

The apparent involvement of polyphenol oxidase and phloridzin in the production of apple rooting cofactors

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SUMMARY

Polyphenol oxidase (PPO) activity and phloridzin content in vacuum-extracted xylem sap of M.26 apple cuttings increased between mid-December and late January, preceding an increase in endogenous rooting cofactor activity and the rooting of cuttings between late January and mid-February 1979. When tested by the mung bean bioassay in the presence of indolylbutyric acid (IBA) many phenolic substances improved rooting, especially oxidized forms and derivatives. The product of a reaction between polyphenol oxidase (PPO) and phloridzin *in vitro* was markedly active in the mung bean test and showed certain similarities with the extracted endogenous cofactor following chromatography. The cofactor activity of sap extracted during the period of poor rooting in midwinter was increased by adding both PPO and phloridzin and the rooting of IBA-treated cuttings was increased by pre-treatment with the products of a PPO and phloridzin reaction.

MANY phenolic substances, especially after oxidation, promote adventitious rooting (Jankiewicz *et al.*, 1973; Poapst and Durkee, 1967; Poapst *et al.*, 1970) and several workers have extracted from woody plants root promoting cofactors characterized as phenolic (Fadl and Hartmann, 1967; Girouard, 1969; Hess, 1964; Lipecki and Dennis, 1972) and/or water soluble (Bojarczuk, 1978; Kawase, 1971; Tognoni *et al.*, 1977). Phenolic cofactor activity in vacuum-extracted sap from winter cuttings of the M.26 apple rootstock is closely correlated with their seasonal rooting pattern (Bassuk and Howard, 1981b).

Considerations of auxin and phenol chemistry (Leopold and Plummer, 1961) and of the possible mode of action of auxin synergists have led to the suggestion that rooting is directly stimulated by the formation of an auxin-phenol complex, its synthesis requiring the presence of a polyphenol oxidase enzyme (Bouillenne and Bouillenne-Walrand, 1955; Haissig, 1974). Such auxin-phenolic conjugates have not been identified and theories on their mode of action need to embrace

the fact that both indole and non-indole auxins are effective in promoting rooting. Against this background, studies of the mechanism of seasonal rooting fluctuations in M.26 apple cuttings were undertaken to investigate the possible involvement of polyphenol oxidase and phenolic substances.

MATERIALS AND METHODS

General

Sap was extracted from M.26 cuttings at intervals during the winter coincident with propagation tests and stored at -20°C for subsequent investigation (Bassuk and Howard, 1981b). The rooting activity of both extracted and synthesized products was examined by a modified mung bean bioassay (Bassuk and Howard, 1981a).

Polyphenol oxidase

On six occasions between 7 November 1978 and 26 March 1979 inclusive, sap from four replicates, each comprising six to nine cuttings, was analysed for relative PPO activity. Absorbance was measured with a Brinkmann PC 600 colorimeter at 420 nm using as substrate 0.25% catechol in pH 6.5 10^{-2} M phosphate buffer. Readings were taken, including a blank of

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buffered catechol to allow for auto-oxidation, immediately after making up test solutions and after the reaction period, usually 22 h. Results are expressed on a tissue FW basis as follows:

$$\frac{[\text{Final optical density} - (\text{initial sap} + \text{final catechol blank readings})]}{\text{ml sap}} \times \frac{\text{g residual FW}}{\text{g residual FW}} \times 1000.$$

PPO for cofactor synthesis was obtained either freeze dried (Sigma, specification tyrosinase 4000 units, PPO 94 370 units and catecholase 277 600 units mg^{-1} solid) or by macerating 20 g fresh mushrooms with 200 ml 10^{-4} M citric-citrate pH 6.5 buffer at 1°C in a Waring blender before filtering through cheese cloth. When denatured PPO was required, a stock solution (see below) was boiled for 20 min and cooled to 25°C before testing.

