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Role of Phenolics in Auxin Induced Rhizogenesis & Isoperoxidases in Cacao (*Theobroma cacao* L.) Stem Cuttings*

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Effects of phenolic substances, *o*- and *p*-hydroxybenzoic acid (*o*- and *p*-HBA) were studied as synergists of indole-3-butyric acid (IBA) in rooting of single node cuttings of cacao. There was marked enhancement in rhizogenesis by *p*-HBA + IBA combination. The total peroxidase and isoperoxidase activities were higher before rooting started and this declined enormously with onset of rooting. In rooting cuttings, the peroxidase activity was higher in treated cuttings as compared to controls. During rhizogenesis the auxin protector activity was higher in rooted than in non-rooted cuttings. The results are discussed in view of the regulatory role of peroxidases and auxin protectors and auxin-phenol synergism during rhizogenesis.

The balance of various internal and external factors influence the rhizogenesis of stem cuttings in plants. Auxins either naturally occurring or synthetic are required for successful formation of adventitious roots in stem cuttings. The physiological role of phenolic substances in rooting process is not clear. Since the phenols are compartmentalized within vacuoles and cellwall, their direct role in metabolism is controversial. However, there are reports on the regulation of growth and organogenesis by phenolics[1-4].

Peroxidase activity and isoperoxidase patterns have been studied in relation to root initiation of various plant species[5-9]. They might alter the lignin biosynthesis and auxin destroying capacities during rhizogenesis. Phenolic substances which are either inhibitors or promoters of IAA oxidase system could be regulatory in the process. In this paper we present the role of phenolics in indole 3-butyric acid induced rhizogenesis of single node cuttings of cacao.

Materials and Methods

Culture of single node cuttings—Single node cuttings with one subtending leaf from cacao (*Theobroma cacao* L. var Forestero) were treated with phenols, viz. *o* and *p*-hydroxybenzoic acid (*o*- and *p*-HBA) singly or in combination with indole-3-butyric acid (IBA) for 2 hr. High IBA conc (1000, 3000 and 6000 mg/l) were used for quick dip of cuttings. Controls were similarly treated with distilled water. Treated stem cuttings were planted in polybags containing sand and left in mist-chamber. The sprinkling was for 5 sec at 5 min intervals. Each treatment had 25 cuttings. Since the observations on number of roots and root length were recorded from unequal number of cuttings, the data were analysed by completely randomized design. Comparison treatments was done using different CD values.

Separation of peroxidases and assay—For preparation of crude enzyme, 1 g tissue was homogenized in 10 ml K phosphate buffer (*p*H 7), strained through 4 layers of muslin cloth and centrifuged at 12,000 *g* at 0°C. Isoenzymes were separated by polyacrylamide gel electrophoresis and stained with benzidine-H_2O_2 mixture[10].

The preparation of enzyme fractions from different cell components was done as follows: tissue (1 g) from rooted and non-rooted stem segments were homogenized in 10 ml 50 mM K phosphate buffer (*p*H 7). The crude homogenate was centrifuged at 1,000 *g* for 10 min. Supernatant was spun at 18,000 *g* for 20 min to get soluble (supernatant) and membrane (pellet) fractions. The membrane fraction after thorough washings was solubilized in the same buffer and clarified by centrifugation.

The 1,000 *g* pellet was washed and extracted in 1 ml 0.2 *M* K phosphate buffer and centrifuged at 12,000 *g* and supernatant formed ionically wall bound fraction. To release covalently bound peroxidases, the pellet was digested in cellulase-pectinase (0.5 and 1.0%, sigma) in cold at *p*H 6 overnight. After centrifugation at 10,000 *g* the supernatant was used as covalent fraction.

Peroxidase activity was assayed with *o*-dianisidine as hydrogen donor[10]. The assay mixture consisted of 2.5 ml 0.2 *M* Na phosphate buffer (*p*H 6.1), 0.2 ml *o*-dianisidine (0.05%), 0.2 ml H_2O_2 (0.3%) and 0.1 ml enzyme. One enzyme unit (E.U.) changes the absorbance of 1/min/mg protein at 25°C..

