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Promotion of Peroxidase Activity in the Cell Wall of *Nicotiana*

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ABSTRACT

Peroxidase catalyzes the oxidation of indole-3-acetic acid. The primary products of this reaction stimulate growth in plants. Therefore, our concept is that an increase in peroxidase activity will increase the effect of indole-3-acetic acid as a growth hormone. Our objective was to study the effect of 2,3,5-triiodobenzoic acid, a growth regulator, on isoperoxidases in the cell wall and cytoplasm of *Nicotiana*. Isoperoxidases from the cell wall and cytoplasmic fractions were separated by acrylamide gel electrophoresis. We found that 2,3,5-triiodobenzoic acid and indole-3-acetic acid increase peroxidase activity in the cell wall. Since both 2,3,5-triiodobenzoic acid and indole-3-acetic acid increase the activity of the same isoperoxidase, we conclude that 2,3,5-triiodobenzoic acid synergizes rather than antagonizes auxin action, and we suggest that this increase in indole-3-acetic acid oxidase activity sensitizes plant tissues to auxin.

Plant growth is inhibited by TIBA[1] and it also causes epinasty of leaves and stems (7, 21).

This "formative" type of growth inhibition resembles that caused by high concentrations of auxin. Nevertheless, data in the literature indicate that TIBA antagonizes the action of auxin (4). It is suggested that TIBA blocks auxin transport (4) or immobilizes auxin and thus renders it nonfunctional (2). Experiments of Bouck and Galston (3) show, however, that the auxin, blocked by TIBA, is functional and that TIBA stimulates growth caused apparently by endogenous auxin that accumulated above the area where TIBA was applied. Thimann and Bonner (19) also reported that TIBA augments the auxin effect in the pea and *Avena* tests.

Our objective was to study the effect of TIBA on the peroxidase system in *Nicotiana* and relate these findings to the concept that the oxidation of IAA catalyzed by peroxidase in plants yields products that stimulate growth (13). We treated *Nicotiana* with TIBA and isolated cell wall and cytoplasmic peroxidases. Cell wall peroxidases were isolated from exudates of intact tissues. Cytoplasmic peroxidase enzymes were isolated from tissues devoid of cell wall peroxidase. Both extracts were subjected to electrophoresis on polyacrylamide gels. We found that both TIBA and IAA stimulated peroxidase activity in the cell wall but not in the cytoplasm. We postulate that an increase in peroxidase activity caused by TIBA may block auxin transport and, at the same time, increase the sensitivity of TIBA-treated tissues to auxin.

[1] Abbreviation: TIBA: 2,3,5-triiodobenzoic acid.

MATERIALS AND METHODS

Nicotiana tabacum L. cv. Xanthi-nc was grown for 7 to 8 weeks in the greenhouse and then sprayed with 1% polyoxyethylene sorbitan (20) monolaurate (Tween 20[2]), or with 10^{-6} to 10^{-3} M TIBA dissolved in 1% Tween 20. Leaves and apices were collected 1 to 2 days later and assayed for peroxidase. Cell wall peroxidases were separated from the cytoplasmic fraction by the vacuum infiltration method (1).

To extract cell wall enzymes, we removed the midrib of each leaf and placed the leaf halves in a desiccator filled with distilled water. The desiccator was evacuated until the tissues released no more air bubbles (5–10 min). The vacuum was released and the tissues were gently blotted. Several tissues were rolled together and placed, with the cut surfaces pointing down, into centrifuge tubes containing a perforated planchet in the bottom. The tissues were centrifuged at 3000 rpm for 10 min and the exudate collected was saturated to 40% (w/v) with sucrose and subjected to electrophoresis.

