Enzyme Activity During Adventitious Rooting of Stoolbed Propagated Chestnut

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ABSTRACT. Chestnut propagation by the stoolbed technique, after girdling the lower portion of the shoots, has been satisfactorily employed for several years. In order to study the physiological mechanisms that stimulate rooting under these conditions, with special reference to the activity of some enzymes, 10-yr-old stoolbeds of the cultivar `Marigoule' *(Castanea crenata* x C. *sativa)* were subjected to: 1) girdling and etiolation; 2) girdling; 3) etiolation; and, 4) control, or totally untreated stoolbeds. Treatments were made on 20 June, 2 mo after bud break. Shoot samples (basal and sub-apical shoot portions) were collected during the period between 23 May and 30 November, 1 mo after leaf fall, for the determination of the activity of the following enzymes: IAA-oxidase, peroxidase, polyphenoloxidase.

The results of the biochemical analyses showed that enzyme activity in the shoots' basal ends developed according to a pattern in the "girdling and etiolation" treatment (the only one in which adventitious rooting occurred) that was different from the other treatments. The different enzymes had a very similar behavior, with a peak of activity soon after treatment, followed by stagnation in early July, which continued until fall, when enzyme activity dropped to low levels. This course of activity might represent an important component of successful rooting.

Adventitious root formation depends on the anatomical, physiological and biochemical characteristics of the involved plant tissues. Therefore, aspects such as plant age, nutritional status, time of year of tissue excision and its position on the plant must be taken into consideration.

Among the numerous factors that affect rooting, a paramount role appears to be played by some hormonal and enzymic classes, i.e., indole-3-acetic acid (IAA), cytokinins (CK), IAA-oxidases (IAAox), peroxidases (PO) and polyphenoloxidases (PPO) (16). As far as hormonal involvement is concerned, IAA appears to be the most important, although its role is not clearly defined in the literature and opinions differ (13, 25). The bound forms of IAA seem to be related more strictly to root induction than free IAA (24, 32, 34).

The most investigated enzyme classes are PO (4, 12, 21, 22,26,27), IAAox (1, 2, 4) and PPO (3, 4, 21). Their actual role is, however, still unclear although several hypotheses can be found in the literature (3, 4, 26).

With regard to the genus *Castanea,* the information available is scarce (18, 19, 20). The species that belong to this genus have shown satisfactory rooting percentages only when cuttings were made from juvenile plant parts, which are known to have more favorable biochemical and metabolic characteristics for adventitious root formation (16). In order to exploit this peculiarity, a propagation method adopted for other difficult-to-root species, stoolbed layering or mound layering, was successfully utilized with European chestnut and with Euro-Japanese hybrids (7, 8, 14, 31, 33). The scantiness of information available on the role of enzymes in adventitious rooting, especially in chestnut, prompted us to investigate what modifications occurred in the activity of selected enzymes when a ;hoot was induced to form roots.

MATERIALS AND METHODS

Plant material. Suckers were obtained by the stoolbed layering technique on `Marigoule' *(Castanea crenata* Sieb. and Zucc. x *C. sativa* Mill.) stock plants in the Asprofrut experimental field of Spinetta (CN), Italy. One hundred plants were utilized; each produced about 15-20 suckers. The following treatments were applied to the suckers on 20 June 1990: (i) etiolation + girdling; (ii) etiolation; (iii) girdling; and, (iv) control. About 350 homogeneous suckers were chosen for each treatment after the excision of the small ones. Girdling was performed on the suckers with plastic covered iron wire, 2-4 cm from their insertion, and etiolation was obtained by burying the basal part of the suckers with 20 cm of fine, carefully hand-pressed soil. Samples were collected on the following dates: 23 May, 30 May, 18 June, 28 June, 2 July, 5 July, 11 July, 19 July, 25 July, 2 August, 9 August, 21 August, 3 September, 25 September, 5 October, and 30 November. For each treatment and time, 12 samples (3 for each of 4 replicates) were randomly collected and frozen at —20 C until the analyses were performed. From each sampled sucker, a basal part, consisting of a 10 cm portion starting from the point where the shoot was girdled, and an apical part, consisting of the 10 to 20 cm portion from the apex, were excised. Corresponding samples also were collected from non-girdled shoots.

