Zeitschrift: Saussurea : journal de la Société botanique de Genève
Herausgeber: Société botanique de Genève
Band: 24 (1993)

Artikel: Early test using phenolic compounds and peroxidase activity to improve in vitro rooting of Sequoiadendron giganteum (Lindl.) Buchholz
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DOI: https://doi.org/10.5169/seals-1099160

Early test using phenolic compounds and peroxidase activity to improve in vitro rooting of Sequoiadendron giganteum (Lindl.) Buchholz

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ABSTRACT

BERTHON, J.-Y., M. J. BATTRAW, Th. GASPAR & N. BOYER (1993). Early test using phenolic compounds and peroxidase activity to improve in vitro rooting of Sequoiadendron giganteum (Lindl.) Buchholz. *Saussurea* 24: 7-13. In English, English and French abstracts.

Phenolic compounds increased or decreased the *in vitro* rooting frequency of *S. giganteum* depending on the time of application. There was a strong correlation between the effect of phenolics on rooting and their effect on the peroxidase activity of extracts made from shoots before induction. These results were applied toward increasing the rooting frequency of *S. giganteum*. We propose that the application of these results could be extended to other difficult-to-root species.

RÉSUMÉ

BERTHON, J.-Y., M. J. BATTRAW, Th. GASPAR & N. BOYER (1993). Test préliminaire utilisant des composés phénoliques et l'activité peroxydasique pour améliorer l'enracinement in vitro du Sequoiadendron giganteum (Lindl.) Buchholz. *Saussurea* 24: 7-13. En anglais, résumés anglais et français.

Les composés phénoliques accroissent ou diminuent la fréquence d'enracinement *in vitro* du *S. giganteum* selon le moment d'application. Il existe une forte corrélation entre l'effet des composés phénoliques sur l'enracinement et leur effet sur l'activité peroxydasique d'un extrait de boutures avant induction. Ces résultats sont appliqués à l'accroissement de la fréquence d'enracinement de *S. giganteum*. Ces données pourraient être étendues à d'autres espèces difficiles à enraciner.

Introduction

Micropropagated woody shoots, particularly those of conifers, generally do not root *in vitro* when cultured on a single medium containing auxin as the sole growth regulator (BONGA & DURZAN, 1987). Rooting of these recalcitrant species has been improved by using at least two successive media (SMITH & THORPE, 1975; MONCOUSIN &

CODEN: SAUSDH
ISSN: 0373-2525
24 7 (1993)

GASPAR, 1983). *In vitro* root formation by shoots of *Sequoiadendron giganteum* has been obtained by culturing shoots in an auxin-based medium and then subsequently transferring the shoots to a culture medium without auxin but supplemented with rutin (BERTHON & al., 1987; BERTHON & al., 1991). The duration of the culture period in the first medium was determined with reference to a proposed induction period that ends with a peak in peroxidase activity. Indeed, a peak of peroxidase activity has been found during the rooting process of many plants (DRUART & al., 1982; MONCOUSIN & GASPAR, 1983; BHATTACHARYA, 1988; MATO & al., 1988; MONCOUSIN & al., 1988), and roots never appear before this peak. Such a peak was also found for *S. giganteum* at day 7 on the auxin-containing medium (BERTHON & al., 1987). Physical and chemical factors which increased the peroxidase activity during the induction phase, or decreased the peroxidase activity during the rooting stage, enhanced the percentage of root formation (DRUART & al., 1982; MONCOUSIN & GASPAR, 1983). It has also been observed that the content of soluble phenolic compounds in plants undergoing rooting varies inversely with the level of peroxidase activity (DRUART & al., 1982; MATO & al., 1988). Hence, phenolic compounds might be controllers of peroxidase activity.