Phloridzin

Seasonal changes of phloridzin were estimated by measuring UV absorbance at 283 nm after descending chromatography (see below). After drying for 24 h the phloridzin spot was identified by comparison with a phloridzin marker under UV light of 365 nm and eluted in methanol, re-run and finally eluted for 25 h in 5 ml ethanol. Absorbance, typically the mean of four replicates, is expressed in relation to tissue FW as follows:

$$\text{Absorbance} \times \frac{\text{ml sap}}{\text{g residual FW}} \times 1000.$$

Cofactor synthesis

0.01 g PPO (Sigma) was dissolved in 100 ml 10^{-4} M citric acid-sodium citrate pH 6.5 buffer at 1°C . 1 ml of this stock solution was added to 1 ml 8×10^{-3} M phloridzin prepared by dissolving phloridzin in distilled water using a hot plate-stirrer and then cooling to 25°C . The mixture was applied to mung bean cuttings in the presence of 2 ml of 4 ppm indolybutyric acid (IBA) as the ammonium salt. Final concentrations were 400 units tyrosinase activity, 2×10^{-3} M phloridzin and 10^{-5} M (2 ppm) IBA. In addition to a 'single' reaction in which PPO and phloridzin were mixed together and allowed to react for periods up to 24 h, a 'continuous' reaction was set up in the hope that this would reflect better the balance of cofactors produced *in vivo*. 100 ml of 8×10^{-3} M phloridzin and PPO at 0.01 g in 100 ml buffer were simultaneously pumped into a beaker at the rate of 2 ml of solu-

tion h^{-1} using a Watson-Marlow peristaltic pump. Over a 21-h period 2 ml aliquots of the beaker contents were removed at intervals and added to 8 ml methanol to stop the reaction. Samples were then concentrated for chromatography and bioassay.

Chromatography

The products of single and continuous PPO-phloridzin reactions were run on chromatograms, prior to bioassay after evaporation under nitrogen, while being heated in a water bath at 50°C until reduced to 0.2 ml. Whatman 3MM paper was used with *n*-butanol-acetic acid-water (63-10-27) as solvent followed by 24 h drying. Chromatograms were inspected under UV light after fuming with ammonia and the margins of coloured bands marked lightly with pencil, cut out and eluted with a 2 ppm solution of IBA in the bioassay vial.

Application of cofactor to M.26 cuttings

In 1977-78 typical M.26 cuttings were stood to a depth of 2 cm in solutions comprising 100 ml IBA (K salt) at 150 ppm, 50 ml 8×10^{-3} M phloridzin and 50 ml of 100 000 ppm crude mushroom tyrosinase to give final concentrations of 75 ppm IBA, 2×10^{-3} M phloridzin and 25 000 ppm tyrosinase extract. Where the experiment required a component to be omitted the volume was made up with distilled water. Cuttings were removed from the solutions after 24 h and allowed to dry for 1 h before planting.

In January 1979 a saturated solution of phloridzin (400 ml 10^{-2} M) was reacted with 40 ml PPO (Sigma, 0.04 g/40 ml) for 24 h at 25°C and evaporated to dryness under vacuum. The products were taken up in 25 ml 50% aqueous acetone and a dilution series prepared $\times 1$, $\times 0.5$, $\times 0.25$, $\times 0.125$ the original concentration. An equal volume of 5000 ppm IBA in 50% aqueous acetone was added to each solution to halve all concentrations and cutting bases were treated for 5 s.

RESULTS

Activity of phenolic compounds

The positive response of sap components to diazotized *p*-nitroaniline, ferric chloride and Salkowski reagent together with their fluorescence or absorption under UV suggested that they were phenolic compounds. Therefore 32 phenolics were dissolved in methanol and run on

a chromatogram in parallel with apple sap, eluted to give a concentration of 2×10^{-3} M, and tested in the mung bean bioassay. Many promoted rooting (Table I), especially phloridzin derivatives such as phloretic acid and phloroglucinol, but only phloridzin and chlorogenic acid corresponded well with constituents of apple sap.