Enzyme extraction. Samples were prepared from the frozen material after removing the oxidized ends and the ice present on the cork surface. Thin sections then were dissected from each sample and the frozen material was homogenized by grinding in a chilled mortar with liquid nitrogen (LN2). One g fresh weight frozen powder was added to 10 ml of ice-cold 200 mM phosphate buffer, pH 7.5, 5 mM disodium EDTA (Na2EDTA), 1 g polyvinyl-

polypyrrolidone (PVPP). The slurry was hand-homogenized with a spatula and maintained in an ice bath for 30 min; it was then filtered through four layers of nylon mesh and the liquid was centrifuged for 20 min at 14,500xg at 4 C. The volume of the supernatant was measured, and represented the crude extract for enzyme analyses.

Enzyme determination. IAAox *activity*. In our assay, the substance acting as the indicator of IAA catabolism was 3-methylenoxindole, with maximum absorbance at 254 nm. IAA-oxidase activity was determined according to a procedure adapted from Rubery (29) and Ricard and Job (28). The reaction was triggered by the addition at regular intervals (30 sec) of 100 ul of the enzyme extract to 3 ml of a mixture of: a) 50 mM phosphate buffer (NaH2PO4); b) 1mM MnC12 x 4H20; c) 100µM paracoumaric acid; and, d) 50uM indole-3-acetic acid (first dissolved in 100% methanol, and then, slowly, in distilled water, to form an hydroalcoholic solution, with 80% water). The mixture was adjusted to pH 4.5 with orthophosphoric acid. The test tubes containing the reaction mixture and the enzyme extract were kept in a 35 C thermostatic bath for 30 min. Determination of the enzyme activity was made spectrophotometrically (Perkin Elmer spectrophotometer, model 124, Oak Brook, Ill.), at regular intervals (30 sec), with the reaction mixture as a blank.

Peroxidase activity (PO). Hydrogen peroxide (H202) was utilized as substrate with guaiacol as the donor (10). The reaction was triggered by the addition of 10 ,u1 of 40 mM peroxide to a mixture of 1 ml of 10 mM, pH 7, monophosphate buffer, 2 ml of 20 mM guaiacol, and 100 ,u1 of enzymic extract. The test tubes were then placed in a 20 C bath, and absorbance was read at 470 nm 10 min after the start of the reaction. A blank consisted of the reaction mixture without the addition of enzyme extract.

Polyphenoloxidase activity (PPO). Pyrogallol was used as a substrate, by adapting the procedure of Canal et al. (9), and Shinshi and Noguchi (30). The reaction mixture in this case was made of 2.8 ml of pH 7, 100 mM phosphate buffer (Na2PO4), ,u1 of 25 mM pyrogallol, and 100 l of enzyme extract. Spectrophotometric readings were made at 420 nm after 30 min at 20 C.

Protein content. The determination of the extract's protein content was made in accordance with the Bradford procedure (6), and using the Bio-Rad solution (Bio-Rad laboratories, Munich, Germany). The protein standard was Sigma crystallized bovine albumin (Sigma, St. Louis, Mo.).

RESULTS

Only girdled and etiolated shoots produced roots (69.3% of rooted suckers). The first growing roots could be detected, immediately above the girdle, as late as the end of August or early September.

IAA oxidase. The activity of this enzyme in the basal part of the shoot, above the girdle, often appeared to be higher in the "girdling and etiolation" and "girdling" treatments (Figure 1); more specifically, a stronger activity was apparent on 28 June for "girdling and etiolation," and in the last samples for both girdling treatments. Only very late in the season (30 November) was a sharp drop of enzyme activity apparent in the "girdling and etiolation" treatment, while in the other treatments such activity kept increasing steadily. In the apical part of the shoot, a marked drop of IAA oxidase took place in the etiolated shoots, soon after the treatment. Later on, the differences among treatments were less dramatic (Figure 2).

