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CHANGES IN PEROXIDASE ISOENZYMES OF *PHASEOLUS MUNGO* HYPOCOTYL CUTTINGS DURING ROOTING

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Key Word Index—*Phaseolus mungo*; Leguminosae; black gram; peroxidase isoenzymes; rooting; indolylacetic acid; cycloheximide.

Abstract—A time course study of changes in the pattern of peroxidase isoenzymes shows that two new isoenzymes *a* and *b* appeared in hypocotyl cuttings in all culture media except in cycloheximide. Roots were also initiated in all media except in cycloheximide. The time of appearance of these isoenzymes in different cultures synchronized with the time of root initiation. Two other isoenzymes *c* and *d* disappeared, while a new fast migrating isoenzyme appeared in cuttings cultured in media containing cycloheximide, even in combination with IAA and sucrose. As the development of initiated roots remained arrested in the latter medium, isoenzymes *c* and *d* appear to be associated with root development.

INTRODUCTION

THE SIGNIFICANCE of peroxidase in growth and differentiation has been reported by a number of workers.[1-4] It has also been shown that a complex of hormonal interactions is involved in root initiation[5] and that a proper balance between auxin and nutrition is needed for the optimal production of adventitious roots[6] suggesting the involvement of both hydrolyzing and oxidative enzymes in the process. A study of the changes in isoenzyme patterns during the period of initiation and development of roots on hypocotyl cuttings of *P. mungo* constitute the subject-matter of this paper.

RESULTS

Rooting response

Table 1 shows that roots were initiated at 48 hr on hypocotyl cuttings in IAA, sucrose, and IAA + sucrose as compared to 96 hr in water (control) and IAA + sucrose + cycloheximide. While 3 roots produced on control cuttings were confined to the lower 0·1 cm portion, 8 produced on hypocotyl cuttings in IAA + sucrose + cycloheximide arose from the upper 1·5 cm portion. On cuttings cultured in IAA, sucrose, and IAA + sucrose roots were distributed all over the hypocotyl, being profuse in sucrose and more so in IAA + sucrose. Hypocotyl cuttings did not root in cycloheximide till the termination of the experiment.

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Peroxidase activity

The activity of peroxidase expressed as units per mg of protein, presented in Fig. 1 was low initially (0 hr). While in the control the activity increased only slightly, in cuttings cultured in IAA + sucrose and even in IAA alone it increased to a higher level within 48 hr. While in IAA alone the activity did not change, in IAA + sucrose it increased further at 96 hr. It may also be noted that in both sucrose and cycloheximide cultures the increase was more marked during the period 48–96 hr and reached a level even higher than that of the control, although the cuttings did not root at all in cycloheximide.

TABLE 1. ROOTING RESPONSE OF HYPOCOTYL CUTTINGS OF *Phaseolus mungo* AFTER 7 DAYS*

Treatment	Segments rooted	No. of roots per rooted segment	Time of microscopic root initiation (hr)
Water (control)	4	3·0 ± 0·4	96
IAA 5 mg/l.	10	7·8 ± 0·6	48
Sucrose 1%	10	28·7 ± 1·2	48
IAA 5 mg/l. + Sucrose 1%	10	46·3 ± 1·2	48
Cycloheximide 1 mg/l.	0	—	—
IAA 5 mg/l. + Sucrose 1% + cycloheximide 1 mg/l.	8	8·0 ± 0·5	96

* Average of 10 replications.

Peroxidase isoenzyme pattern

The results of the time course study of isoperoxidases in cuttings cultured in various test solutions are presented in Fig. 1. Eight isoenzymes could be observed in cuttings