TABLE I
Mung bean response to phenolic compounds, all with
2 ppm IBA added

Compound	Mean No. of roots per cutting
*control (2 ppm IBA)	7.8
*phloroglucinol	21.6
*phloretin	19.5
*phloretic acid	33.5
ferulic acid	20.3
vanillic acid	11.5
*p-coumaric acid	24.3
3, 4-dihydroxybenzoic acid	15.5
umbelliferone	25.5
*phloridzin	11.0
*caffeic acid	29.0
aesculin	12.3
methylumbelliferone	18.7
cinnamic acid	25.3
coumarin	11.0
phenylpropionic acid	22.5
naringenin	13.5
*catechol	17.2
methyl catechol	17.5
syringic acid	18.0
umbellic acid	7.8
quinic acid	8.4
gallic acid	18.5
o-coumaric acid	12.0
3, 4 methylenedioxycinnamic acid	15.0
*trilobatin	11.5
seiboldin	15.0
chlorogenic acid	13.0
juglone	13.5
melilotic acid	11.0
quinol	14.0
quinone	17.0
3-hydroxyphloretin	15.5

For the best replicated compounds marked * the value of the lowest replicate was greater than that of the best control replicate, except for phloridzin and trilobatin.

Seasonal assessment of PPO and phloridzin

PPO activity and phloridzin content increased significantly ($P < 0.001$ and $P < 0.01$ respectively) in late January and early February 1979, preceding the rise in M.26 rooting and cofactor activity in sap (Figure 1).

Reaction of PPO and phloridzin in vitro

All relevant combinations of PPO (active and denatured), phloridzin and IBA were tested on mung beans, each with six replicates of four

cuttings. Rooting was slightly but significantly increased by many treatments, especially where IBA was present, but a threefold increase in mung bean rooting was seen in the presence of PPO, phloridzin and IBA compared to the response obtained when the PPO was denatured (Figure 2).

PPO and phloridzin were reacted for 15 min, 1 h and 21 h, the products separated by chromatography and assessed for root-promoting activity by the mung bean bioassay in the presence of IBA. Many of the products were similar in colour and location to those from endogenous sap (see below) and are described here using the code previously suggested (Bassuk and Howard, 1981b). Synthesized cofactors 2A, 2B, 3A and 3B maintained or increased their activity with time, except for 2A at 1 h. The activity of cofactor 1 decreased ($P < 0.001$) between 15 min and 21 h (Figure 3, top). The continuous reaction, in which PPO and phloridzin were pumped into a beaker, was stopped at 15 min, 1 h and 21 h and it was found that the activity of cofactor 1 did not decrease (Figure 3, bottom). Six other phenolic substances were tested separately and in all combinations with PPO and IBA. Trilobatin, which differs from phloridzin only in the position of the glucose attachment, gave a similar large increase in activity in the presence of PPO and IBA to that previously obtained with phloridzin. Other substances also reacted positively with PPO and IBA, but IBA alone was frequently equally or almost equally effective (Figure 4).

Comparison of synthesized and endogenous cofactors

Products of the PPO-phloridzin reactions were compared after chromatography with those of the endogenous sap from cuttings with good rooting potential (22 March 1979) in the mung bean bioassay. Good correlation was obtained up to R_f .51 (Figure 5). The first two sections of the chromatograph of synthesized products were cut identically to those of endogenous sap because they lacked UV detectable coloured bands. The considerable similarity of activity from R_f .20 to .51 was supported by close correlations between orange coloured segments from both chromatograms at R_f .33-.41 and .45 to .51. Towards the solvent front coloured segments from the two sources differed with the exception of the absorbent spot corresponding to phloridzin at R_f .69-.80 and .69-.81 for endogenous and