Peroxidase. Peroxidase activity increased sharply through the season in all treatments above the girdling point, particularly in the September—October girdled treatments (Figure 3). At the end of November, a marked reduction of activity was noticed in the "girdling and etiolation" treatment. In the apical part of "etiolated" shoots, peroxidase activity dropped soon after treatment (end of June). The reduction in peroxidase activity was markedly less than IAA oxidase. The levels of activity remained low for all treatments on 2 July, with the exception of the untreated control, however, the differences among treatments throughout the season were never very great.

Polyphenoloxidase. Above the girdle, a sharp increase in this enzyme's activity was evident in the "girdling and etiolation" treatment, soon after the treatment was made 28 June (Figure 4). The activity of this enzyme appeared to be very similar to that of IAA oxidase, with regard to the two girdling treatments. In the late summer—early

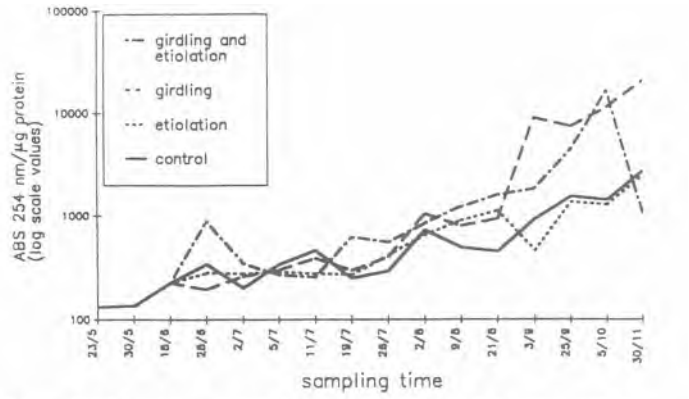

Figure 1. IAA-oxidase activity in the basal portion of chestnut suckers.

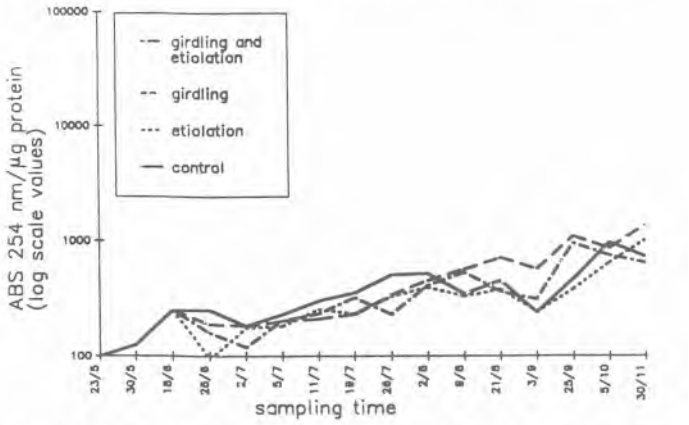

Figure 2. IAA-oxidase activity in the apical portion of chestnut suckers.

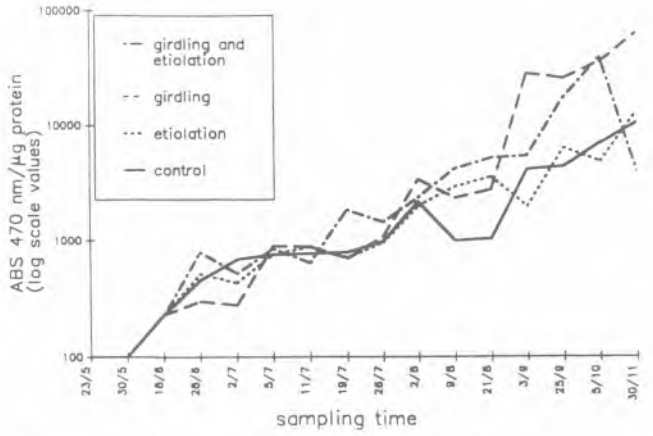

Figure 3. Peroxidase activity in the basal portion of chestnut suckers.

