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Practical uses of peroxidase activity as a predictive marker of rooting performance of micropropagated shoots *

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Summary — A peak of specific peroxidase activity (increase in enzyme activity followed by decrease) has generally been found in crude extracts of stem- or shoot-cuttings prior to root formation. Effectors, some of them of a phenolic nature, play a role in these changes in peroxidase activity. This may explain discrepancies in some investigations dealing with partially purified extracts or cell fractions. The peroxidase peak does not terminate the rooting inductive period as proposed some years ago, but rather the initiative phase before identification and growth of endogenous root primordia. Depending upon the materials studied, characteristics of the peroxidase peak such as precocity of appearance, height, and velocity of decrease have been correlated with ulterior rooting performance. Physical and/or chemical factors which were able to modulate the peak also had effects on subsequent rooting rate. An early test using peroxidase activity of extracts made from shoots before transferring to rooting media determined the phenol compounds capable of enhancing the rooting process and the moment of application in relation to the peroxidase peak.

peroxidase / rooting

Résumé — Utilisation pratique de l'activité de la peroxydase pour prédire les performances d'enracinement de tiges. *Un pic d'activité peroxydase spécifique (augmentation de l'activité de l'enzyme suivie par une décroissance) a été trouvé en général dans les extraits bruts de boutures de tiges avant la formation de racines. Des effecteurs, dont certains sont de nature phénolique, jouent un rôle dans ces variations de l'activité peroxydase. Cela peut expliquer les désaccords entre des travaux sur des extraits ou des fractions cellulaires partiellement purifiés. Le pic de peroxidase ne termine pas la période d'induction de l'enracinement, comme on le proposait il y a quelques années, mais plutôt la période d'initiation avant l'identification et la croissance de primordia de racines endogènes. Suivant le matériel étudié, des caractéristiques du pic de peroxydase comme la précocité de son apparition, sa hauteur, et la vitesse de diminution ont été associées aux performances ultérieures d'enracinement. Les facteurs chimiques ou physiques qui modulent le pic ont aussi des effets sur la proportion d'enracinement subséquent. Un test précoce, utilisant l'activité peroxydase d'extraits de tiges avant de les transférer au milieu d'enracinement permet d'identifier les composés phénoliques capables de favoriser le processus d'enracinement et d'en déterminer le moment d'application par rapport au pic de peroxydase.*

peroxydase / enracinement

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HISTORICAL BACKGROUND

The early observation that exogenously applied natural or synthetic auxins favour rooting of cuttings has been repeatedly confirmed and auxins have been used in conventional horticulture for a long time. Vegetative propagation of several plants through tissue culture, *ie*, micropropagation, also exploits the rooting properties of auxins (Gaspar and Coumans, 1987). The rooting properties of exogenously applied auxins have automatically (and unfortunately ?) led to 2 concepts in the mind of plant physiologists: first, that cuttings or shoots must necessarily accumulate a certain amount of natural or synthetic auxins in order to be able to root; and second, that the applied auxins simply increase the bulk of endogenous auxins. Until recently, experiments aiming at establishing a relationship between (a high endogenous) auxins level and rooting led to very different results (Gurumurti *et al,* 1984; Haissig, 1986; Jackson, 1986; Davis *et al,* 1988; Gaspar and Hofinger, 1988). These discrepancies may be explained by:

– degradation and/or loss to varying degrees of the extracted endogenous auxin, depending upon the different techniques used;

– the various biological or biochemical estimation tests which differed greatly in their sensitivity;

– estimations which were made without reference to advancement of the rooting process.

Synthetic auxins may not use the same auxin receptors or act *via* the same pathways as endogenously produced auxins. As a matter of fact, it is quite clear that synthetic auxins may initiate a series of signals from the exterior of the cells before their penetration (Millet and Greppin, 1990).

Several papers have reported an auxin-content regulating role for the so-called auxin-oxidase system – thus an inverse relationship between the endogenous IAA level of a plant tissue or organ and its IAA-oxidase activity (Pilet and Gaspar, 1968); many researchers therefore investigated the changes in IAA-oxidase activity in relation to rooting (Mato and Vieitez, 1986; see ref in Bhattacharya, 1988; Moncousin, 1991). The activity of the so-called IAA-oxidase system was later attributed to one or several peroxidases (Gaspar *et al,* 1982). Because the measurement of peroxidase activity is simpler than that of IAA-oxidase activity, relationships between different aspects of rooting and changes in peroxidase and isoperoxidase activity have been investigated by many authors.