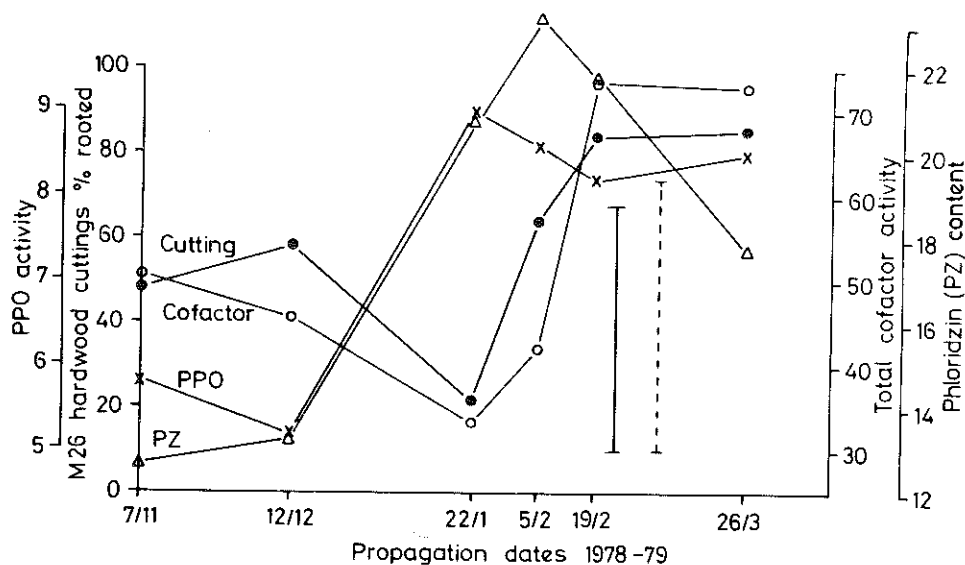


FIG. 1
Polyphenol oxidase activity and phloridzin content related to cofactor activity and rooting of M.26 cuttings
Bars = LSD ($P < 0.05$) (solid PPO, broken PZ)
Rooting-cofactor correlation coefficient $P < 0.001$

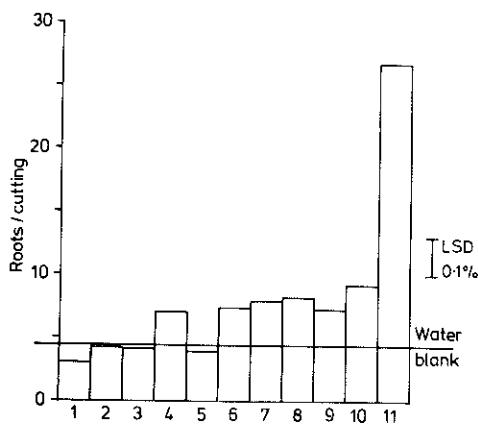


FIG. 2
Mung bean response to polyphenol oxidase, phloridzin and IBA

1. Phloridzin (PZ) 2×10^{-3} M
2. Denatured polyphenol oxidase (PPO) 400 units
3. PPO
4. Indolybutyric acid (IBA) 2 ppm
5. PZ + denatured PPO
6. PZ + PPO
7. PZ + IBA
8. IBA + denatured PPO
9. IBA + PPO
10. IBA + PZ + denatured PPO
11. IBA + PZ + PPO

synthesized products respectively. Root-promoting activities of the cofactors from both sources were of the same relative order of magnitude, the largest differences being the reduced activity of the first and third segments from the synthesized products.

Amelioration of winter sap by the addition of PPO and phloridzin in the presence of IBA

400 units of PPO with and without 2×10^{-3} M phloridzin were added to low activity sap from poorly rooted cuttings collected on 19 January 1978 and the resulting activity in the mung bean bioassay after chromatography compared with that in sap from cuttings collected on 26 March 1979 when rooting was high. The addition of phloridzin alone increased activity ($P < 0.001$) at R_f .57-.69 and .69-.80 (cofactors 3A and 3B). The addition of PPO alone also increased activity ($P < 0.001$) at R_f .33-.41 and .45-.51 corresponding to the two orange bands of cofactors 2A and 2B. By adding both substances to winter sap, activity increased as before and also in the position of cofactor 1B at R_f .06-.20 (Figure 6).

