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FORMATION OF PEROXIDASES IN RESPONSE TO INDOLE-3-ACETIC ACID IN CULTURED TOBACCO CELLS

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Abstract—Tissue cultures of *Nicotiana tabacum* (var. White Burley), grown on an agar medium with either IAA or 2,4-D as the auxin, display peroxidase forms which are dependent on the auxin used for growth. Two enzyme forms are found only in cells whose growth is stimulated by IAA, while all other observed forms are common to cells grown with either auxin. In suspension cultures supplied both IAA (2 ppm) and 2,4-D (0·1 ppm), the forms induced by IAA are found exclusively in the culture medium, apparently completely secreted as exoenzymes.

INTRODUCTION

THE MULTIPLE nature of peroxidases has been described for many plant tissues, most notable of which are horse-radish,[1] pea,[2,3] *Pelargonium*[4] and tobacco.[5,6] Tobacco pith contains multiple forms which are characteristic of this plant part; however, the number of these forms, as detected by electrophoresis, changes with the development of a callus from the pith.[6] The multiple forms thus obtained are further dependent on the absence or presence of indole-3-acetic acid (IAA) which, along with kinetin, resulted in optimum development of a callus from the pith explant. In this instance, IAA causes the induction of one form and the repression of two others.

The investigation reported here demonstrates that in cultured cells of *Nicotiana tabacum* (var. White Burley), the formation of particular forms of peroxidase is dependent on IAA, a phenomenon which is not similarly effected by 2,4-dichlorophenoxyacetic acid (2,4-D).

RESULTS

Electrophoretic separations of tobacco callus extracts demonstrated the multiplicity of peroxidases in these tissues. However, the forms in the cells are dependent upon the auxin used to sustain growth of the callus on an agar medium (Fig. 1). Cells grown using 2,4-D (2 ppm) in the medium contained only cathodic forms of peroxidase (I_c–V_c), while those grown using IAA (2 ppm) showed in addition two anodic forms (I_a and II_a). The presence of the anodic forms in IAA-grown callus cells was independent of whether the callus was initiated from explants originally grown on 2,4-D or IAA.

The callus cultures maintained on agar media, when transferred to liquid media containing IAA (2 ppm) and 2,4-D (1 ppm or 0·1 ppm), disassociated sufficiently to result in growing

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cell suspensions. The peroxidases in these suspension cultures were found to be distributed in both cellular extracts and culture media and the enzyme forms present in cells and medium

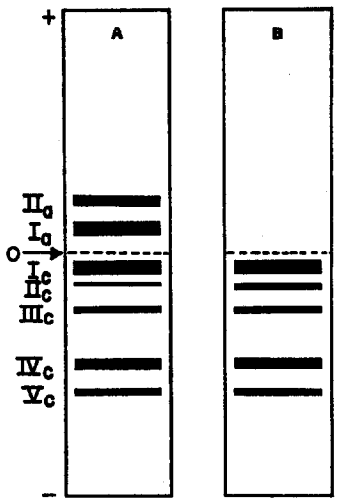

FIG. 1. ELECTROPHORETIC COMPARISON OF PEROXIDASE FORMS IN CALLI GROWN IN PRESENCE OF IAA AND 2,4-D.

A, IAA (2 ppm) present in culture medium, B, 2,4-D (2 ppm) present in culture medium. Electrophoresis and staining were performed as described in the Experimental section.

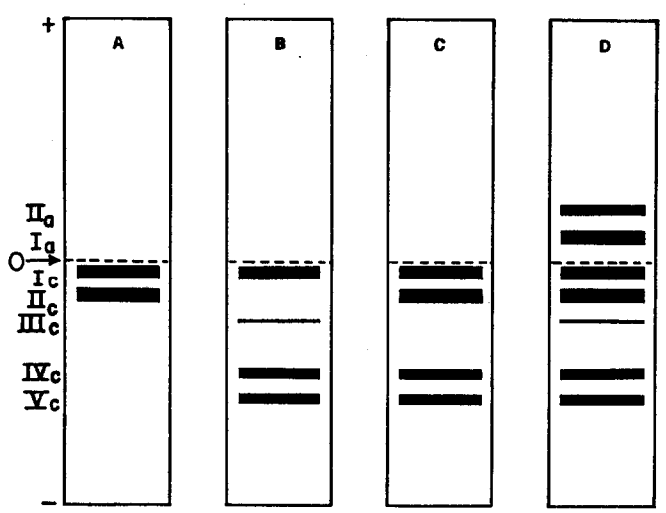

FIG. 2. DISTRIBUTION OF PEROXIDASE FORMS BETWEEN CELL AND MEDIA IN SUSPENSION CULTURE AFTER 36 hr (A AND B) AND 3 weeks (C AND D).