A PEROXIDASE ACTIVITY PEAK PRECEDING ROOTING. THE CONCEPT OF AN INDUCTIVE PROCESS

Analyses of changes in total peroxidase activity and in the isoperoxidase spectrum in the course of rooting in microcuttings of different *Prunus* (Quoirin *et al,* 1974) and *Asparagus* (Van Hoof and Gaspar, 1976) species, and in epidermal layers of tobacco (Gaspar *et al,* 1977; Thorpe *et al,* 1978) led to the production of a general curve (Gaspar, 1981). It appeared that root formation occurred after the cutting had reached and passed a peak of maximum enzyme activity. The activity and/or number of acidic isoperoxidases increased continuously during the course of the process which means that the peak of peroxidase activity was due to an inverse variation in the activity of the basic isoenzymes before and after the peak (fig 1). Such changes in peroxidase activity in the course of root formation, most of them involving a passage through a peak, were measured in many other materials (Chandra *et al,* 1971; Molnar and Lacroix, 1972; Dalet and Cornu, 1986, 1989; Gebhardt, 1986; Berthon *et al,* 1987; Mato *et al,* 1988; Gonzalez *et al,* 1991; Kevers and Gaspar, 1992). They apparently corresponded to parallel changes in IAA-oxidase activity (Mato and Vieitez, 1986; refs in Bhattacharya, 1988; Ben-Efraim *et al,* 1990). In some cases, peroxidase activity of the shoot-cuttings dropped immediately after transfer from a multiplication to an auxin-based rooting medium. This was followed by the rapid emergence of root primordia (Quoirin *et al,* 1974; Van Hoof and Gaspar, 1976). It was later shown that the elevation and peaking of peroxidase activity preceding rooting might already take place at the end of the foregoing multiplication/elongation cycle (Druart *et al,* 1982; Hausman *et al,* 1990).

There was an apparent coincidence between the peroxidase peak and the first visible signs of differentiation (Gaspar *et al,* 1977) which led to the hypothesis that the period of peroxidase elevation up to the peak corresponded to an inductive or preparatory phase of rooting occurring before any visible morphological or histological event (Gaspar, 1981). The recognition of such an inductive phase for rooting was relatively new and rarely discussed before that time, although

