of individual shoots was counted after 3 weeks on rooting medium and plotted against the activity of basPox in the basal 3 mm of the same shoot measured at the time of transfer (Fig. 1b). If the nonrooted shoots were omitted from the calculation, there was a significant positive correlation ($p < 0.001$). If the nonrooted shoots were included, the correlation was not significant ($p < 0.1$).

3.2. The activity of basPox in shoots on rooting medium

Shoots from both subclones were cultured on rooting medium for increasing periods of time. In both subclones, the activity of Pox in the basal 3 mm of the stem increased sharply up to 14 days and remained constant thereafter (Fig. 2). Most of the activity measured was basPox (guaiacol is the preferable substrate for basPox, diaminobenzidine for acidic Pox; de Klerk and ter Brugge, unpublished data). The activity of Pox was higher in E1.

After 15 days on rooting medium, basPox was determined in individual rooted and nonrooted shoots. In the nonrooted shoots, basPox also increased sharply and reached the same level as basPox in rooted shoots (Fig. 3). In rooted shoots, there was no correlation between the number of roots and the activity of basPox (data not shown).

We also determined basPox in the basal parts of shoots on rooting medium, propagation medium and hormone-free medium (Fig. 4). This

experiment was carried out with a clone of 'M 9'. All media resulted in an increase of basPox. Shoots on hormone-free medium showed the lowest increase. It should be noted that in 'M 9' the activity of basPox at the time of transfer was high.

3.3. Outgrowth

Many times, nonrooted shoots formed callus. It might be that no root primordia had formed at all but only callus, or that root primordia had grown into callus instead of developing into roots. Therefore, we determined the number of root primordia microscopically at days 7 and 10, and the number of roots at day 21 (Fig. 5). The number of initiated root primordia was 60-80% higher in E1 than in E2, but the percent outgrowth was the same. The rooting percentages were the same as the percentages of shoots with root primordia. When IBA was not removed after 1 week, outgrowth was inhibited up to 50%.

Fig. 5 indicates that IBA was taken up slowly. In other tissue-culture systems, though, auxin is taken up rapidly (for instance, 80% uptake after 7 days; Smulders *et al.*, 1988). This prompted us to examine the rate of uptake of auxin by the shoots. The experiment was carried out with ^{3}H-IAA. During the first days of the culture, the *Malus*-shoots took up 0.7% of the total available IAA per day. About 50% of the total label in the shoots was present in the basal 3-mm portion of the stem (Fig. 6).

4. Discussion

To our knowledge, the relation between Pox-activity at the time of transfer to rooting medium and the number of roots has been studied only by Patience and Alderson (1987). They observed no relation. In our experiments, the basal portions of individual shoots had very different activities of basPox (Fig. 1a). These levels of basPox correlated with the number of roots formed (Fig. 1B). The correlation only existed when the nonrooted shoots had been omitted from the calculation. We suggest that the high activity of basPox is related with the capability of cell division and that the increased number of cell divisions may either result in more roots or in more callus. It should be stressed that we found no correlation between basPox and the number of roots formed in another experimental system (shoots transferred to rooting medium at various points of time in the propagation cycle; G.J. de Klerk & J. ter Brugge, unpublished results).

Many authors reported a sharp initial increase of basPox during rooting of shoots followed by a sharp decrease (references in Gaspar *et al.*, 1985). The maximum concurs with the formation of the root primordia and the value of the maximum is related with the rooting percentage and the number of roots formed (*e.g.*, Moncousin and Ducreux, 1984). Fig. 2 shows an increase of basPox, but not a subsequent decrease after formation of the primordia (according to microscopical observations at ± day 7). The hard-to-root subclone had a lower activity of basPox than the easy-to-root subclone (Fig. 2), but in other experiments we found very similar activities. We also observed a sharp increase of basPox in nonrooted shoots on rooting medium (Fig. 3) and in shoots on propagation medium or hormone-free medium (Fig. 4). We suggest that the increase of basPox is related to cell divisions, which may either result in the forma-

tion of roots (often with additional callus formation) or in the formation of callus.

