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# An Ethylene Biosynthesis-Inducing Endoxylanase Elicits Electrolyte Leakage and Necrosis in *Nicotiana tabacum* cv Xanthi Leaves[1]

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## ABSTRACT

**We have previously demonstrated that a protein purified from xylan-induced culture filtrates of *Trichoderma viride* contains β-1,4-endoxylanase activity and induces ethylene biosynthesis in tobacco (*Nicotiana tabacum* cv Xanthi) leaf discs. When the ethylene biosynthesis-inducing xylanase (EIX) was applied to cut petioles of detached tobacco leaves, it induced ethylene biosynthesis within 1 hour and extensive electrolyte leakage and necrosis were observed in tobacco leaf tissue within 5 hours. Ethylene-pretreatment (120 microliters per liter ethylene for 14 hours) of tobacco leaves enhanced ethylene biosynthesis in response to EIX by more than threefold and accelerated development of cellular leakage and necrosis. In intact plants, similar symptoms could be induced in leaves that were distant from the point of the enzyme application. The evidence suggests that EIX is translocated via the vascular system and elicits plant responses similar to those observed in a hypersensitive response.**

Ethylene is released during the process of protoplast formation by cell wall digesting enzyme preparations (1, 10, 12). Fuchs *et al.* (11) identified a β-1,4-endoxylanase which copurifies with the primary ethylene biosynthesis inducing factor in a commercial enzyme preparation, Cellulysin.[2] The EIX[3] has been purified directly from xylan-induced *Trichoderma viride* cultures (7). Xylanases with similar physical characteristics, including cross-reactivity with EIX antibodies, have been identified in culture filtrates of plant pathogenic fungi (6) as well as a number of commercial enzyme preparations (6, 11).

Hydrolytic enzymes and their biologically active products have been demonstrated to elicit diverse responses in plants besides ethylene production (18, 20), including necrosis (8, 14, 17, 20), lipid peroxidation (17), electrolyte leakage (14, 17) and biosynthesis of secondary products (20), and hydroxyproline-rich glycoprotein (18). It is possible that EIX induces ethylene biosynthesis by mechanisms similar to those involved in other elicitor-dependent plant defense responses (5, 6) but, we have no evidence that EIX generates heat-stable components responsible for this bioactivity (10).

Recently, Ishi (13) demonstrated that a macerating xylanase isolated from a commercial preparation of *T. viride* origin was toxic to rice cell cultures. Lotan and Fluhr (16) observed the induction of pathogeniesis-related proteins (*i.e.* chitinase) in tobacco plants in response to EIX treatment. These observations provide increasing evidence that EIX acts as an elicitor of plant defense responses. Results presented here demonstrate that in addition to induction of ethylene biosynthesis in intact *Nicotiana tabacum* L. cv Xanthi leaves, EIX causes necrosis and electrolyte leakage. All of these symptoms are commonly observed plant responses to exogenously applied elicitors or inoculation with plant pathogens.

## MATERIALS AND METHODS

### Plant Materials

Greenhouse grown tobacco plants (*Nicotiana tabacum* L. cv Xanthi), 25 to 30 cm tall which had not initiated flower development were used in intact plant studies. Detached leaf experiments used fully expanded leaves 10 to 15 cm long from similar plants.

### Enzymes and Chemicals

Ethylene inducing xylanase was purified from xylan-induced *Trichoderma viride* cultures as previously described (7). The AVG was a gift from Dr. A. Stempel, Hoffmann La-Roche, Nutley, NJ, while the remaining chemicals were of commercial origin.

### Detached Leaf Treatment

Detached leaves with an average weight of 2 g were incubated in the dark for 14 h in a humid chamber under an atmosphere of 120 μL/L ethylene or an atmosphere purged of ethylene with 10 g of the organic absorbant, Purafill II. EIX, dissolved in 5 μL 200 mM ammonium acetate as a result of the last purification step, was applied as a hanging drop to

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

[3] Abbreviations: EIX, ethylene biosynthesis-inducing xylanase; ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine.

freshly cut petioles of detached leaves. After the solution was absorbed by transpiration-driven uptake, the petioles were placed in assay medium containing 10 mM Mes (pH 6.0), 250 mM sorbitol, 50 μg/mL streptomycin sulfate, and 50 units/mL penicillin G. The effect of EIX concentration on ethylene induction was determined using a preparation containing 0.2, 1.0, and 5.0 μg of protein. Controls included aliquots of boiled EIX or nonboiled 200 mM ammonium acetate. The treated leaves were maintained under light and temperature conditions on a laboratory bench until ethylene production or electrolyte leakage were measured.