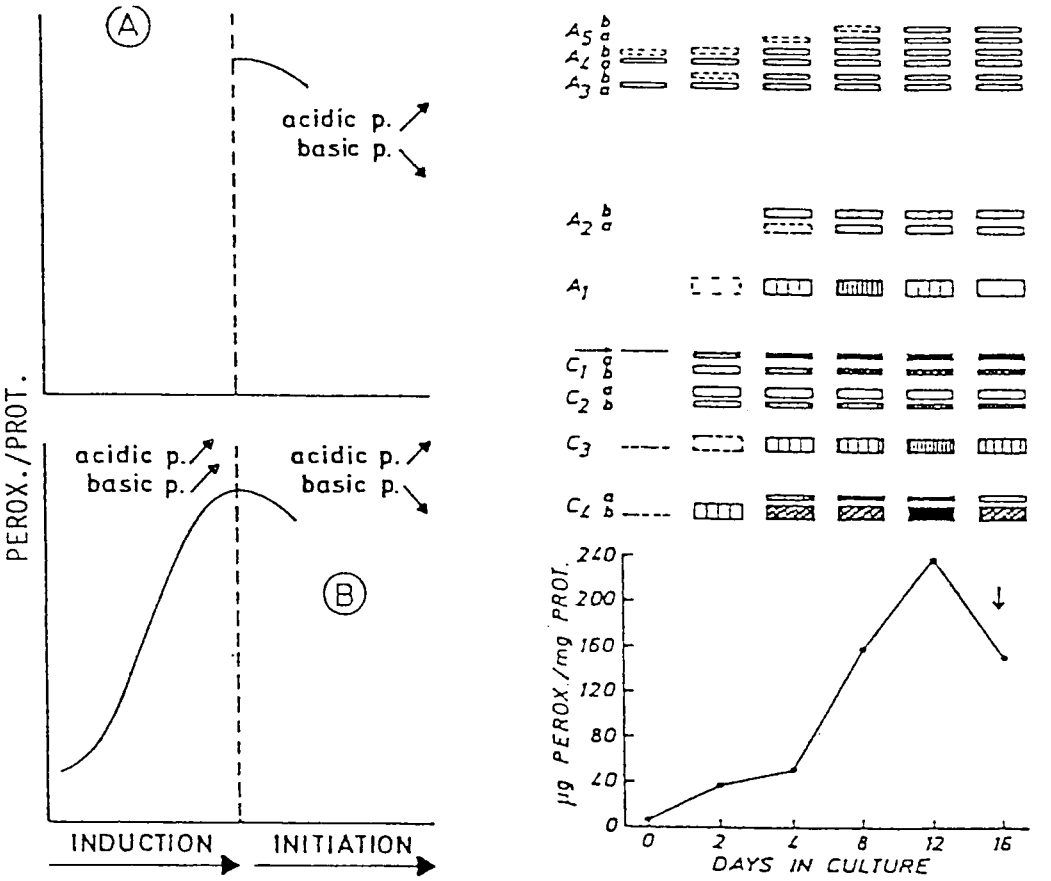


Fig 1. Left : schematic representation of the variation in specific peroxidase activity during the inductive and initiative phases of rooting by preinduced **(A)** and non-preinduced **(B)** explants (from Gaspar, 1981). Right : peroxidase zymograms and changes in total specific peroxidase activity during root formation from epidermal layers of tobacco cultured for 16 d. Arrow indicates the time of visible appearance of roots (from Thorpe *et al,* 1978).

such an induction concept had long been acquired for the flowering process.

That the peroxidase peak was associated with the first cell divisions, *ie* that the peroxidase peak terminated the inductive period of rooting, was questioned by Jarvis (1986). This author placed the end of the rooting–induction phase (corresponding to the biochemical changes preceding cytological and histological events) before the peroxidase peak. As described hereafter, our further studies confirmed this view (Moncousin *et al,* 1988; Gaspar *et al,* 1990).

Although the increase in peroxidase activity preceding rooting has generally been noticed, some research workers were unable to demonstrate the peak and subsequent decrease in enzyme activity (Gebhardt, 1985; Pythoud and Buchala, 1986; Patience and Alderson, 1987; de Klerk *et al,* 1990). The explanation of these discrepancies may stem from the fact that in these studies, peroxidase activity was measured in purified extracts and/or not expressed per unit protein. When well evidenced, the (specific) peroxidase activity peak was measured in crude extracts where (poly)phenols apparently modulated the enzyme activity (Druart *et al,* 1982; Mato *et al,* 1988; Gaspar *et al,* 1990; Berthon *et al,* 1990b). We have further shown (Aghmir *et al,* 1991) that none of the cell fractions (extracellular, soluble, membrane, wall ionic, wall covalent) separated from a crude extract (of Rhododendron shoots) showed the typical increase and subsequent decrease of peroxidase activity, whereas the total extract did. This indicates that the peroxidase peak was not only the result of changes in proteinic enzyme activities but also of changes in their effectors. The results of Curir *et al* (1989) indirectly confirmed this view.

ROOTING AS A SERIES OF SUCCESSIVE PHYSIOLOGICAL PHASES. REFINEMENT OF THE KINETIC VARIATION OF PEROXIDASE ACTIVITY. RELATIONSHIP WITH THE ENDOGENOUS AUXIN LEVEL

Rooting has been described to occur in at least 6 successive interdependent phases (Favre, 1973; Mitsuhashi-Kato *et al,* 1978a, b; Moncousin *et al,* 1988; Berthon *et al,* 1990a; Gaspar *et al,* 1990): induction (the period preceding visible cytological events); nucleus swelling; transverse and longitudinal first divisions of pericycle or cambial cells; continued cell divisions, without increase in gross volume, constituting morphogenetic radical fields (clusters of cells which show no polarity); volume increase of cell clusters by cell expansion and identification of radical meristems; internal growth of root primordia and root protrusion.

Table I shows results from leaved and nonleaved node cuttings of vine shoots raised *in vitro,* illustrating how these 2 cutting types differently entered into 5 well defined ontogenetic stages of root formation. Maximum rooting by leaved (98%) and nonleaved (95%) cuttings was reached after 156 and 168 h respectively. Leaved cuttings started the 5 successive ontogenetic stages somewhat earlier: swollen nuclei were seen in 40% of leaved cuttings at 33 h; in 40% of nonleaved cuttings at 40 h. Note that radical meristems did not occur before 110 h. Evolution of specific peroxidase activity in the cuttings was characterized by an initial reduction during the first 12 h, followed by an increase which culminated at 72 h. Peroxidase activity decreased later, while the roots were appearing (fig 2A). No real difference could be observed between the leaved and the nonleaved cuttings. There was a clear increase in the content of free IAA in both types of cuttings, somewhat greater in the leaved ones, during the first 12 h (fig 2B). The IAA-level then began to decrease sharply to a minimum at 36 h. This minimum was followed by a slow progressive increase.