Application of synthesized cofactors to M.26 cuttings

In the first experiment the basal 2.5 cm of

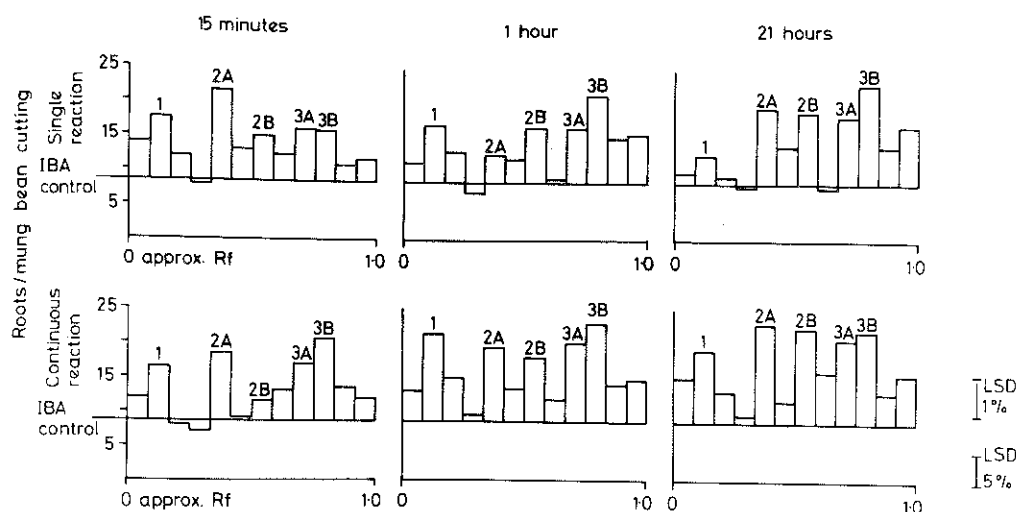


FIG. 3
Mung bean response to synthesized cofactors from a single (top) and continuous (bottom) reaction of polyphenol oxidase and phloridzin

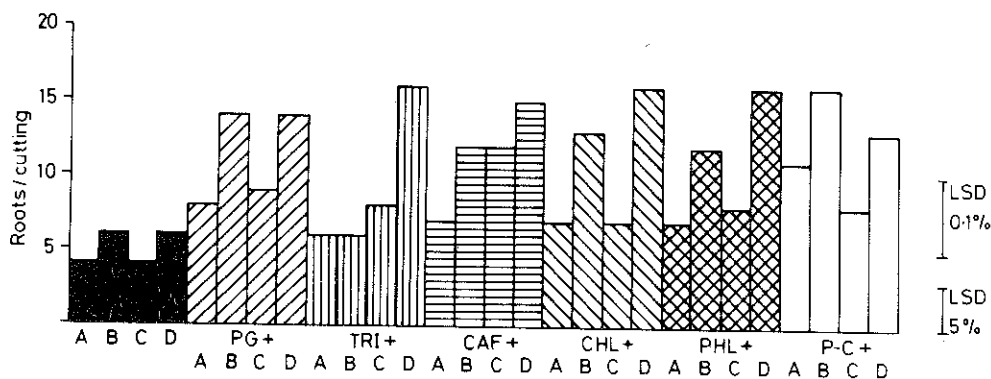


FIG. 4
Mung bean response of 6 phenolic substances (all at 2×10^{-3} M) with and without polyphenol oxidase (PPO) and indolybutyric acid (IBA)
PG = phloroglucinol, TRI = trilobatin, CAF = caffeic acid
CHL = chlorogenic acid, PHL = phloretin, P-C = paracoumaric acid.
A = H₂O, B = IBA 2 ppm, C = PPO 400 units, D = IBA + PPO

M.26 cuttings were soaked for 24 h in aqueous solutions of the following, prepared as described under Materials and Methods:

- (1) 75 ppm IBA,
- (2) mushroom extract,
- (3) IBA and mushroom extract,
- (4) IBA, mushroom extract and phloridzin (2×10^{-3} M).

An additional control (5) in the form of the standard 5-s treatment with 2500 ppm IBA in 50% acetone was included. Five replicates, each

of 10 cuttings, were used for each treatment, applied on 27 February 1978 which subsequently proved to be a period of good rooting. Mushroom extract with or without IBA significantly ($P < 0.05$) improved rooting over IBA alone, while treatment with mushroom extract, phloridzin and IBA further improved rooting ($P < 0.001$). The standard IBA treatment of 2500 ppm IBA gave the higher rooting however (Figure 7, top).