### Ethylene Measurement

For ethylene measurements, treated detached leaves were sealed in jars (475 mL) for various time intervals and gas samples were taken for analysis through rubber septa affixed to the jar lids. Ethylene production by leaf discs taken from detached leaves was determined by cutting six leaf discs (1 cm diameter, average total weight 85 mg), and placing them in a 25 ml Erlenmeyer flask containing 1 mL of assay medium or deionized distilled water. Leaf discs were cut from leaves at 1 h intervals after EIX treatment, and the flasks were sealed with rubber septa for 15 to 60 min before taking gas samples. Ethylene was quantified by gas chromatography (15). The responsiveness of ACC synthase to EIX was demonstrated by inclusion of 0.1 mM AVG, an inhibitor of ACC synthase, in the assay medium. The effect of osmoticum was determined by eliminating sorbitol from the assay medium.

### Electrolyte Leakage

Six leaf discs, cut at 1 h intervals from EIX-treated detached leaves, were placed in 1 mL deionized distilled water. The pH and conductivity of the water, expressed as micromhos, was determined after 1 h using a Markson SensiMark Analyzer, model 5504.

### Gel Electrophoresis and Western Blotting

To determine whether EIX moved from the point of application, 1 cm discs were taken at 3 cm intervals along the leaf midrib. Protein was extracted by grinding in SDS-PAGE sample buffer (50 mM Tris-HCL [pH 6.8], 12% glycerol, 4% SDS, 0.01% Coomassie brilliant blue G-250, 2:1 w/v). The samples were boiled 2 min and subjected to Tricine-SDS-PAGE (19), using 13% gels (5×8×0.075 cm). Proteins from gels were electroblotted to Polyvinylidene difluoride membranes (1 h at 100 V), and EIX bands were identified by Western blot analysis using the antibody raised against 22 kD EIX polypeptide as previously described (6). The gel lanes were loaded using equal volumes (5 μL) of extract since the protein concentration of the extracts increased from the petiole outward to the leaf tip. The maximum protein loaded per lane was 5 μg.

### Whole Plant Experiments

Whole plants were exposed to 120 μL/L ethylene for 14 h in sealed chambers after which 50 μg EIX was applied to the midvein of a leaf petiole midway up the stem using a 200 μL microcapillary pipet tip. The uptake of 50 μL of a solution containing EIX required approximately 15 min. The development of necrotic lesions was observed for at least 48 h after EIX was applied.

## RESULTS AND DISCUSSION

### Ethylene Induction in Intact Leaves

Previous studies of the response of tobacco to EIX or Cellulysin utilized a leaf disc bioassay. Ethylene production is a common response to wounding of some plant tissues (22), but not in tobacco leaves. However, it is possible that other wound-induced effects in tobacco leaf discs, such as callose synthesis or vascular occlusion, might impede EIX penetration of the tissue, and thus complicate our understanding of the induction of ethylene biosynthesis by EIX. Consequently, we developed a detached leaf bioassay to minimize any wound effects introduced by cutting of tobacco leaf discs.

Using the detached leaf bioassay, an increase in ethylene production was observed in leaves within 1 h of EIX application. The rate of ethylene production peaked after 3 to 4 h (Fig. 1), decreased between 4 and 5 h, but remained elevated even after 8 h. A previous study also reported enhanced ethylene production in tobacco leaf discs within 1 h after application of Cellulysin as the source of EIX (4). Ethylene production rates increased in response to higher EIX concentration; however, the response was not linear.

Pretreatment of detached leaves with ethylene resulted in three- to fourfold higher rates of ethylene production compared to leaves incubated in an atmosphere depleted of ethylene (Fig. 2). Stress caused by incubating detached leaves in an ethylene-depleted atmosphere did not enhance ethylene production compared to freshly harvested leaves, indicating