The peroxidase peak preceding the emergence of root primordia found in other materials (see above) was again found here but this more critical analysis showed a prior passage through a minimum peroxidase activity (at 12 h). This minimum corresponded to an early acute momentary peak of auxin level (fig 2B). The peak of peroxidase activity that culminated at 72 h (fig 2A) corresponded to a larger period of auxin depletion (fig 2B; see also Berthon *et al,* 1989). These results may explain the discrepancies in the results of investigations aimed at establishing a relationship between the amount of endogenous auxin-like substances in cuttings and their rooting performance (Nakano *et al,* 1980; Fouret

Table I. Mean time (h) required by different percentages of the leaved (LC) and nonleaved (NLC) cuttings to reach the 5 ontogenetic status.

Ontogenetic status	*LC*			*NCL*		
	40%	*60%*	*80%*	*40%*	*60%*	*80%*
A	33	39	45	40	52	59
B	45	54	68	57	64	76
C	78	81	92	83	90	99
D	106	115	126	111	119	130
E	128	136	142	143	145	157

A: nucleus swelling; B: swollen nucleus and beginning of periclinal divisions in the cambium; C: presence of morphogenetic fields; D: presence of radical meristems; E: emergence of first root (from Moncousin *et al,* 1988).

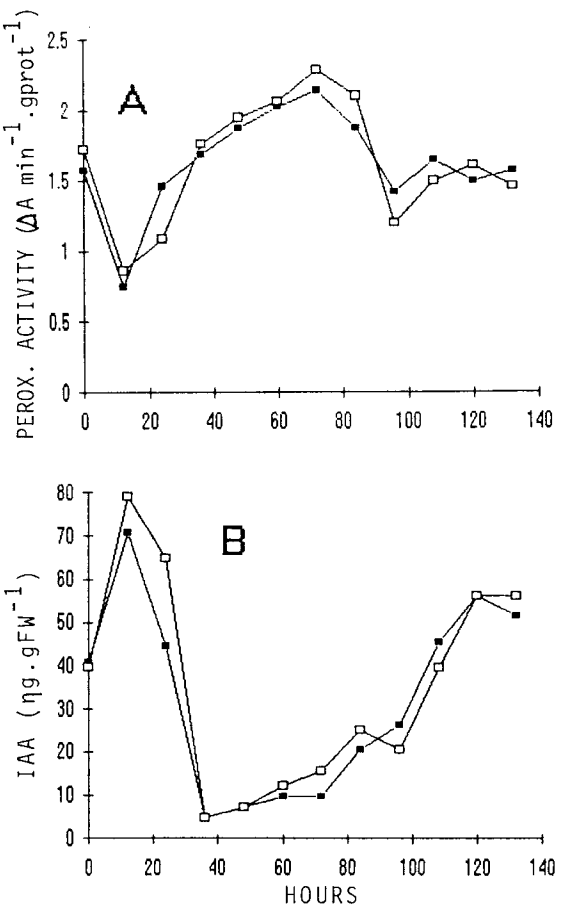


Fig 2. Evolution of specific peroxidase activity **(A)** and endogenous IAA level **(B)** in leaved (◊) and nonleaved (◆) vine cuttings during the first 132 h on rooting medium (redrawn from Gaspar *et al,* 1990).

et al, 1986; Welander and Snygg, 1987; Gaspar and Hofinger, 1988). The above analyses made in association with the ontogenetic phases of root formation indicate that the rooting inductive phase already terminated with the minimum of peroxidase activity at 12 h, *ie* with the maximum auxin level; histological events began from that time and not as previously hypothesized from the peroxidase peak occurring later at 72 h. The period of time between the peroxidase minimum (level of auxin maximum), and the peroxidase maximum might be qualified as the initiative phase of rooting, itself preceding an expressive one (Berthon *et al,* 1990a).

