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EFFECTS OF IAA AND 2,4-D ON POLYPHENOL OXIDASE IN TOBACCO TISSUE CULTURES

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Abstract—When tobacco tissue cultures are grown on agar medium containing either IAA (2 mg/l.) or 2,4-D (2 mg/l.) there is eight to ten times the polyphenol oxidase activity in the IAA-grown tissue as in the 2,4-D-grown tissue when chlorogenic acid is the substrate. This difference in enzyme activity is directly correlated with a pigmentation difference in the cultures on the two auxins: dark brown on IAA and pure white on 2,4-D. The presence of IAA in the growth medium can induce polyphenol oxidase activity in tissue which has been previously growing on 2,4-D and is very low in polyphenol oxidase activity. Chromatograms of ethanolic extracts of both tissues reveal a strongly fluorescent spot in the IAA-grown tissue which is entirely absent from the 2,4-D-grown tissue. Possible modes of action for IAA are discussed.

INTRODUCTION

DIFFERENTIAL effects of kinetin, indole-3-acetic acid (IAA), and 2,4-dichlorophenoxyacetic acid (2,4-D) upon the activity of enzymes in cultured plant tissues have been described.[1–4] During the development of a callus from excised tobacco pith, multiple forms of peroxidase are found, the appearance or the repression of which are dependent upon the presence or absence of kinetin and IAA.[1,2] In established callus cultures of tobacco which have either IAA or 2,4-D in the growth medium, different forms of peroxidase can be detected in the presence of IAA or 2,4-D.[3] Different isozymes of polyphenol oxidase (PPO) are present in tobacco pith tissue before and after culture in the presence or absence of kinetin and IAA.[4] After 1–2 days in culture in the presence of IAA, one isozyme of PPO, very active using 3,4-dihydroxyphenylalanine as substrate, appeared. Added kinetin in the growth medium tended to prevent this IAA effect.

While growing several established tobacco callus cultures on a modified White's medium containing either IAA or 2,4-D, we made several rather interesting observations on the appearance of the tissues. In particular, tissue strain H-196 was quite white when grown on 2,4-D medium, but quite dark brown (sometimes almost black) when grown on IAA medium. Growth on the two media was, however, equal. The simplest explanation for this difference in pigmentation was that the IAA-grown tissue possessed more PPO activity than did the 2,4-D-grown tissue. The investigations presented here indicate that when chlorogenic acid is used as the substrate for PPO, there is indeed more PPO activity in tissues grown on IAA.

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RESULTS

Preliminary experiments were conducted to determine the best method of preparing the enzyme. Palmer[5] has found that PPO activity in banana fruits is associated mostly with the particulate matter. The enzyme could be solubilized by homogenizing the tissue in a buffer containing a non-ionic detergent. Acetone powders of whole tissue have also been recommended in order to prevent irreversible denaturation of the enzyme by the products of the reactions it catalyzes. Tobacco tissue (H-196) ground in buffer with detergent exhibited slightly less activity than tissue homogenized without detergent. Thus, it would appear that the enzyme in tobacco tissue is not particulate bound. Acetone powders were prepared by homogenizing H-196 tissue in acetone at −15°, filtering, washing the residue on the paper with cold acetone and air-drying. Calculated quantities of the dried residue were then stirred at 0–2° with ice-cold phosphate buffer for one hour, centrifuged, and the supernatant assayed. There was no substantial difference in activity between the supernatants prepared from acetone powders and those prepared by simply homogenizing the tissue in cold buffer. Therefore, all the enzyme extracts were prepared by homogenizing the tissues in cold buffer.

The striking pigmentation difference in H-196, pure white when grown on 2,4-D and brown when grown on IAA, was observed, to a lesser degree, in other tissues growing on IAA or 2,4-D. H-239 and M-222 show about the same differences as H-196 while G-252 is almost white on IAA medium and light yellow green on 2,4-D. When these tissues were assayed for PPO activity, consistently higher values were found in the tissues grown on IAA (Table 1).

TABLE 1. POLYPHENOL OXIDASE ACTIVITIES OF VARIOUS TISSUE CULTURES GROWN ON IAA AND 2,4-D MEDIA

Tissue	Activity (units/ml) IAA	2,4-D
H-196	170	18
H-239	36	2
M-222	21	4
G-252	168	30

TABLE 2. EFFECT OF 2,4-D OR EXTRACTS OF 2,4-D-GROWN TISSUE ON POLYPHENOL OXIDASE

Addenda*	Activity Units/ml
None	90
2,4-D (10^{-3} M)	87
Extract of 2,4-D-grown tissue	50
Volume of H_2O equal to tissue extract	50

* Substances added to a standard enzyme prepared from H-196 tissue grown on IAA medium.