In many studies, it has been shown that auxin is necessary during the first phases of the rooting process, but inhibits the growth of roots. In our experiments we have shown that in rooting medium in which auxin remained present, the outgrowth of root primordia is inhibited up to 50%, whereas no such inhibition was observed when IBA was removed by photooxidation by riboflavin (Gorst *et al.*, 1983). Contrary to other tissue-culture systems, auxin was taken up very slowly (Fig. 6). This was likely due to the small wound area. It has been reported that the uptake via the epidermis of the stem is very low (Collet and Le, 1987).

The easy-to-root subclone formed twice as many root primordia as the hard-to-root subclone, whereas the percent outgrowth was the same in both subclones (Fig. 5). Apparently, the hard-to-root subclone was inhibited early, *i.e.*, prior to the formation of root primordia. Presently, we examine the relation between this inhibition and the low activity of basPox in more detail.

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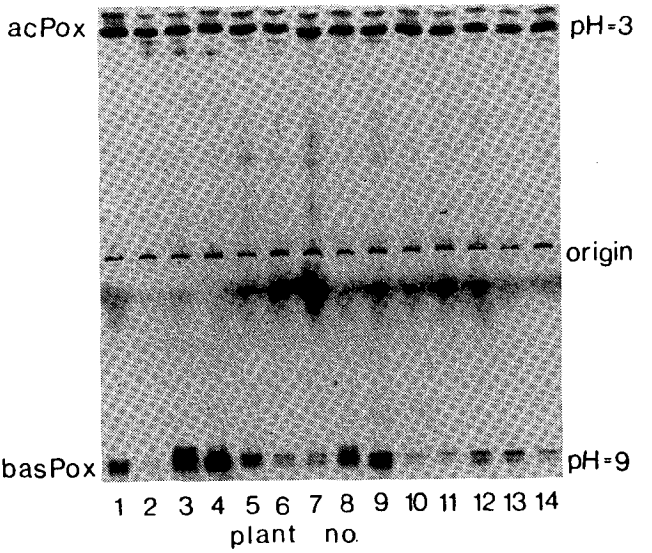
1a



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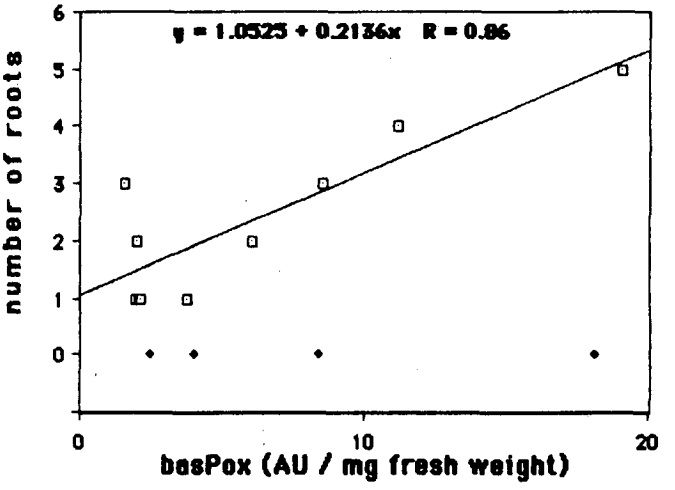

Figure 1a - Isoelectric focussing of peroxidases in the basal 3-mm portion of 14 individual *Malus* shoots (E2) at the time of transfer to rooting medium.

b - Root numbers of individual shoots after 21 days on rooting medium plotted against the activity of basPox at the time of transfer to rooting medium. The shoots are the same as in Fig. 1a. The correlation was calculated using the data of the rooted shoots only.