PEROXIDASE ACTIVITY AS MARKER AND PREDICTOR OF THE ROOTING PERFORMANCE. PRACTICAL MANIPULATIONS TO IMPROVE ROOTING

Sorting out species or donor-plants with varying rooting abilities

Before *in vitro* culture, buds from *Prunus* species known for their greater rooting ability were characterized by a higher peroxidase activity than species which rooted with difficulty (Quoirin *et al,* 1974). There was a similar relationship between the rhizogenetic potential of *Asparagus* donor plants and the peroxidase activity of their cuttings (Gaspar and Van Hoof, 1976). A positive correlation also occurred between the activity of (basic) peroxidases in stem of shoots of apple at the time of transfer to rooting medium and the number of roots formed later (Gus'kov *et al,* 1988; de Klerk *et al,* 1990).

Physical and chemical treatments preparing rooting at the foregoing multiplication or elongation phase

Although a direct relationship between the height of the peroxidase peak preceding rooting and the rooting rate was not always evident, treatments which enhanced peroxidase activity during the multiplication or elongation stage favoured rooting after transfer of the shoots to rooting medium. Benzyladenine used instead of kinetin in the multiplication medium of *Asparagus* provided a shoot population with a higher peroxidase activity and a higher rooting percentage (Gaspar and Van Hoof, 1976). Continuous darkness instead of a 16–8 day–night cycle during elongation and root initiative phases of apple shoots favoured successive increase and decrease of peroxidase activity, and resulted in higher percentages of rooted plantlets (Druart *et al,* 1982). On the contrary, enhancement of peroxidase activity of globe artichoke shoots was favoured by continuous light, whereas a reduced photoperiod or darkness was necessary to achieve the decrease in peroxidase activity and to obtain rooting (Moncousin and Gaspar, 1983). The peroxidase peak appeared as a pivot time indicating when, most often, physical and/or chemical factors should be favourably changed. However, the requirements for increasing the peroxidase activity peak and principally for further lowering it as far and rapidly as possible (see hereafter), differ from plant to plant.

Substances which modify the rooting properties of exogenously applied auxins have been classified as auxin synergists or antagonists. Since the peroxidase (mediated IAA-oxidase) system has been shown to vary in an inverse manner throughout the phases of the rooting process (see above), such substances should have varying effects during the successive phases. We indeed have shown that compounds such as gibberellic acid and polyphenolic acids inhibit or stimulate root formation depending on the moment and duration of application (Gaspar *et al,* 1977). A simple classification between auxin synergists and antagonists thus no longer holds true (Gaspar, 1989).

Precocity and performance of rooting in relation to peroxidase activity

Results from investigations on *Kalmia latifolia* presented in table II and figure 3 allow the following conclusions to be made (Kevers *et al,* 1990, 1991; Kevers and Gaspar, 1992):

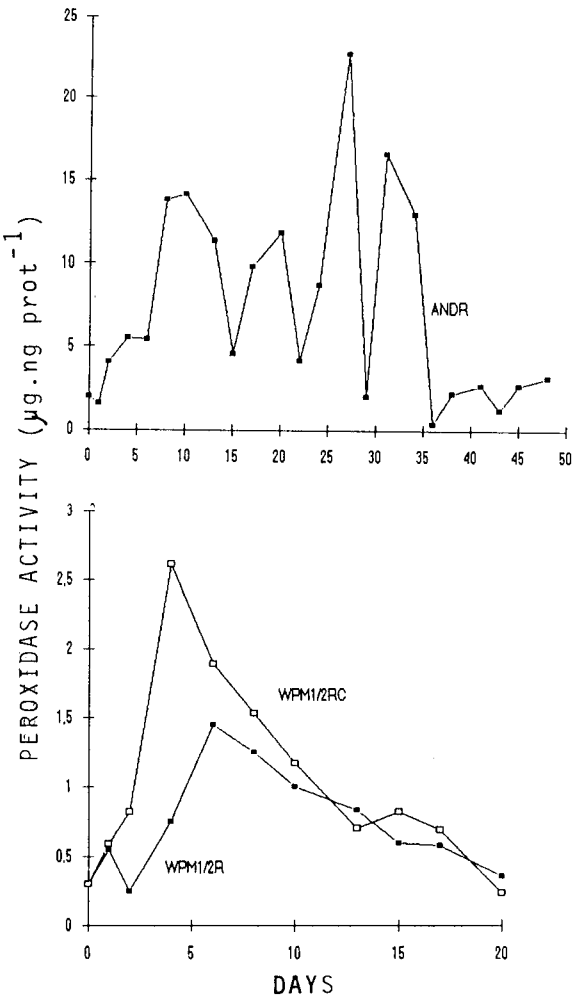
– peroxidase activity of the shoots transferred to rooting medium always increased to a peak;

– on bad rooting media (AND), the peroxidase peak was never completely reduced but was followed by several other peaks. Such a situation was also encountered with shoots from juvenile and mature clusters of *Sequoiadendron giganteum* (Berthon *et al,* 1987);

– on good rooting media (WPM 1/2) the peroxidase peak was unique and fell fairly rapidly;

Table II. Characteristics of peroxidase activity peak in relation to the precocity and rooting percentage of *Kalmia latifolia* shoots (from Kevers and Gaspar, 1992).