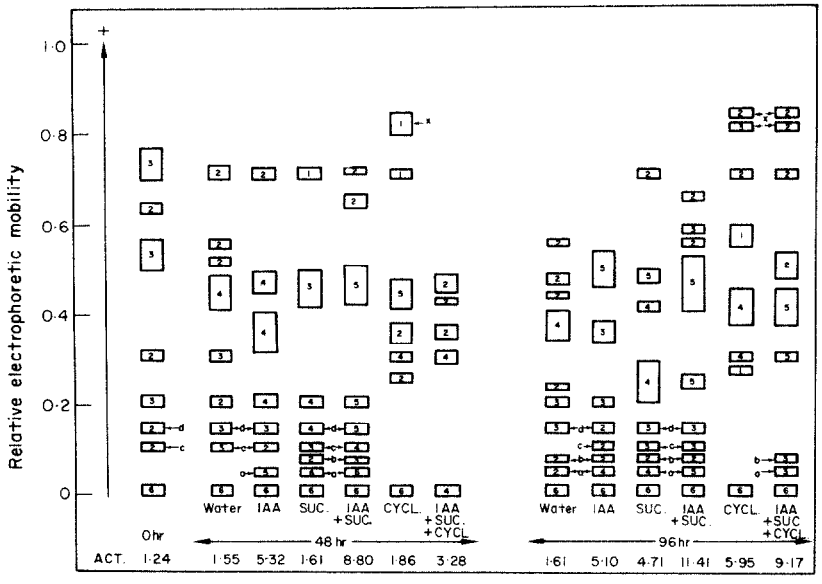

FIG. 1. PEROXIDASE ISOENZYME PATTERN OF *Phaseolus mungo* HYPOCOTYL CUTTINGS AT 0, 48 AND 96 HR OF CULTURE IN DIFFERENT TEST SOLUTIONS.

Number inside the bands indicates the intensity of the isoenzyme; and figures at the base, the activity of the enzyme per mg protein.

initially. While a maximum of 10 isoenzymes was observed at 96 hr in cuttings cultured in water and IAA + sucrose, the minimum of 5 was resolved at 48 hr in those cultured in IAA + sucrose + cycloheximide.

A survey of the isoenzyme patterns in hypocotyl cuttings cultured in different test solutions revealed significant changes only in the isoenzymes designated as *a*, *b*, *c*, *d* and *x* in Fig. 1. The description here, therefore, is restricted to these isoenzymes. It is seen that isoenzymes *c* and *d* that were present initially continued in hypocotyl cuttings cultured in water, IAA, sucrose or IAA + sucrose at 48 hr as well as at 96 hr, but disappeared in those cultured in cycloheximide alone or in combination with IAA + sucrose. However, two new isoenzymes, *a* and *b*, appeared in cuttings cultured in sucrose alone or in combination with IAA at 48 hr, but in water or IAA + sucrose + cycloheximide at 96 hr. In IAA cultures, while isoenzyme *a* appeared at 48 hr, *b* could be detected only at 96 hr. Neither of these two isoenzymes developed in cuttings cultured in cycloheximide alone, in which *c* and *d* also disappeared.

Another interesting point is that isoenzyme *x* with high electrophoretic mobility was detected in cuttings cultured in cycloheximide alone as a faint band at 48 hr which resolved into two by 96 hr. This isoenzyme was detected also in cuttings cultured in IAA + sucrose + cycloheximide at 96 hr.

DISCUSSION

The results presented in this paper show that isoenzymes *a* and *b* develop in culture solutions which enhance root initiation, but not in cycloheximide which inhibits rooting completely. This is suggestive of their association with adventitious root formation in this system. Streptomycin which inhibits rooting in mung bean hypocotyls is also reported to suppress some peroxidase isoenzymes that are associated with rooting.[7]

The isoenzymes *c* and *d* although present in cuttings at initial stages (0 hr), disappeared in those cultured in cycloheximide alone or in combination with IAA + sucrose. The isoenzymes *a* and *b*, however, appeared in the latter case at 96 hr. As stated earlier, root initiation also occurred in IAA + sucrose + cycloheximide at 96 hr although root development remained arrested. The isoenzymes *c* and *d* thus appear in some way to be associated with the development of roots.

The appreciable increase in rooting in sucrose over control but only slight in IAA, indicates that the level of endogenous auxin in these cuttings was adequate to trigger the synthesis of new proteins (isoenzymes) concerned in root initiation. An increase in the activity of peroxidase as a whole in cuttings cultured in IAA substantiates this hypothesis. Exogenously supplied IAA is known to promote the synthesis [8–10] as well as activity[1,11] of peroxidases. The limited number of roots that are produced, however, may be ascribed to inadequate nutrition, as is evident from the fact that rooting of cuttings cultured in IAA + sucrose increased markedly. It has been already shown that a proper balance between auxin and nutrition is necessary for optimal production of adventitious roots.[12,13] In contrast to IAA, cycloheximide causes the inhibition of fresh synthesis of