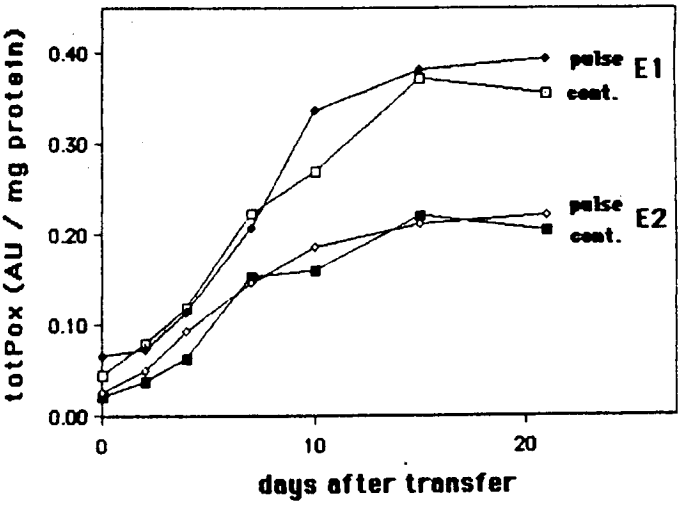

Figure 2 - Activity of total Pox in the basal 3 mm of *Malus* shoots (E1 and E2) during culture on rooting medium. IBA was present continuously (cont.) or only for 7 days (pulse). In the latter case, IBA was destroyed by riboflavin upon transfer to the light.

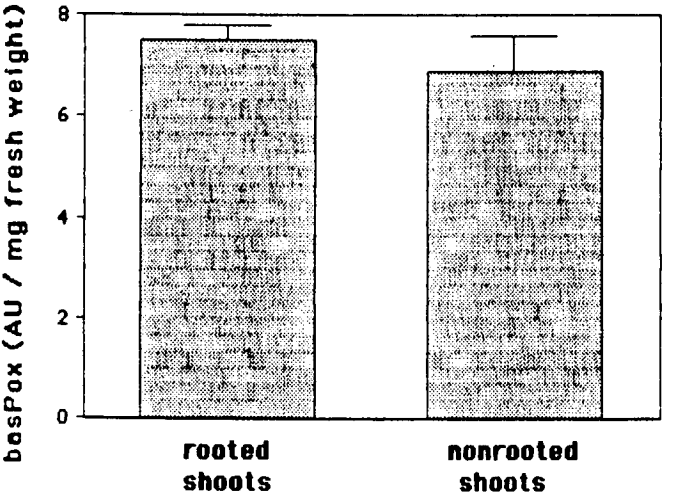

Figure 3 - Activity of basPox in the basal 3 mm of rooted and nonrooted *Malus* shoots (E2) after 15 days on rooting medium. For rooted or nonrooted shoots, resp. 8 or 6 shoots were analysed individually.

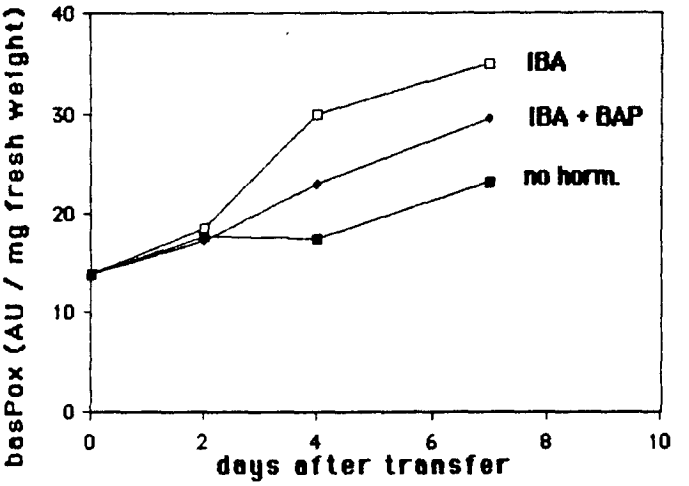

Figure 4 - Activity of basPox in the basal 3 mm of *Malus* shoots ('M 9') during culture on rooting medium (IBA), propagation medium (IBA + BAP) or hormone-free medium (no horm.).