Multiplication media	*Rooting media*	*Timing (d)*	*Peroxidase peak intensity (1st peak)*	*% decrease (in 4 d)*	*10% rooting at: (d)*	*% rooting (6 wk)*
AND X	AND R	10–20	14.12	–	–	7
	WPM 1/2 R	10	6.83	25	22	73
	WPM 1/2 RC	6	6.19	36	17	67
WPM X	AND R	10–17	11.25	–	29	13
	WPM 1/2 R	6	2.22	62	15	100
	WPM 1/2 RC	4	2.22	67	12	100
AND Y	AND R	6–13	5.34	–	–	7
	WPM 1/2 R	6	1.45	31	16	60
	WPM 1/2 RC	4	2.61	41	14	60
WPM Y	AND R	2–13	8.34	–	38	13
	WPM 1/2 R	13	4.71	72	22	100
	WPM 1/2 RC	6	5.64	62	15	100



– there was no relationship of the percentage of rooting with the height of the peroxidase peak but with the velocity of the decrease of peroxidase activity. Shoots raised on thidiazuron instead of a cytokinin might fail to root adequately and showed an accompanying improper peroxidase decrease;

– there was also a relationship between the precocity of the peroxidase peak and the precocity of rooting.

These studies have again demonstrated that root formation does not depend only on the rooting media but also on the multiplication media used previously.

An early test using phenolic compounds and peroxidase activity to improve rooting

Investigations made on shoots of *Sequoiadendron giganteum* raised *in vitro* indicated that phenol compounds increased or decreased the *in vitro* rooting frequency depending on the time of

Fig 3. Changes in peroxidase activity of *Kalmia latifolia* shoots on poor (AND R) and good (WPM 1/2 R, WPM 1/2 RC) rooting media. Rooting performance : WPM 1/2 RC > WPM 1/2 R > AND R (from Kevers and Gaspar, 1992).

application, *ie* before the peroxidase peak (during so-called but inappropriately (see before) rooting-inductive period) or starting with the peroxidase peak onwards (during the initiative–expressive phase of rooting) (Berthon *et al,* 1992). The same phenol compounds were also evaluated for their effects on peroxidase specific activity of extracts made from shoots before transferring to rooting media. Some of the compounds increased the specific activity, while other compounds decreased it. There was a strong correlation between the effect of phenolics on rooting and their effect on peroxidase activity (fig 4). For example, phenolic compounds that increased the peroxidase specific activity of shoot extracts also caused an increase in the rooting percentage when applied during the "induction" (phase corresponding to the elevation of peroxidase activity) phase of rhizogenesis, and *vice versa.* It has been proposed that this predictive assay might be used to increase the rooting percentage of other difficult to root species.

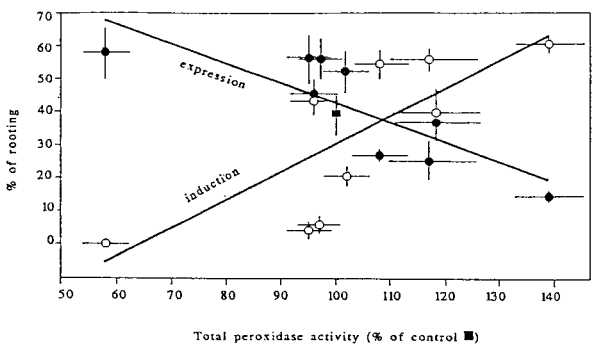


Fig 4. Correlation between the effect of phenol compounds on rooting and the effect of phenol compounds on peroxidase specific activity. Orthogonal regressions and correlations between the effect of phenol compounds on the rate of rooting, when applied at the induction (O; $R = 0.77$; $P = 0.991$) or expression phases (O, $R = 0.81$; $P = 0.996$) of rhizogenesis, and the effect of the phenol compounds on peroxidase specific activity when added to an extract made from shoots before the induction of rhizogenesis. Bars represent the standard error (from Berthon *et al,* 1992). ■: control in the absence of phenolic compound.

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