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isoenzymes *c* and *d* and these disappear in such cultures as soon as the isoenzymes present initially are lost through enzyme decay. The loss of enzyme through decay has been shown by some workers.[14]

An interesting point that emerges from this investigation is that cycloheximide treatment which does not allow the synthesis of isonenzymes *a*, *b*, *c* and *d*, causes the production of a fast migrating isoenzyme *x*, not seen in any other treatment. The production of a totally new kind of isoperoxidase in a system in the presence of cycloheximide, a potent inhibitor of protein synthesis, is rather novel and a little harder to understand. It appears that cycloheximide is not an inhibitor of all proteins. This isoenzyme may be an IAA oxidase which may decrease the auxin content to a level lower than is needed for the initiation of roots. The fact that root initiation did occur in IAA + sucrose + cycloheximide cultures though delayed to 96 hr, supports this contention. Endo[15] and Yoneda and Endo[16] have shown that certain peroxidase isoenzymes act as IAA oxidases. Similar effects of cycloheximide have been reported by other workers.[17–19] In fact, Nanda *et al.*[20] have shown that cycloheximide causes stimulation of GA_3-induced differentiation of leaves and floral buds in *Impatiens balsamina*.

EXPERIMENTAL

Material. Healthy uniform seeds of black gram were germinated in the dark on a thin layer of cotton in sterilized Petri dishes (15 cm dia.). After 6 days, when the hypocotyls were 5 cm long, cuttings were prepared by excising them 3 cm below the cotyledonary node. The shoot apex, the leaves and the cotyledons were also excised, retaining about 3 cm of the epicotyl with the hypocotyl.

Treatments. The cuttings were planted in holes on tin foils stretched over specimen tubes (3 × 7·5 cm), each containing 25 ml of the following test solutions and kept in the dark for 8 days: (a) (control); (b) IAA 5 mg/l.; (c) Sucrose 1%; (d) IAA 5 mg/l. + sucrose 1%, (e) Cycloheximide 1 mg/l.; (f) IAA 5 mg/l. + sucrose 1% + cycloheximide 1 mg/l. All test solns were prepared in 30 μM chloramphenicol, including the H_2O control and the test solns were changed every 24 hr to prevent microbial growth.

Anatomical studies. Hand sections of the hypocotyl samples were cut from each test soln at 12 hr intervals and were stained with safranin fast green to note the precise time of microscopic root initiation. Observations on the number of rooted cuttings and roots were recorded after 7 days and the analyses of peroxidases were made on samples at 24 hr intervals. The data of only 48 and 96 hr have been included in this paper.

Enzyme preparation. The 3 cm portions below the cotyledonary nodes (from which roots are initiated) of 80 hypocotyl cuttings from each treatment were washed and homogenized separately at 4° and the crude enzyme was extracted in 0·1 M Tris buffer pH 8·0 containing 17% sucrose, 0·1% ascorbic acid and 0·1% cysteine hydrochloride. The extracts were centrifuged at low speed and the supernatant clarified by centrifuging at 16000 *g* for 20 min. All operations were carried out at 5°. The extraction procedure is similar to that of Staples and Stahmann.[21] The resultant cell-free extract was assayed for total peroxidase activity in a colorimeter at 710 nm using benzidine-hydrogen peroxide as substrate[22] and is expressed as units of activity per mg of protein that was determined, using Folin reagent.[23]

Enzyme assay. Gel electrophoresis was effected with a disc system (running pH 8·3 with Tris–glycine 0·01 M buffer in electrode compartments in 10% polyacrylamide gel at 4°). The method is essentially similar to that of Ornstein[24] and Davis.[25] All enzyme preparations on the gels were normalized with respect to their total protein content and the space above the gels was overlayered by 60% urea solution. Each run was terminated when

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the tracking dye reached the lower ends of the vertically oriented gels. Gels were removed by rimming and stained with 0·3% benzidine in 25% HOAc mixed with an equal vol. of 0·01% H_2O_2. The intense blue bands that developed were recorded immediately. The results of electrophoretic separation of isoenzyme assay on the gel matrix have been presented schematically, with mobilities calculated as the relative distance traversed by the band with respect to the distance travelled by the bromophenol dye (= 1·0). The experiment was repeated 3 × with similar results. The intensity of isoenzyme bands in Fig. 1 is given as score numbers arbitrarily fixed on the basis of visual observations. The higher the score number, the higher is the intensity of the isoenzyme.

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