To extract cytoplasmic enzymes, we homogenized the remaining tissues in 0.4 M tris-glycine buffer (1 g tissue/2 ml of buffer) at pH 8.0 and centrifuged the mixture at 10,000 rpm for 10 min. The supernatant fluid was saturated to 40% with sucrose and subjected to electrophoresis. Total peroxidases were obtained from the supernatant of 1 g of fresh tissue ground in 5 ml of tris-glycine buffer. Protein content of the extracts was determined by the Lowry method (12). The cell wall fractions were checked for malic acid dehydrogenase activity to ascertain whether the exudates were free of cytoplasmic components. The method used was that of Laycock *et al.* (10).

Electrophoretic Separations. Immediately after extraction, the samples (10 μl) were fractionated by zone electrophoresis in 7.5% acrylamide gels submerged in water-cooled 0.4 M tris-glycine buffer at pH 8.0. A current of 2.5 ma per gel was applied until the indicator band of bromophenol blue reached within 5 mm of the bottom of the gel.

Enzymatically active bands were identified by flooding the gels for 15 to 30 min with 0.05% *o*-dianisidine. The gels were then developed for 10 to 15 min in 0.03% H_2O_2 and rinsed in distilled water. The color intensity of the developed bands was measured at 500 nm in a Gilford linear transport gel scanner.

Bioassay. The interaction between TIBA and IAA on growth was examined with the *Avena* first internode test of Nitsch and Nitsch (17). Routinely, ten 4-mm first internode sections were floated in a test tube for 12 hr in 2 ml of 1% sucrose plus TIBA or IAA dissolved in 50 mM phosphate buffer. The tubes were placed in a roller tube apparatus and rotated at a speed of 4 rpm to prevent curving of the sections.

[2] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

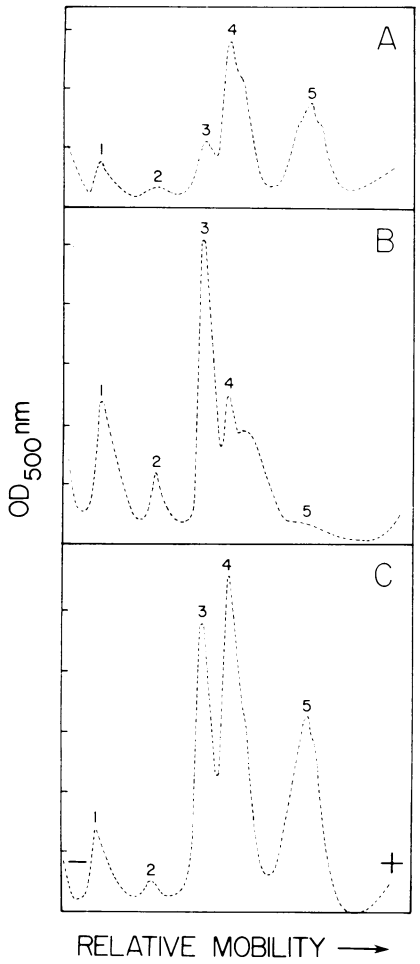

FIG. 1. Scan of cytoplasmic isoperoxidases (A), of cell wall peroxidases (B), and of a homogenate (C), from young leaves 8 to 10 cm long.

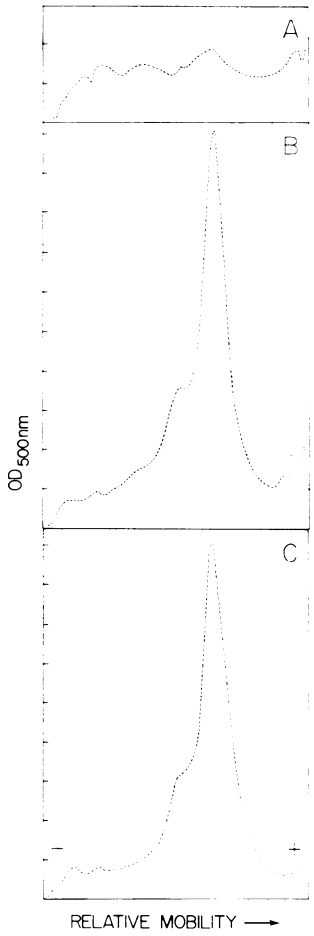

FIG. 2. Malic acid dehydrogenase activity in cell wall fraction (A), cytoplasmic fraction (B), and in homogenates (C), from young leaves 8–10 cm long.