Activity of the enzyme extracted from the tissues varied from sample to sample, but the ratios of the activity were approximately the same. Thus, H-196 tissue grown on IAA medium might show 66 units per ml enzyme extract while the comparable 2,4-D-grown tissue might exhibit 6 units of activity. Then, on another occasion, the same tissues, extracted later in the growth period of the cultures, show an activity of 170 units for IAA-grown tissue and 18 units in 2,4-D-grown tissue. The ratio however, remained the same. Because the pigmentation difference was so striking and the ratio of activity so constant between the IAA- and 2,4-D-grown tissues, H-196 tissue was utilized for the remainder of this study.

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The question then arose as to the effect of 2,4-D on the enzyme: does 2,4-D inhibit the activity of the enzyme or does it cause the production of an inhibitor in the tissue? To investigate this, 2,4-D, at a final concentration of 10^{-3} M, was added to an enzyme of known activity prepared from IAA-grown tissue and then the enzyme was assayed in the usual manner. An additional enzyme extract was prepared from 2,4-D-grown tissue and added to an aliquot of the enzyme from IAA-grown tissue. The resulting mixture was assayed in the normal manner. A control had a volume of water added to the standard IAA tissue extract to compensate for the dilution. The data in Table 2 show that neither 2,4-D nor the extract of the 2,4-D-grown tissue have any effect on the activity of the IAA tissue enzyme. Thus, the effect of 2,4-D is not one of direct inhibition of the enzyme or in the production of an inhibitor.

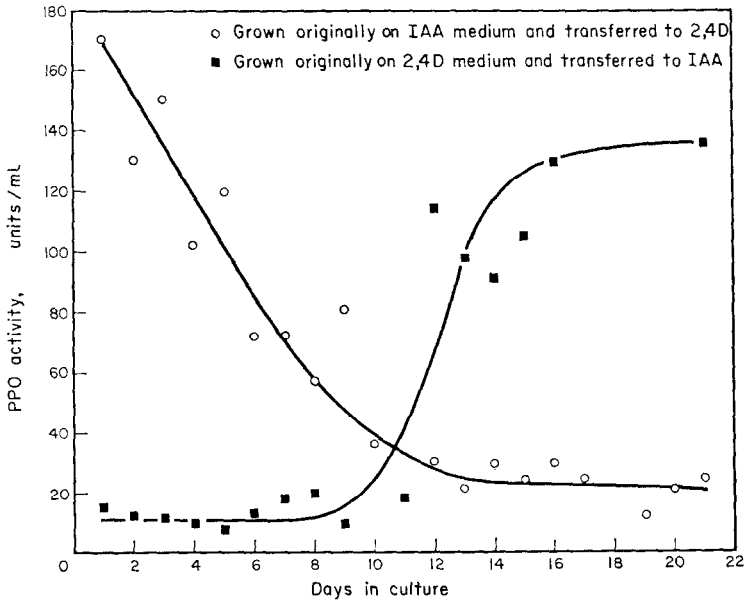

FIG. 1. CHANGES IN PPO ACTIVITY OF H-196.

These results suggested that the effect of IAA was to induce the formation of the enzyme. To test this, H-196 tissue grown on 2,4-D was incubated with a solution of IAA, 10^{-3} M, for up to 20 hr and then assayed. There was no increase in activity. However, since 2,4-D might interfere with the production of enzyme and the piece of 2,4-D-grown tissue might still have a residuum of 2,4-D sufficient to inhibit synthesis of the enzyme, a 20-hr incubation period might not be sufficient to dilute out the 2,4-D. This possibility was investigated by transferring 2,4-D-grown tissue to IAA agar medium. As a control, IAA-grown tissue was transferred to 2,4-D agar medium. Samples of both tissues were then assayed approximately every day for 21 days. From the data in Fig. 1, it is apparent that there is almost complete reversal in enzyme activity in the tissues grown on the two media. Thus, if the 2,4-D-grown tissue remains in contact with the IAA medium long enough, PPO activity will increase.

There are several explanations for this effect of IAA. IAA could be inducing the enzyme directly, but this seems unlikely because IAA is not a substrate for the enzyme. In addition,

it would be extremely difficult to test this in agar grown tissues. A more feasible explanation for the action of IAA would be that it mediated the production of a substance, perhaps a substrate for the enzyme, which then induced synthesis of the enzyme. Perhaps the 2,4-D-grown tissue needed the presence of an appropriate substrate to produce enzyme. In an attempt to answer these questions, several experiments were tried. Extracts of IAA-grown tissue were incubated with 2,4-D tissue enzyme to determine if pigment could be produced from the added substrate. Tissue grown on 2,4-D was incubated with extracts of IAA-grown tissue to see if the enzyme activity of the 2,4-D tissue would be enhanced. Tissue grown on 2,4-D was incubated with chlorogenic acid to determine if enzyme activity was enhanced. Extracts of IAA-grown or 2,4-D-grown tissues were chromatographed to see whether there were differences in the presence of phenolic substances.

The first three experiments gave negative results. However, chromatography revealed a striking qualitative difference. Viewed under short wave UV light, chromatograms showed a brilliant bluish-green fluorescent spot in the IAA tissue extracts which was entirely absent from the extracts prepared from 2,4-D-grown tissue. While we have not yet investigated its chemical nature or its inductive ability, it is tempting to speculate that this is the substance which induces the formation of PPO after its formation has been mediated by IAA.