Extract of cells, A, and concentrated culture medium, B, from a 36-hr culture; and cellular extract, C and medium concentrate, D, from a 3 week culture were subjected to electrophoresis and stained for peroxidase activity.

varied as well (Fig. 2). Most noteworthy, however, was the finding that in cultures in which growth was sustained by normal levels of IAA (2 ppm) and low 2,4-D (0·1 ppm), the anodic species, found only in the medium, were the same as those present in callus cell cultures

grown with IAA (Fig. 1). The presence of the anodic exoenzymes was not evident after 36 hr, but was evident after 3 weeks in conditions where the 2,4-D concentration was low. Also, when the higher 2,4-D concentration was used in the medium, the presence of these forms was not evident, even after 4 weeks. It is thus suggested that formation of the anodic exoenzymes is dependent on IAA, but that 2,4-D at 1 ppm inhibits this effect.

The question which then arose was whether the IAA-induced forms of peroxidase might be related to the oxidative metabolism of IAA, a peroxidase-catalyzed function. Prior to studying this, it was necessary to obtain cell extracts essentially free of inhibitory polyphenols. Extracts of callus tissues obtained with insoluble polyvinylpyrrolidone (Polyclar AT) demonstrated IAA oxidase activity with a minimum of lag period and at a faster rate compared with that observed in extracts obtained using buffer alone (Table 1). Addition of soluble polyvinylpyrrolidone to the reaction cuvette considerably reduced the inhibitory effects of substances in the buffer-only extract, presumably by selective absorption of polyphenols.

TABLE 1. EVALUATION OF PROCEDURES FOR PROTEIN EXTRACTION AND ANALYSIS OF IAA-OXIDASE ACTIVITY

Extraction condition	Reaction condition*	Min before linear rate	Reaction rate ΔOD at 254 nm/min
Buffer only	No PVP	10·0	0·006
Buffer only	PVP added	7·5	0·01
Polyclar and buffer	No PVP	5·5	0·008
Polyclar and buffer	PVP added	5·0	0·009

* Equal quantities of tissue used and volumes of extract equal. Other details given in Experimental.

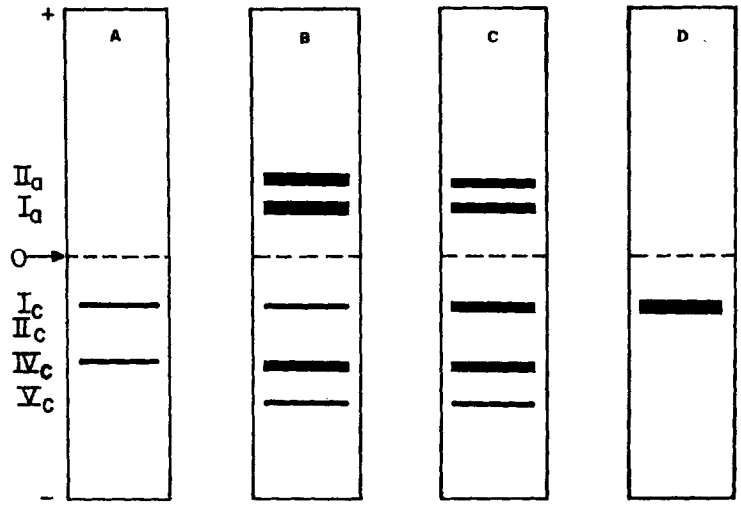

FIG. 3. ELECTROPHORETIC DETECTION OF PEROXIDASE FORMS OBTAINED BY $(NH_4)_2SO_4$ PRECIPITATION FROM EXTRACT OF CALLUS CELLS GROWN USING IAA IN MEDIA.

Diagrams represent peroxidases in precipitates from A, 0–40%, B, 40–60%, C, 60–80% and D, 80–100% saturation with $(NH_4)_2SO_4$.