RESULTS

Extracts of tobacco tissues are resolved into five major isoperoxidase enzymes (Fig. 1C). When we analyzed the cell fraction separately from the cytoplasmic fraction, we found that isoperoxidase 3 was the major component of the cell wall peroxidase fraction (Fig. 1B). The cytoplasmic fraction contained most of the isoperoxidase 4 and 5 activity (Fig. 1A).

The specific activity of isoperoxidase 3 in the cell wall fraction (Fig. 1B), was 2.57 u/mg protein and in the cytoplasmic fraction the activity was only 0.02 u/mg protein.[3] To ascertain that the cell wall fractions were devoid of any cytoplasmic components, we assayed these fractions for malic acid dehydrogenase activity, a soluble enzyme in the cytoplasm. As shown in Figure 2, the malic acid dehydrogenase activity was found primarily in the cytoplasmic fraction (Fig. 2B) and little, if any, was detected in the cell wall fraction (Fig. 2A). Figure 2C represents the malic acid dehydrogenase activity from leaf homogenates.

If we freeze the tissues and thus destroy the cytoplasmic membrane, we find that cell wall fractions contain considerable amounts of malic acid dehydrogenase. A comparison of the malic acid dehydrogenase activity found in cell wall fractions from frozen and fresh tissues is shown in Figure 3.

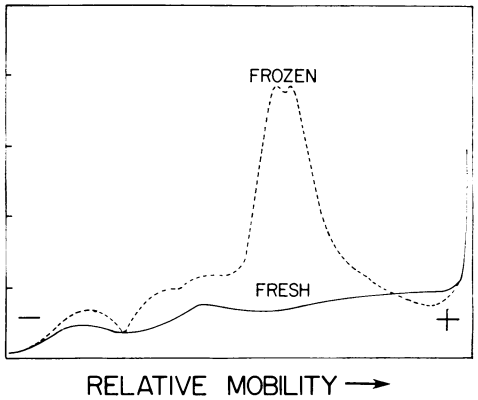

FIG. 3. Malic acid dehydrogenase activity in cell wall fraction of frozen and fresh tissues. Each division on the ordinate axis equals 0.1 $A_{500\ nm}$.

TIBA increases peroxidase activity and, as shown in Figure 4, the increase is attributable entirely to the activity of isoperoxidase 3. Peroxidase activity increases within 8 hr after TIBA treatment and, as shown in Figure 5, it increases logarithmically with increasing TIBA concentrations. TIBA does not change isoperoxidase activity in the cytoplasmic fraction. Figure 6 shows that isoperoxidase 3 activity is also increased by IAA and that the TIBA effect is synergistic.

[3] u: ΔA_{500} min^{-1}.

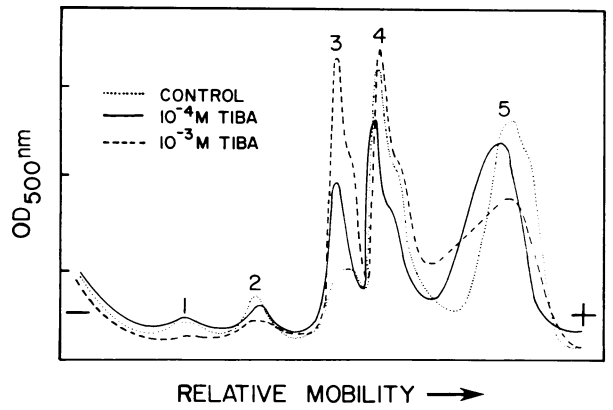

FIG. 4. Effect of TIBA on isoperoxidases isolated from apices which were harvested 8 hr after treatment.