DISCUSSION

The effects of the auxins on a number of enzymes *in vitro*, including respiratory enzymes, have been reviewed by Bonner and Bandurski[6] and Freed *et al.*[7] The effects are generally non-specific; at some concentrations inhibition or enhancement of activity of a number of enzymes may be found. In some cases there is no effect until levels considerably higher than those considered to be physiological are reached. Stafford and Galston[4] have reported the appearance of a well defined isoenzyme of PPO after culture of freshly excised tobacco pith explants on a modified White's medium containing IAA. We now report that, in established tissue cultures of tobacco, the presence of IAA in a modified White's medium maintains a consistently higher PPO activity than can be demonstrated in tissues grown on 2,4-D. Since neither 2,4-D, at the concentration used, nor an extract of 2,4-D-grown tissue had any effect on PPO activity *in vitro* it was concluded that 2,4-D, *per se*, was not acting by inhibiting enzyme activity. The possibility still remains that 2,4-D is somehow repressing the synthesis of PPO. The length of time required for IAA to cause an increase in enzyme activity in 2,4-D-grown tissue which had been transferred to the IAA (agar) medium suggests a residuum of 2,4-D which was effective in repressing enzyme synthesis until it had been diluted out by growth. However, this lag could also be interpreted as the time required for IAA to diffuse into the tissue from the agar, mediate the production of any intermediates necessary for PPO induction, and then synthesis of the enzyme. The chromatographic evidence, showing at least one major phenolic component present in IAA-grown tissue which is absent in 2,4-D-grown tissue, and the fact that a doubling time in these tissues is $1\frac{1}{2}$–2 weeks (unpublished data) tend to support the latter explanation.

While the higher PPO activity in IAA-grown tissues may be correlated with the brown coloration observed in H-196, Leonova *et al.*[8] have found an inverse correlation between the concentration of α-naphthylacetic acid (NAA) in the liquid growth medium and the

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polyphenol content of tobacco callus cultures. In cultures lacking NAA, browning would occur during fixation in neutral alcohol. In addition, Stafford and Galston[4] report that the browning of protein extracts from tobacco tissue is lower in the presence of added IAA. Thus it is difficult to draw a firm conclusion from the correlation between the presence of IAA, increased PPO activity and the brown pigmentation of our strain of tobacco tissue cultures.

EXPERIMENTAL

The tissues used in this study were originally isolated as single cell clones by A. C. Hildebrandt at the University of Wisconsin. Subcultures of the tissues were obtained from Wisconsin after the clones had been established in culture for several years. Tissue cultures H-196 and H-239 were derived from a hybrid cross of *Nicotiana tabacum* × *N. glutinosa*, while G-252 was derived from *N. glutinosa*, and M-222 was isolated from *N. tabacum* by G. Morel and subsequently cloned by Hildebrandt. The cultures were maintained on a modified White's agar medium[9] to which 2 g/l. acid hydrolyzed casein and 2 mg/l. IAA or 2,4-D were added. The cultures were maintained at 27 ± 1° under fluorescent lighting.

Extraction. Tissue was blotted between paper towels to rid it of excess H_2O. 5 g of blotted tissue were homogenized in 25 ml of ice-cold 0·1 M phosphate buffer, pH 7·0, for 1 min at 'high' speed in a VirTis '45' homogenizer. The brei was centrifuged at 20 000 *g* for 20 min at 0–2° and the supernatant was retained as the enzyme preparation. The assay for PPO is essentially that described by Sisler and Evans[10] which utilizes chlorogenic acid as a substrate for the enzyme. The reaction mixture consisted of 7 ml of 0·1 M phosphate buffer, pH 7·0, 1 ml 10^{-2} M NaEDTA, 1 ml $5{\cdot}7 \times 10^{-4}$ M chlorogenic acid and 1 ml enzyme preparation. A portion of this mixture was quickly added to a 3 ml cuvette and the decrease in absorbance at 326 nm with time was followed. Blanks contained the reaction mixture with 1 ml H_2O substituted for the enzyme. Enzyme activity is expressed as units per ml of enzyme preparation where one unit is defined as that amount of enzyme causing a decrease in absorbance at 326 nm of 0·001 per min. Thus, these units are directly proportional to a standard fr. wt. preparation of the tissue and may be compared on this basis. For chromatography, tissue was extracted by grinding in 80% (v/v) EtOH, filtering the extract with Whatman 1 paper, and concentrating the extract in a flash evaporator. The concentrated extracts were spotted on acid-washed sheets of Whatman 1 paper and developed by descent in *n*-BuOH–HOAc–H_2O (4:1:5, top layer). The bottom layer was used for chamber equilibration. Visualization of the chromatograms under short wave UV light was aided by spraying the papers with a 1% (w/v) methanolic solution of Neu's 'natural product reagent A' (β-aminoethyl diphenylborate, Fluka Co., Buchs SG, Switzerland).[11]

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