There are reports of both beneficial and antagonistic effects on rooting when shoots are treated with phenolic compounds in addition to auxin (MACHACKOVA & al., 1975; STONIER & al., 1979; JAMES & THURBON, 1981; WELANDER & HUNTRIESER, 1981). However, the significance of the effect of phenolic compounds has not yet been clearly established because rooting was generally not considered as a sequence of processes, or when it was, the effect of phenolic compounds was only assayed during one phase of the rooting process.

An objective of the present work was to determine whether phenolic compounds which act as inhibitors or activators of peroxidase activity (TOMASZEWSKI & THIMANN, 1966; MACHACKOVA & al., 1975; STONIER & al., 1979; GASPAR & al., 1982; LEE & al., 1982) may improve or retard the frequency of rooting of shoots of *S. giganteum*, depending on the time of their application in relation to the peak in peroxidase activity. In addition, the relationship between the effect of phenolic compounds on rooting and their effect on the peroxidase activity in extracts made from shoots was investigated in order to develop an assay that may be used to predict the effect of other phenolic compounds on rooting.

Materials and methods

Growing conditions and rooting media. — Shoots of one year-old *Sequoiadendron giganteum* (Lindl.) Buchholz plants were micropropagated as described previously (BERTHON & al., 1991) and then transferred to an elongation medium (BERTHON & al., 1987) for two months before performing rooting assays. Ten mm-long shoot tips were then transferred to the first rooting medium (further referred to as induction medium) consisting of half-strength Knop's salts plus 2 mg/l glycine, 1 mg/l nicotinic acid, 1mg/l pyridoxine, 1 mg/l thiamine, 50 mg/l myo-inositol, 10 mg/l Vitamin D2, 5 g/l sucrose, 1 mg/l NAA, with the pH adjusted to 5.5 with KOH before the addition of 6 g/l bacto-agar. After 7 days on induction medium, the shoots were transferred to expression medium (induction medium minus NAA and Vitamin D2). After 15 days on expression medium, 24 plants were used for the determination of each rooting percentage. Three repetitions were made. Phenolic compounds were added at 1 mM, to the induction and expression media, prior to pH adjustment. Cultures were maintained at 22° C under a 16 h photoperiod of a 17 W/m^2 irradiance produced by Sylvania Grolux fluorescent tubes.

Peroxidase activity. — Whole shoots, growing on elongation medium, were removed and 300 mg fresh weight were ground in 0.6 ml 0.01 M phosphate buffer solution, pH 7.0. The resulting homogenate was centrifuged at 20,000 g for 20 min. The supernatant was recentrifuged at 10,000 g and used as the extract for the peroxidase assay. The peroxidase assay was performed in the presence of 0.02 M $H_2 O_2$ and 0.16M guaiacol (BERTHON & al., 1987). Peroxidase activity was expressed as the increase of absorbance at 470 $nm.min^{-1}.mg\ protein^{-1}$. Protein was assayed using Coomassie blue (SPECTOR, 1978). Phenolic compounds were added to the enzyme extract at concentrations of 0.1 mM. The effects of the phenolic compounds were compared to a control without phenolic compounds. Nine phenolic compounds were evaluated and three replicates of each assay were performed with the same enzyme extract. Three different enzyme extracts were used.

Statistical analysis. — The properties of a phenolic compound's capacity to inhibit or stimulate the rhizogenesis during the first phase and during the second phase of the rooting process were analysed. For each compound, we tested its capacity expressed in the percentage of rooted explants. The relations between these two properties were tested in the aggregate for all the compounds combined by the means of a linear correlation (RL). We mentionned the value of this correlation and the level of the associated significance. For being accurate on the significance of the relation of the measured properties, we have calculated the Spearman coefficient of rank correlations (RS) which is not sensible to the deviations to the normality.

Linear correlation and Spearman coefficient of rank correlations were also calculated for the relations between phenolic compounds effects on the rhizogenesis percentage and their effects on the peroxidase specific activity of extracts made from shoots, before induction of rhizogenesis.