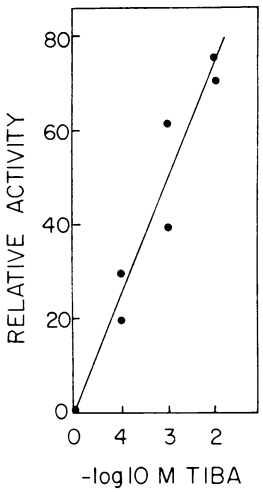

FIG. 5. Activity of isoperoxidase 3 isolated from young leaves 2 days after plants were treated with TIBA. Averages of the experiments.

When TIBA was tested in the *Avena* growth test, we found that 0.1 μM TIBA doubled the IAA-induced growth of the 4-mm *Avena* sections (Fig. 7). Lower concentrations of TIBA were less effective and higher concentrations inhibited IAA-induced growth (Fig. 8). The slight growth stimulation with TIBA in the absence of added IAA might be attributed to residual endogenous IAA.

DISCUSSION

Galston and Dalberg (5) were the first to suggest that the peroxidase enzyme IAA oxidase is an adaptive enzyme. They showed that various auxins and TIBA induce the formation of IAA oxidase in pea plants. In subsequent studies with tobacco pith tissue Galston and his co-workers attributed the auxin-induced increase in peroxidase activity to the increase in the formation of a specific isoperoxidase (9). Our results with IAA support their findings that auxin stimulates the formation of a specific isoperoxidase but we did not observe any repression of the formation of other members of the peroxidase isozyme complex. This difference in results might be because of the difference in plant materials used. The effect of TIBA on peroxidase enzymes was confirmed by Audus and Bakhsh (2), who reported that TIBA more than doubled the rate of oxidation of IAA, and that the increase in IAA oxidase activity was due to the adaptive formation of this enzyme.

Our data also show that TIBA increases peroxidase activity in plants, but, in addition, our results indicate that this increase occurs only in the cell wall. We isolated the cell wall peroxidases from exudates of water-infiltrated tissues. We distinguished the cell wall fractions from the cytoplasmic fractions by the malic acid dehydrogenase activity which is present as a soluble enzyme only in the cytoplasmic fraction. We did not find any increase in isoperoxidase 3 activity in the cell wall fraction when we intentionally destroyed the cytoplasmic mem-

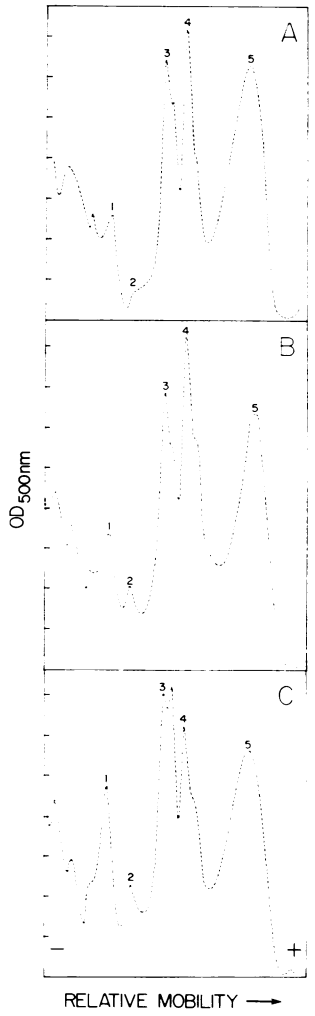

FIG. 6. Scans of isoperoxidases from Xanthi treated with 1 μM TIBA (A), 1 μM IAA (B), and 1 μM TIBA plus 1 μM IAA (C).

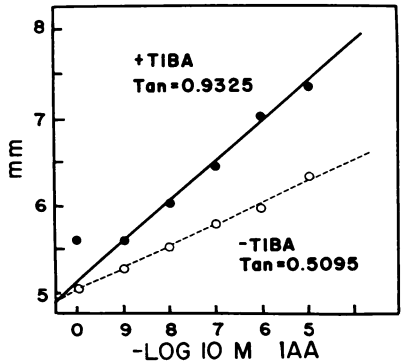

FIG. 7. Effect of 0.1 μM TIBA on IAA-induced growth of first internode sections of *Avena*.