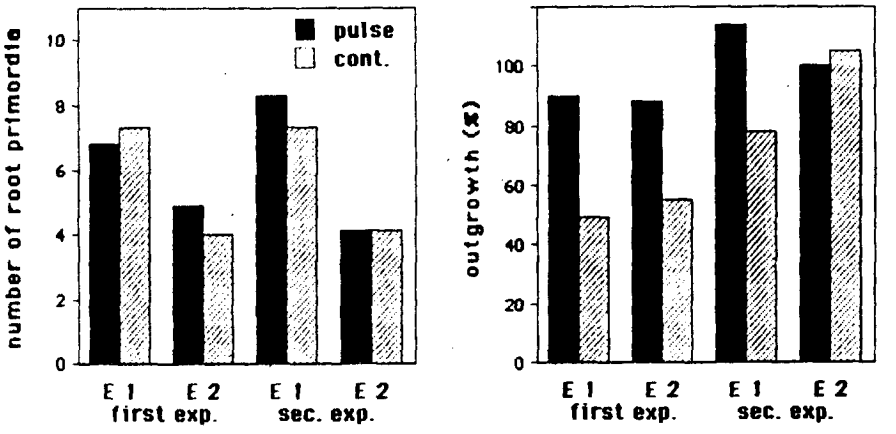

Figure 5 - Number of root primordia after 7-10 days on rooting medium (a) and outgrowth of the root primordia (b). The number of root primordia was determined in 12 shoots. Outgrowth was determined by dividing the number of roots (in a sample of 30 shoots cultured in a parallel experiment) by the number of root primordia.

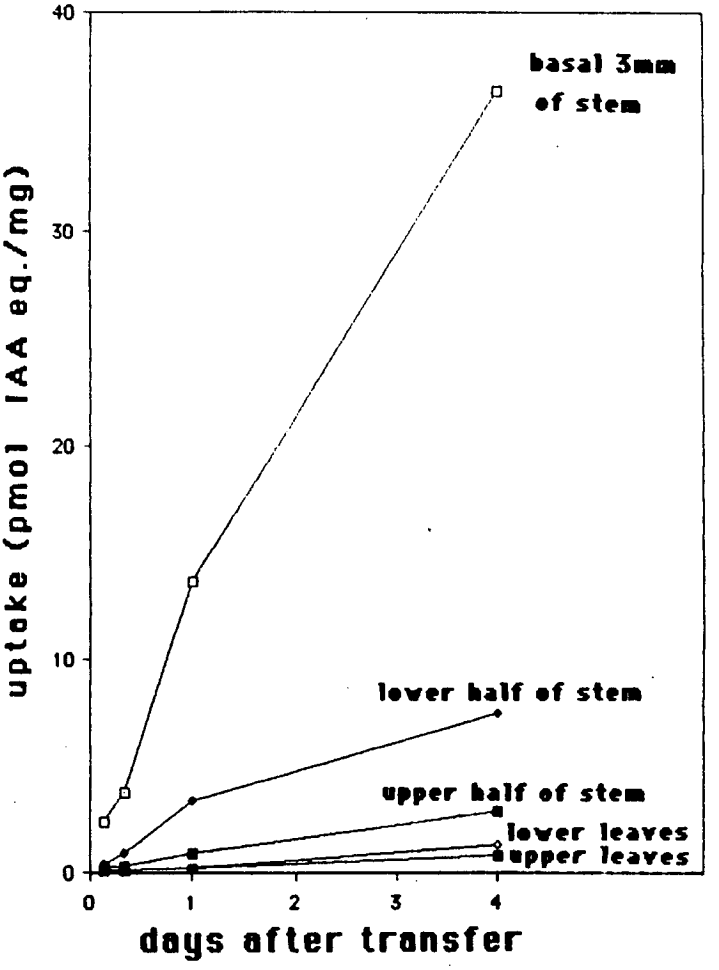

Figure 6 - Uptake of ^{3}H-IAA by various parts of *Malus* shoots during culture on rooting medium with IAA instead of IBA.