Results

The effects of the nine phenolic compounds on rooting, when applied during the induction or expression phases of rhizogenesis, are shown in Fig. 1. We distinguished three groups of phenolic compounds based on their effect on rooting. The first group increased the percentage of rooting when added during the induction phase of rhizogenesis, the second group increased the percentage of rooting when added during the expression phase, and the third group had no effect on the rooting percentage when applied at either phase. Those compounds that increased the percentage of rooting during one phase of rhizogenesis decreased the percentage of rooting during the other phase ($RL = -0.93$, $P < 0.001$, $RS = -0.975$, $P < 0.001$).

These same phenolic compounds were also evaluated for their effect on peroxidase specific activity of extracts made from shoots before tranferring to rooting media (Table I). Some of the compounds increased the specific activity, to as much as 139% of the control level, while other compounds decreased the specific activity, to as little as 58 % of the control level.

A correlation was found between the effect of phenolic compounds on the rooting percentage (Fig. 1) and the effect of the compounds on the peroxidase specific activity in a shoot extract (Table I). This relationship is presented as an orthogonal regression in Fig. 2. A positive correlation ($RL = 0.77$, $P = 0.01$, $RS = 0.812$, $P < 0.01$) was seen between the effect of phenolic compounds on rooting percentage, when applied during the induction phase of rhizogenesis, and the effect of the compounds on the peroxidase specific activity of an extract made from shoots, before the induction of rhizogenesis.

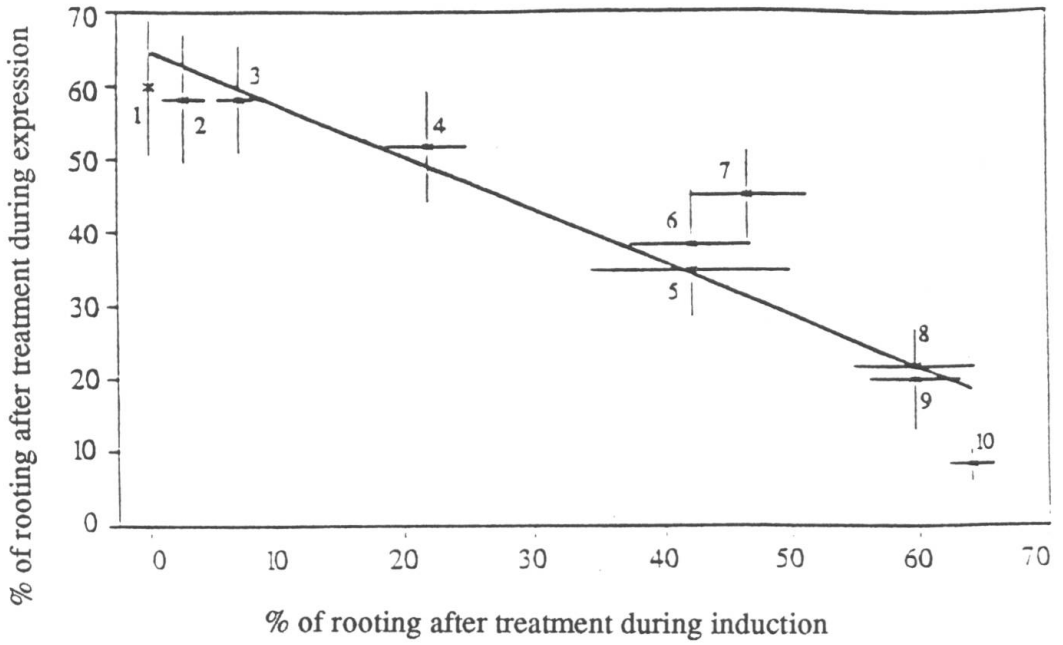

Fig 1. — Effect of phenolic compounds on rooting when applied during induction or expression phases of rhizogenesis. Orthogonal regression and correlation (RL = —0.93, P < 0.001; RS = —0.975, P < 0.001) obtained after treatment with phenolic compounds during the induction or expression phases of rhizogenesis. Phenolic compounds: rutin, 1; quercetin, 2; chlorogenic acid, 3; caffeic acid, 4; ferulic acid, 5; no phenolics applied, 6; naringenin, 7; protocatechuic acid, 8; coumaric acid, 9; gallic acid, 10. Bars represent the standard error.