Extraction of auxin protector and assay—Tissue (1 g) was homogenized in 10 ml 60 mM Na phosphate buffer (*p*H 6.1), squeezed through muslin cloth and

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centrifuged at 16,000 *g* for 20 min. This crude extract was used for auxin protector assay. The crude extract (8 ml) was filtered through a sephadex G-100 column (2.5 × 15 cm), and 10 ml fractions were collected. The total volume of eluating buffer (60 mM Na phosphate *p*H 6.1) was 150 ml. Protector activity was assayed at 30°C in a shaker-water bath. Ten ml reaction mixture contained in final conc; 35 mM Na phosphate buffer (*p*H 6.1) 0.1 mM each of IAA, $MnCl_2$ and 2,4-dinitrophenol, 0.3 mg horseradish peroxidase (HRP, type VI, Sigma) and 200 μg fresh wt equivalent of extract or 0.5 ml of gel-filtrate. Aliquots of 0.5 ml were withdrawn at time intervals and added to 2 ml Salkowski reagent (1 ml 0.5 M $FeCl_3$ in 50 ml 35% PCA), and absorbance was read at 535 nm after 30 min[9,11].

Protein estimation—Protein was determined by the folin phenol reagent[12].

Estimation of phenols—Tissues were extracted in boiling methanol, filtered and aliquots used for estimating total phenols by Folin Denis reagent[13] and o-dihydric phenols by Arnow's reagent[14].

Results and Discussion

Among the various treatment to single node stem cuttings, IBA 500, IBA 6000 and IBA 500 + *p*-HBA 10 were superior in inducing rhizogenesis (Table 1), the last showing highest rooting per cent. IBA alone however produced maximum number of roots significant over that of IBA + *p*-HBA. The IBA + *p*-HBA combination resulted in longer roots than IBA alone. Both these treatments showed significant differences in number and length of roots compared to control. Though the phenolic compounds were weak stimulants for rooting, their synergistic action with auxin was remarkable. The dihydric phenols stimulated rhizogenesis of bean cuttings in presence of IAA[3] and rooting was enhanced by *p*-HBA and IBA in stem cuttings of some forest trees[15]. Similarly synthetic aryl esters of IAA and IBA enhanced greatly the rooting of bean and jack pine cuttings[16]. The phenol alone as such may not be effective but acted synergistically with an auxin in rhizogenesis process. The increased biosynthetic activity during differentiation required m-RNA and protein synthesis and is discernible from the increase in protein content (Table 2).

The phenolic substances affect the physiological process by one of the 2 mechanisms: (1) interference with IAA biosynthesis, (2) affect IAA oxidase activity. The first possibility is not tested here though there is evidence for phenolic involvement in shikimic acid pathway[1,3]. Peroxidases are the major components in the IAA oxidase systems in plants and their activity was assayed (Table 2). Before rooting started, there were no appreciable differences in the activities between treatments. The total peroxidase activity declined sharply when the cuttings rooted. Further the activity was appreciably higher than control in *p*-HBA, IBA, IBA + *p*-HBA and IBA + *o*-HBA at this stage. This was accompanied by changes in anodic isoperoxidases (Fig. 1). In before rooting phase 4 isoperoxidases were detected and some of these disappeared with onset of rhizogenesis. An inverse variation in cathodic and anodic isoperoxidases was reported in the IBA induced rooting of *Prunus* and *Asparagus*, i.e. anodic peroxidases increased while cathodic ones decreased[6,7]. Our results are in agreement with these reports, as the total peroxidase

Table 1—Effect of IBA and Phenolics Singly and in Combination on Rhizogenesis of Stem Cuttings