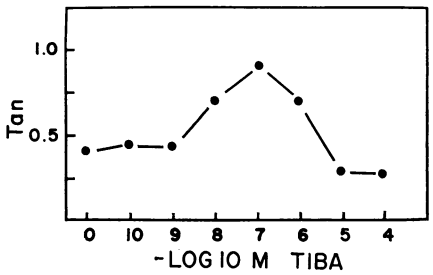

FIG. 8. Effect of various concentrations of TIBA on IAA-induced growth of first internode sections of *Avena*. Each point represents the tangent of the angle of the slope of an IAA concentration response curve similar to that shown in Figure 7.

brane by freezing or treating the tissues with methyl decanoate emulsions, a contact type compound that destroys cytoplasmic membranes (16, 18). These results assured us that the cytoplasmic membrane did not rupture during the isolation of the cell wall fraction and that the isoperoxidase 3 is contained in the cell wall.

The cell wall fractions contained 3 to 4% of the total protein which increases to about 5% when the tissues are treated with TIBA or IAA. More than 94% of the isoperoxidase 3 activity is in the cell wall (Fig. 2), and most of the faster migrating isoperoxidases (isoperoxidase 4 and 5) are found in the cytoplasmic fraction. When plants are treated with TIBA or IAA, only the concentration of isoperoxidase 3 in the cell wall is increased. The activity of isoperoxidases 4 and 5 in the cytoplasm does not change. This suggests that TIBA and IAA react outside of the cytoplasmic membrane.

Since treatments with TIBA and IAA both stimulate peroxidase synthesis, it might be argued that the increase in peroxidase activity is induced by endogenous IAA inhibited from transport by TIBA. This argument is not valid, however, because TIBA increases peroxidase activity in mature tissues in which little or no free auxin is present. Furthermore, other growth regulators such as 2,4-dichlorophenoxyacetic acid; 2,4,5-trichlorophenoxyacetic acid (11); and various benzoic acid derivatives that promote growth (15) also increased peroxidase activity. The increase of isoperoxidase 3 activity was not observed when plants were treated with derivatives of benzoic acid or phenoxyacetic acid that did not promote growth (11, 15).

If the foregoing conclusion has physiological significance, then any increase in peroxidase activity should increase the effectiveness of IAA as a growth hormone. When internode sections of *Avena* plants are treated with 0.1 μM TIBA, the requirement for IAA to induce a given amount of growth is reduced in half (Fig. 7). At higher concentrations of TIBA, the exogenously applied IAA inhibits growth (Fig. 8). These results agree with the observations of Thimann and Bonner (19), and we concur with their interpretation that TIBA "enables a small amount of auxin to bring about a disproportionate amount of growth." We attribute this increase in the oligodynamic effect of IAA to the increase in the oxidative transformation of IAA in the tissues.

This interpretation is also supported by earlier observations that intermediate oxidation products of IAA stimulate growth more than IAA itself (13).

We cannot discard the possibility, therefore, that the interaction between these various growth stimulants is contingent upon (*a*) the presence of auxin, (*b*) the ability of these hormones to induce or promote IAA oxidase activity, and (*c*) the presence of specific receptor sites for the oxidation products of IAA to bind to. The latter requirement is deduced from observations that oxidation products of IAA bind readily to macromolecules in plants (6) and react readily to form condensation products with aldehydes *in vitro* (14).

The stimulation of IAA oxidation by TIBA and the binding of the reaction products may explain how TIBA inhibits auxin transport. At low TIBA concentrations, the increase in the oxidative transformation of IAA may enhance auxin-induced growth. When higher concentrations of TIBA, or super-optimal concentrations of IAA are used, the oxidation of IAA increases and the production of reactive IAA molecules may thus exceed the number of available receptor sites that can accommodate optimal growth. Under these conditions it is conceivable that "formative" growth inhibition may be induced by excess reactive IAA molecules competing for the same receptor sites.

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