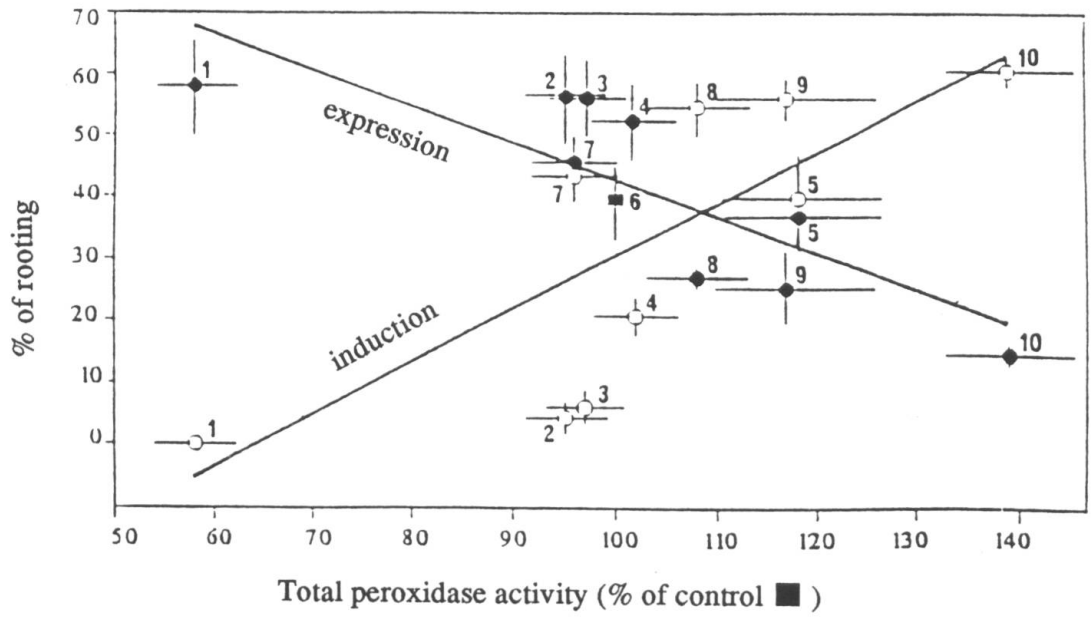

Fig 2. — Correlation between the effect of phenolic compounds on rooting and the effect of phenolic compounds on peroxidase specific activity. Orthogonal regressions and correlations between the effect of phenolic compounds on the rate of rooting, when applied at the induction (RL = 0.77, P = 0.001; RS = 0.812, P < 0.01) or expression phases (RL = —0.82, P = 0.004; RS = —0.887, P < 0.01) of rhizogenesis, and the effect of the phenolic compounds on peroxidase specific activity when added to an extract made from shoots before the induction of rhizogenesis. Phenolic compounds: rutin, 1; quercetin, 2; chlorogenic acid, 3; caffeic acid, 4; ferulic acid, 5; no phenolics applied, 6; naringenin, 7 protocatechuic acid, 8; coumaric acid, 9; gallic acid, 10. Bars represent the standard error.

Table 1. — The effect of phenolic compounds on peroxidase specific activity when added to an extract made from shoots before transfer to rooting media. Specific activity values are represented as a percentage of the specific activity obtained when no phenolic compounds are added to the extract (specific activity of control was 3.1 ± 0.2 U.mn.mg protein).