No.	Treatment	Percent rooting	No. of roots/ cutting	Length of root (cm)
1	Control	11.1	2.6	4.78
2	IBA 500**	85.7	12.3	5.13
3	IBA 1000*	11.1	0.5	0.50
4	IBA 3000*	27.8	8.8	9.10
5	IBA 6000*	72.2	5.7	9.94
6	*o*-HBA 10	33.3	2.2	3.68
7	*o*-HBA 30	22.2	6.0	5.63
8	*o*-HBA 10 + IBA 250	33.3	4.7	4.78
9	*o*-HBA 10 + IBA 500	50.0	4.9	9.44
10	*p*-HBA 10	33.3	4.5	7.78
11	*p*-HBA 30	22.2	4.0	13.95
12	*p*-HBA 10 + IBA 250	33.3	3.2	8.17
13	*p*-HBA 10 + IBA 500	94.4	8.1	10.17

CD ($P = 0.05$) for No. of roots: To compare 1 and 13-5.88
1 and 2 -7.18
CD ($P = 0.05$) for root length: To compare 1 and 13-7.87
1 and 2 -9.60
2 and 13-5.22

*Quick dip method; **Figures are conc in mg/l.

Table 2—Peroxidase Activity and Protein Content

	Peroxidase (E.U.)			Protein (mg/g fresh wt.)		
	15 (stem)	30 (stem)	30 (root) days	15 (stem)	30 (stem)	30 (root) days
Control	16.13	0.22	—	1.40	14.52	—
IBA 500	18.19	0.82	6.88	2.00	4.93	4.53
o-HBA 10	28.57	0.11	—	1.76	11.59	—
o-HBA 10 + IBA 500	11.50	0.96	3.18	2.13	5.63	4.99
p-HBA 10	25.22	1.27	—	2.26	6.73	—
p-HBA 10 + IBA 500	21.55	1.19	3.61	2.07	4.60	4.59
CD($P = 0.05$)	NS	0.74	1.85	0.05	6.20	1.90

activity was higher in hormone treatments and also number of isoperoxidases were greater (Fig. 1).

The rapid decline in the peroxidase activity from high (non-rooted) to very low levels (rooted) should result in sparing the auxin from being oxidized[3,6,7]. Those isoperoxidases which disappeared during rhizogenesis could probably be the most active IAA oxidases. However in the present investigation, the characterization of individual isoenzymes has not been presented and such specific isoperoxidases have been described in other plants[10,17]. Another role of peroxidases is in the process of lignification. The higher peroxidase activity in IBA and *p*-HBA treated stems might take part in the lignification during rhizogenesis[8,18].

The lower levels of total and *o*-dihydric phenols in rooting cuttings (Table 3) by IBA and phenolic treatment probably suggest that endogenous phenolics were metabolised effectively during the process of lignification.

A sparing action of IAA oxidation is provided by low or high polymers of *o*-dihydric phenols[3,9,11,19,20]. There was higher protector activity in stems of rooted than non-rooted cuttings (Fig. 3), as evidenced by longer lag period in HRP catalysed IAA oxidation. When the crude extract was passed through sephadex G 100 column single peak of protector activity between 5th and 7th fractions (50-70 ml) having *Ca* molecular weight of 10,000 was detected. The lag period differed in non-rooted and rooted stem cuttings. In non-rooted stems lag periods were 60 and 15 min in fractions 6, and 7; while in rooted stems it was 220, 80 and 20 min in fractions 5, 6 and 7, respectively. This is in agreement with those reported by Kevers *et al.*[9] but does not support the hypothesis of Stonier[21] who reported decline in auxin protector activity during differentiation.

The stem tissue of rooted cuttings showed lower activity of peroxidase in soluble, membrane and cell wall bound fractions (Fig. 2). Isoperoxidase A_3 was typical in being associated with membranes and an interesting feature noted here is the major activity associated with this fraction in rooted stems.