In a second experiment on 22 January 1979, at a time when cuttings were rooting poorly, a 5-s

Apple rooting cofactors

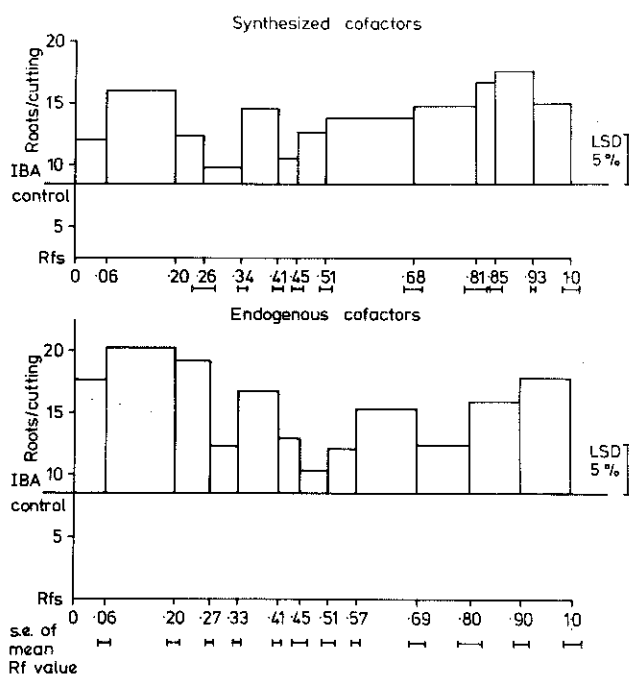


FIG. 5
Mung bean response to synthesized (top) and endogenous (bottom) cofactor

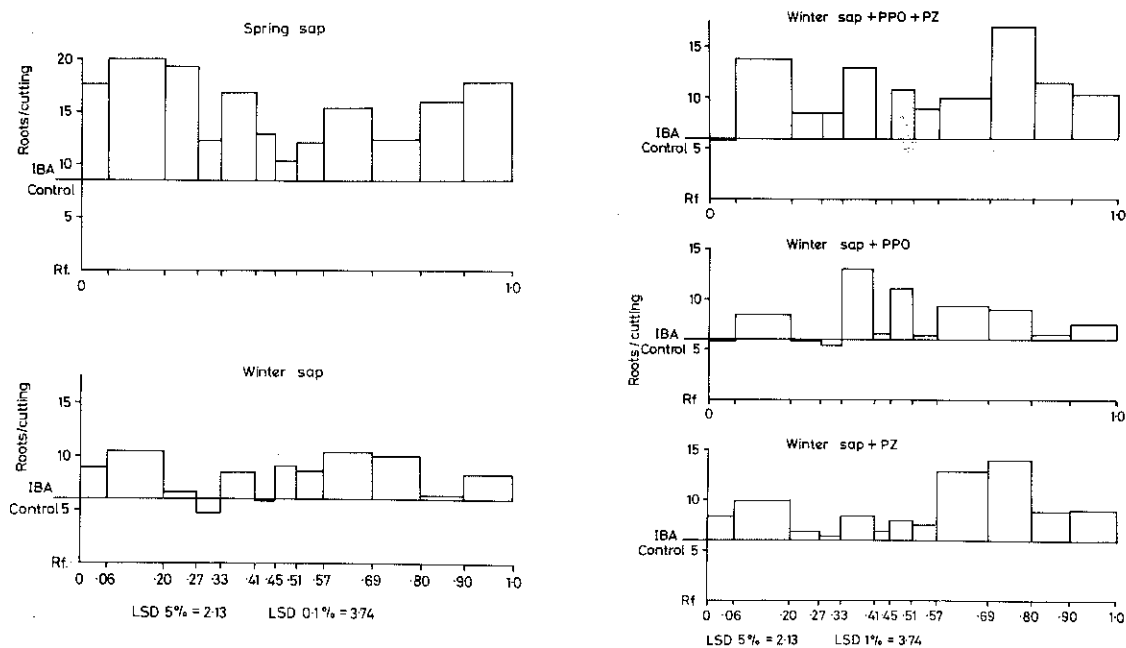


FIG. 6
Mung bean response to M.26 winter-collected sap with polyphenol oxidase (PPO) and/or phloridzin (PZ) added, compared to spring-collected sap

basal dip in 2500 ppm IBA in 50% acetone was given with or without the products of the PPO-phloridzin reaction added in a dilution series (see Materials and Methods).