Phenolic compound	*Peroxidase Specific Activity*
	% of non phenolic-control
rutin	58.1 ± 3.9
quercetin	95.5 ± 1.7
chlorogenic acid	97.2 ± 2.5
caffeic acid	102.2 ± 2.8
ferulic acid	117.5 ± 2.7
no phenolics applied	100
naringenin	96.3 ± 4.1
protocatechuic acid	108.2 ± 2.2
coumaric acid	116.9 ± 2.1
gallic acid	139.7 ± 4.4

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 of rhizogenesis. A negative correlation ($RL = -0.82$, $P = 0.004$; $RS = -0.887$, $P < 0.01$) was seen between the effect of phenolic compounds on the rooting percentage, when applied during the expression phase of rhizogenesis, and the effect of the compounds on the peroxidase specific activity of an extract made from shoots, before the induction of rhizogenesis. For example, phenolic compounds that decreased the peroxidase specific activity of shoot extracts also caused an increase in the rooting percentage when applied during the expression phase of rhizogenesis.

Discussion

Previous work has shown that rhizogenesis in *S. giganteum* is composed of two main phases, induction and expression, each characterized by distinct biochemical events. (BERTHON & al., 1987). Variation in the basic fraction of peroxidase activity served as marker for the induction and expression phases of root formation. These variations in peroxidase activity have been shown in several species (QUOIRIN & al., 1974; CHIBBAR & al., 1979; BHATTACHARYA, 1988).

It has previously been observed that phenolic compounds can influence root formation, as reviewed by DAVIS & al. (1988). Our work also demonstrates that phenolic compounds can affect the rooting process, but also shows that the effect of phenolic compounds have, depends upon what developmental stage the shoot explant is in, when the phenolic compounds are added. We observed that phenolic compounds that increased the percentage of rooting during the induction phase of rhizogenesis, decreased the

percentage of rooting when applied during the expression phase, while phenolic compounds that decreased the percentage of rooting when applied during the induction phase of rhizogenesis, increased the percentage of rooting when applied during the expression phase. These results suggest that phenolic compounds that are known to inhibit root formation (JONES & HOPGOOD, 1979) may actually enhance root formation if applied during the appropriate phase of rhizogenesis.

It has previously been shown that phenolic compounds can increase (TOMASZEWSKI & THIMANN, 1966; MACHACKOVA & al., 1975) or decrease (ZENK & MULLER, 1963; STONIER & al., 1979) the IAA-oxidase-peroxidase activity, as measured during the induction or expression phases of rooting. Our present work shows that there is a correspondence between the effect that phenolic compounds have on rooting, when applied at either the induction or expression phases of rooting, and the effect that phenolic compounds have on the peroxidase activity of extracts made from shoots before rhizogenesis is initiated. For example, compounds that increased the level of peroxidase activity of extracts made from shoots before root induction increased the rooting percentage when applied during the induction phase of rhizogenesis and decreased the rooting percentage when applied during the expression phase.

All phenolic compounds classified as stimulators of root expression were o-dihydric phenols, particularly flavonols like quercetin and rutin. Stimulators of root induction were either monohydric phenols (coumaric acid) or benzoïc acids (gallic acid and protocatechnic acid).

This assay of the effect of phenolic compounds on peroxidase activity of extracts made from shoots before induction was used to predict the effect of applying these compounds during the induction or expression phases of rhizogenesis for *S. giganteum*. This assay allowed us to determine which compound to add, and when it should be added, to increase the rooting percentage of *S. giganteum*. Because both a variation in peroxidase activity in relation to the phase of rooting and a result of increased rooting percentage following the application of phenolic compounds have also been observed for species other than *S. giganteum*, we propose that the predictive assay we used to increase the rooting percentage for *S. giganteum*, may be used to increase the rooting percentage of other difficult-to-root species.

ACKNOWLEDGMENTS

Jean-Yves Berthon is gratefully indebted to the E.E.C. for postdoctoral fellowships. The authors thank Mrs Espagnol and Mrs Faure for their technical assistance.

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