Table 3—Effect of IBA and Phenolics on the Total and *o*-Dihydric Phenols

Treatment	Total phenols (mg/g fresh wt)		*o*-Dihydric phenols (mg/g fresh wt)	
	Stem	Root	Stem	Root
Control	16.80	—	5.50	—
IBA	6.00	5.50	3.50	1.55
IBA (500)+*p*-HBA (10)	8.60	7.40	2.35	1.40
IBA (500)+*o*-HBA (10)	5.00	4.80	1.50	1.30

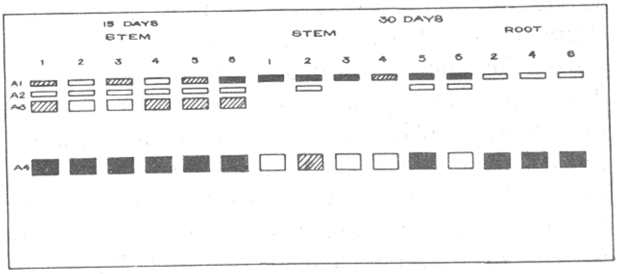

Fig. 1—Isoperoxidase Zymogram in stem and root before rooting (15 days) and after rooting (30 days). Control (1); IBA (2); *o*-HBA (3); IBA + *o*-HBA (4); *p*-HBA (5); IBA + *p*-HBA (6).

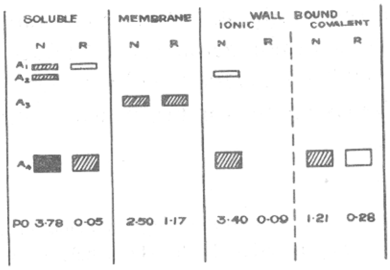

Fig. 2—Isoperoxidase Zymogram (above) and peroxidase activity (below) in soluble, membrane and wall bound fractions in stem tissue of non-rooted (N) and rooted (R) cuttings.

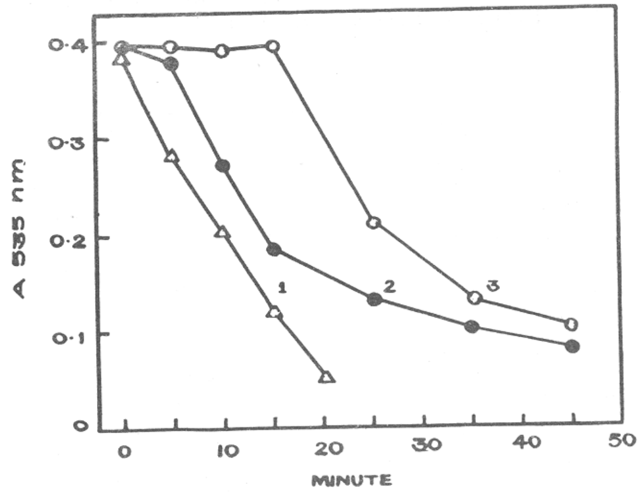

Fig. 3—Auxin-protector activity of non-rooted (2) and rooted (3) stem extracts. Control curve (1).

The results obtained on the whole is very interesting. Two phases in peroxidase variations during the process of rooting in general could be recognized. The first is an increase which corresponds to the inductive phenomenon, while no histological changes can be observed. Second is the decrease which commences with root primordia initiation, thus with the initiative phase. The necessity of auxin requirement is long recognised for rooting process, but has been erroneously thought that external application of an auxin will simply enhance endogenous auxin levels without modifying normal metabolism[22]. The variations in the peroxidase levels would result in decrease in endogenous auxin level to a minimum during induction of rooting which is a new concept. The levels of auxin protectors, i.e. low during inductive (non-rooted) and high during initiation phase (rooting) reflects their role in maintaining high auxin levels during latter phase. Thus, the balance between synthesis and destruction of auxin in which peroxidase is an important component determines rhizogenesis of cuttings. Phenolic compounds take part in these initiative processes by protecting destruction of auxin.

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