There was no effect due to concentration and so analysis of variance was carried out for IBA only (10 replicates of 5 cuttings) and for IBA with PPO-phloridzin products (20 replicates), the latter treatment significantly ($P < 0.03$) improving rooting (Figure 7, bottom).

DISCUSSION

Phenolic substances were found generally to promote rooting in the presence of IBA. Although phloridzin, the major glycoside of apple (Williams, 1964), was not particularly root-promoting, its derivatives were very active, as is also the case *in vitro* (Jones and Hatfield, 1976). While it is claimed that phloretic acid, and sometimes phloroglucinol, have been identified in apple and other plants (Durkee and Poapst,

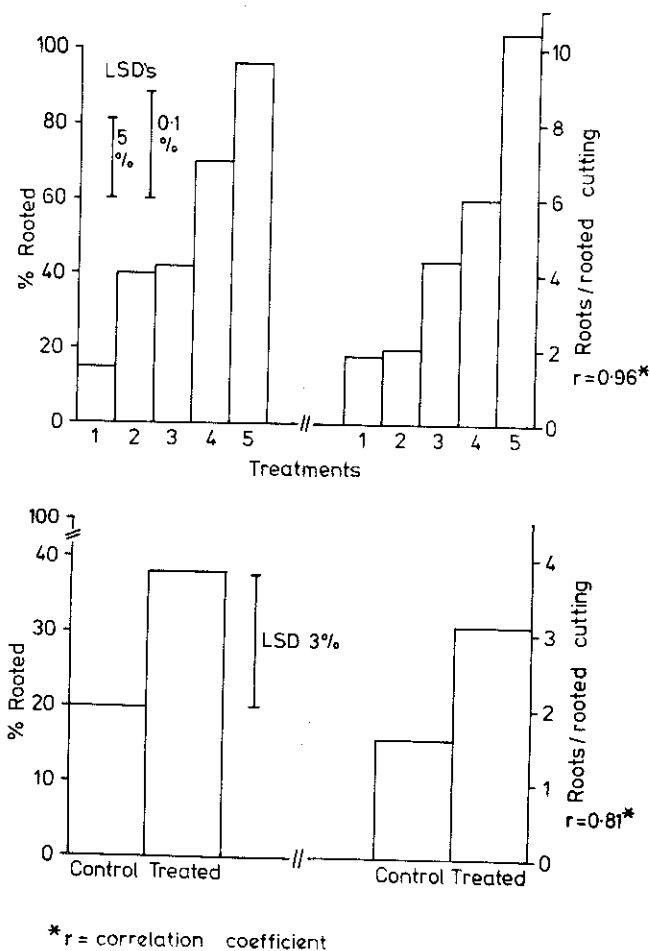


FIG. 7
 M.26 cutting response to combinations of mushroom tyrosinase (PPO enzyme), phloridzin (2×10^{-3} M) and indolylbutyric acid (IBA), from 27 February 1978 (top) and to the products of the reaction applied in 50% acetone together with IBA at 2500 ppm from January 1979 (bottom). 1 = IBA 75 ppm, 2 = mushroom extract, 3 = IBA and mushroom extract, 4 = IBA, mushroom extract and phloridzin, 5 = IBA 2500 ppm/50% acetone

1965; Grochowska, 1967; Ibrahim and Towers, 1960) it is generally accepted that if full precautions are taken to stop enzyme activity only phloridzin will be found. Nevertheless, the rise in PPO activity in vacuum extracted sap, coincident with the increase in phloridzin, suggests a causal relationship with cofactor activity which correlated well with subsequent rooting of cuttings (Bassuk and Howard, 1981b). A complex system of (apple) enzymes is involved in the production of such metabolites as quinones, phloroglucinol and phloretic acid from phloridzin (Hunter, 1975). Following tissue damage at cutting collection enzyme and substrate release could result in metabolite production. Several of these metabolites, when tested on mung beans with IBA present, increased the levels of rooting by factors of from two to four over the levels obtained with IBA alone (Table I). Although an indole-phenol complex could result, such a substance has not been found *in vivo* and it may be sufficient that auxin and cofactor is each available at the rooting site in adequate amounts.