Ammonium sulfate fractionations were employed to effect enrichment of the anodic peroxidase forms from IAA-grown tissues. The precipitations were successful only if Polyclar was included during extraction. Attempted fractionations using extracts obtained without the use of Polyclar resulted in protein fractions which floated rather than precipitated. The electrophoretic patterns in Fig. 3 show that the anodic forms can be precipitated between 40 and 60 per cent $(NH_4)_2SO_4$ saturation. Although some cathodic forms are also separated, this procedure resulted in an enrichment of these forms relative to the whole extract.

DISCUSSION

The formation of specific forms of peroxidase in cultured tobacco tissue in response to IAA is evident. It appears that IAA specifically induces the synthesis of these forms, since growth in the presence of an unnatural auxin alone does not result in their formation. Our studies do not yet exclude the possibility that 2,4-D represses the formation of the IAA-induced forms in conditions where 2,4-D is present at higher concentrations. The exclusive presence of the IAA-induced forms in the suspension medium was most surprising. It does suggest, however, that these forms, as well as others found in the medium, are secreted as exoenzymes shortly after they are synthesized in the cell. That some forms are present, in both cell and medium, may indicate that there is some selection made in the cell for retention of particular forms.

It is well established that peroxidases also exhibit IAA oxidase activity[6,7] and that removal of the heme from horse-radish peroxidase results in an apoenzyme having only IAA oxidase activity.[6,8] The major product of the enzyme-catalyzed oxidation *in vitro*[7] and *in vivo*[9,10] is 3-hydroxymethyloxindole, which rapidly dehydrates to 3-methyleneoxindole. However, since there are several forms of peroxidases in plant tissues, a fact substantiated by our results, it becomes of interest which of these forms is kinetically favorable for the catalytic oxidation of IAA *in vitro*. It has been suggested by Tuli and Moyed[9,10] that the oxidation products may actually be responsible for the observed hormonal effects, so that the rates of IAA oxidation are important in the regulation of growth. The alternative, of course, is that the levels of IAA in the cell are closely regulated by the oxidase enzyme. The fact that IAA and not 2,4-D induces the synthesis of specific peroxidases makes these forms prime suspects for the *in vivo* oxidation of this important plant hormone.

EXPERIMENTAL

Cultures of *Nicotiana tabacum* (var. White Burley) have been maintained on two different agar media. The first is a modification of that described by Wolter and Skoog,[11] in which coconut milk (10%) and yeast extract (0·1%) are substituted for kinetin, myo-inositol, pyridoxine HCl and 1 ppm 2,4-D. The second medium used is that described by Linsmaier and Skoog[12] with 2,4-D or IAA at 2 ppm and kinetin at 0·2 ppm. Cell suspensions for this study were initiated from calli grown on the modified Wolter and Skoog agar medium. The liquid medium was composed of this modified preparation less agar with 2 ppm IAA and either 0·1 or 2 ppm 2,4-D added to sustain growth. Suspension cultures were grown at 25 ± 1° on a gyrorotary shaker in 250-ml flasks with 100 ml of medium.

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Extraction

Callus tissue was homogenized in an Omni-mixer (Sorvall) using phosphate buffer, pH 5·8, and 0·5 g of purified insoluble polyvinylpyrrolidone per g of fresh tissue.[13] The slurry was filtered through 90-mesh silk bolting cloth and centrifuged at 22,000 *g* for 30 min. Medium and cells from the suspension cultures were separated by filtering, with suction, through Whatman No. 40 filter paper. The cells were extracted as above and the medium concentrated for appropriate assays. Dilute enzyme solutions were concentrated using a Diaflo membrane (UM 10 and UM 20-E).

The IAA oxidase system included 1×10^{-4} M IAA, 1×10^{-4} M $MnCl_2$, 1×10^{-4} M 2,4-dichlorophenol, freshly prepared 0·1 % polyvinylpyrrolidone (mol. wt. 40,000), and 0·1 ml of enzyme solution. The volume was adjusted to 3 ml with 0·02 M phosphate buffer, pH 5·8. The reaction was measured by observing the change in absorbance at 254 nm with time using a Beckman Model DK-2A equipped with a time drive attachment.

Electrophoretic separation of extracts were carried out on cellulose polyacetate strips (Sepraphore) in a Gelman electrophoresis chamber for 1 hr at 1 mA/strip. Protein extracts were applied across the center of the strip to facilitate identification of anodic and cathodic proteins. The strips were strained for peroxidase activity using a mixture of 30 ml ethanol, 20 ml 0·1 M acetate buffer, pH 5·2, 1 ml 30% H_2O_2 and 0·05 g benzidine HCl.[14]

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