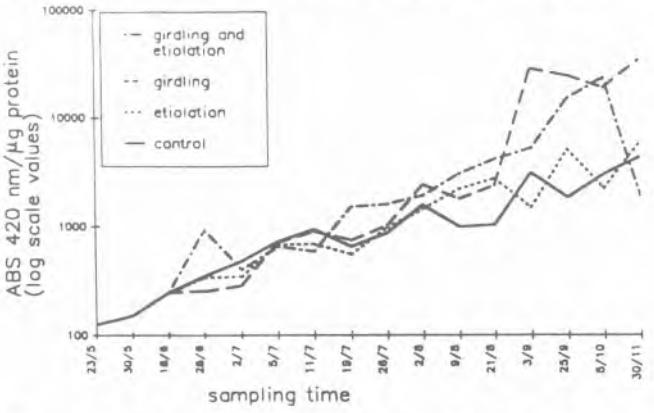

Figure 4. Polyphenoloxidase activity in the basal portion of chestnut suckers.

autumn period, the two girdling treatments markedly increased their polyphenoloxidase activity, but the "girdling and etiolation" treatment showed a drop in October—November. At the distal end of the shoot, the increase of activity was gradual throughout the season, and the differences among treatments were not significant (data not shown). The only interesting event was a drop in activity during the 2 wk following the treatment for all treatments, except the untreated control.

DISCUSSION

A reaction was apparent in all tested shoot portions in the 10-day period following the treatments. Above the girdling point, the enzyme activity was strongly enhanced in the "girdling and etiolation" treatment, especially with reference to IAA-oxidase and PPO, while the opposite occurred in the "girdling" treatment. "Etiolation" alone did not modify the enzymic pattern in the etiolated zone, but caused a sharp drop in activity in the shoot apex, for all enzymes tested. The effect was similar, but delayed a week in the "girdling and etiolation" treatment. "Girdling" was much less affected, and only with regard to PPO.

During July, the values tended to remain similar, although somewhat lower for "etiolation" and "girdling and etiolation." However, in the second half of the month the effects of treatments became apparent in "girdling and etiolation" where the enzymes became more active, and remained so throughout the season. By the end of July the same occurred with "girdling," although in this treatment enzyme activity was far more fluctuating. Also in "etiolation," we observed a similar fluctuation, but never too far from the control levels.

The increase of enzyme activity in the two girdled treatments may have been related to the fact that girdling hampered the flow of assimilates and hormones towards the roots, and caused an accumulation of such substances above the girdle. This would induce an increase of the enzymic activity aimed at reducing the excessive concentration of certain substances, such as hormones and cofactors (15).

The last important modification in enzyme activity was the reaction to "girdling and etiolation" in October—November, where, unlike "girdling," all enzyme activity dropped to rest levels. The difference might be explained by the presence of young, superficial roots above the girdling point. The mound was very much exposed to the environment, and the roots were very unlikely to perceive water or temperature stress. The plantlets that formed from most of our girdled and etiolated shoots, would therefore readily slow or stop their activity when the weather became unfavorable.

With regard to adventitious rooting, the enzymic activity of the "girdling and etiolation" treatment differed from the others mostly in its increase soon after treatment and in its high levels in the second half of the summer. This type of enzymic activity would agree with the scheme proposed by other authors (1, 4, 11, 17, 23). Accordingly, the induction phase would require low IAAox activity, which would then appear enhanced in the course of early initiation, phase. In our "girdling and etiolation" material, IAAox was significantly lower than in the control on 5 July and 11 July, and dramatically increased in the second half of the month (19 July and 26 July), about 2 wk before the first anatomical signs of the formation of root initials (5). This type of activity might make the difference with regard to the stimulation of new root formation, when compared to the other treatments, including "girdling"; the increase in activity might have been too late and have fluctuated too widely to be effective.

"Girdling" or "etiolation," alone induced minor effects on enzyme activity in the shoot zone where roots are usually formed, at least in the root induction period, and had no effects on rooting. Still, their combination had profound effects on the shoot tissues, and roots were eventually formed; even when the changes appeared to be too early to influence rooting (late June), the impact of the combined treatment proved to be dramatic. Enzyme activity was certainly not the only process to be affected by the treatments and other substances and biological processes will have to be investigated in future studies of adventitious rooting in chestnut. Nevertheless, this research shows that enzyme activity may be a suitable indicator of the regenerative activity within the tissues of a layered shoot.

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