Polyphenol oxidase has been found in various cell organelles in higher plants both in soluble and membrane-bound form. Tomaszewski and Wojciechowska (1973) suggested that fungal mycelia excreted an oxidizing enzyme into the culture medium, and Linderman and Call (1977) found that arctostaphylos cuttings, which show similar seasonal rooting patterns to apple, rooted better in December when the propagation medium had been inoculated with mycorrhizae. Molnar and La Croix (1972a) found that oxidative enzyme activity in stem tissues of *Hydrangea macrophylla* rose dramatically during root development. The fact that this plant contains preformed root initials (Molnar and La Croix, 1972b) may link any causal relationship with root development rather than initiation.

Injury, along with various stress factors, is known to trigger an increase in PPO activity (Mayer and Harel, 1979) and there is the possibility that in the work reported here PPO was released by cell disruption during extraction. However, such an artefact would probably be similar at each date, and investigation so far provides no evidence that it occurred (Harrison-Murray, 1980).

The effectiveness of enzymes in reacting with phloridzin to produce substances with marked bioassay activity in the presence of IBA was clearly shown in these studies (Figures 2, 3, 5)

supported by a similar response from trilobatin (Figure 4) of which the structure is comparable with that of phloridzin and which in the absence of PPO promotes rooting equally weakly (Table I). Poapst and Durkee (1967) demonstrated improved rooting of *Phaseolus vulgaris* cuttings by adding the oxidized forms of various phenolics. The partial similarity of chromatograms from sap and the PPO-phloridzin reaction (Figure 5) supports the view that these are important in the cutting. The powerful cofactor 1 from the reaction *in vitro* was not visible under UV in contrast to that from the sap, which implies that more than one substance is present on the paper in that position. The synthesis of cofactor 1 suggested that it might be short-lived, activity decreasing markedly after 21 h compared with that maintained in the continuous reaction. If a PPO-phloridzin reaction occurs in the cutting it is possible that this ephemeral cofactor, which may be only partially oxidized, is prevented from further oxidation by reducing agents such as ascorbate (Hunter, 1975). It appears that at least five of the eight cofactors tentatively identified in sap (Bassuk and Howard, 1981b) are synthesized *in vitro*, including 1B and 3B which are prominent in the improved rooting of cuttings in late winter and spring.

The greatest increase in activity of winter sap was obtained by adding *both* PPO and phloridzin. Different responses were obtained by adding either PPO or phloridzin, causing an increase in cofactors 2 and 3 respectively, which indicates complex interactions between reactants and products. The fact that cofactor 3B was significantly reduced on the addition of PPO alone and that cofactor 2 was not increased on the addition of phloridzin alone in the presence of the low level of endogenous PPO may indicate that the substrate for this stage is the phloridzin-like substance cofactor 3B. The fact that the low level of endogenous phloridzin did not lead to an increase in cofactor 2 on the addition of PPO alone, may also indicate that cofactor 3 synthesis utilizes PPO to form a partially oxidized form of phloridzin which then inactivates PPO (Hunter, 1975; Mayer and Harel, 1979) or remains in equilibrium with it. Only when PPO is available in relatively large amounts would cofactors 2A and 2B be produced, as was the case when PPO was added.

Although there was no opportunity in these studies to investigate optimum conditions for the

treatment of M.26 cuttings with cofactor, preliminary results are encouraging. Significant improvement in rooting was obtained by aqueous and acetone solutions of either PPO (as mushroom tyrosinase) and phloridzin, or the product of the reaction respectively. The fact that in the first experiment a high concentration of IBA gave better rooting than a low level of IBA

with cofactor is possibly explained by the auxin-induced activity creating a sink for the high levels of endogenous cofactor present in the cutting at that time.

The results are taken from a thesis by Nina L. Bassuk accepted by London University in partial fulfilment of the Ph.D. degree.

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