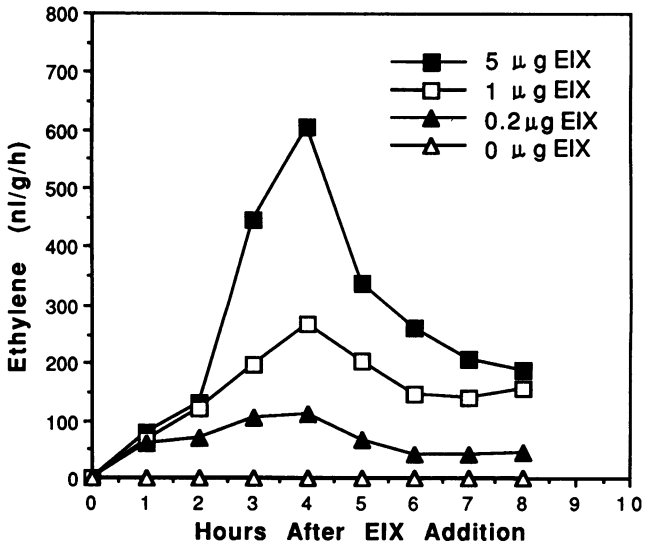


**Figure 1.** Induction of ethylene biosynthesis in detached tobacco leaves treated with EIX. Leaves were pretreated with 120 μL/L ethylene for 14 h, and EIX was applied to cut petioles. At hourly intervals jars containing detached leaves were sealed for 15 min, after which the accumulated ethylene was measured.

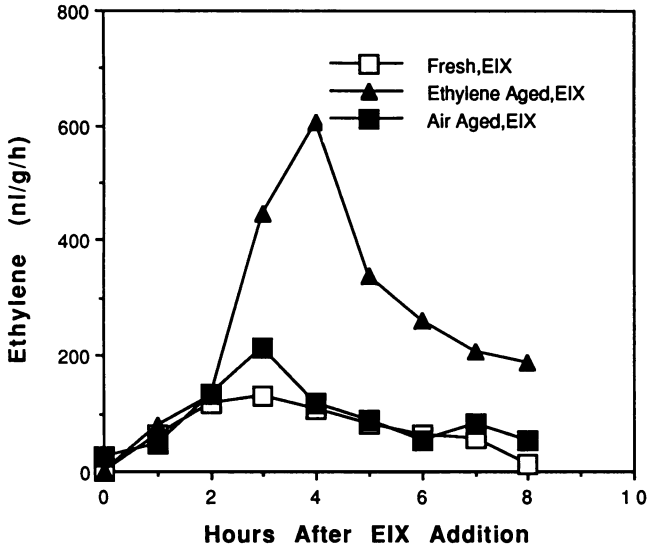


**Figure 2.** Effect of ethylene-pretreatment on ethylene production by detached tobacco leaves treated with EIX (5 μg). EIX was applied to leaves after 14 h incubation in either an ethylene-purged atmosphere or 120 μL/L ethylene or fresh leaves. Detached leaves placed in jars were sealed for 15 min at hourly intervals, after which ethylene levels were determined. Ethylene production was below the limits of detection for control leaves (-EIX) of each treatment.

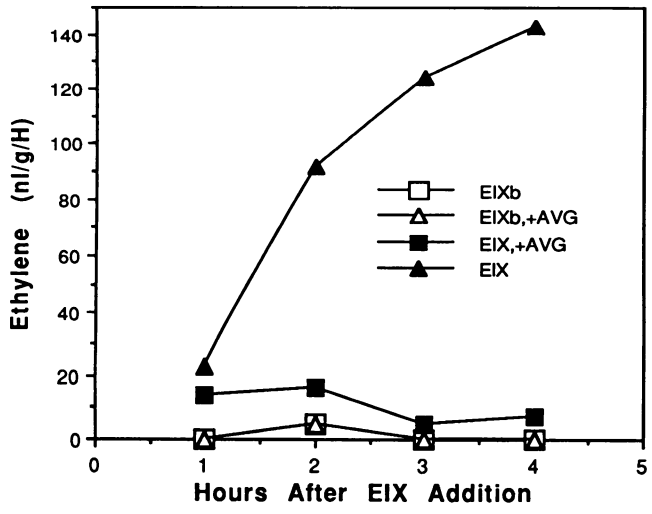


**Figure 3.** Effect of AVG (0.1 mM) on ethylene biosynthesis by leaf discs cut from EIX-treated detached leaves. Detached tobacco leaves were pretreated with 120 μL/L ethylene for 14 h, and 5 μg native EIX or boiled EIX (EIXb) was applied to freshly cut leaf petioles. Ethylene production was determined for leaf discs cut from the leaf midsection 1 h after EIX application. Discs were floated on assay media with or without AVG in 25 mL flasks which were sealed at hourly intervals. Ethylene samples were taken 1 h after the flasks were sealed.

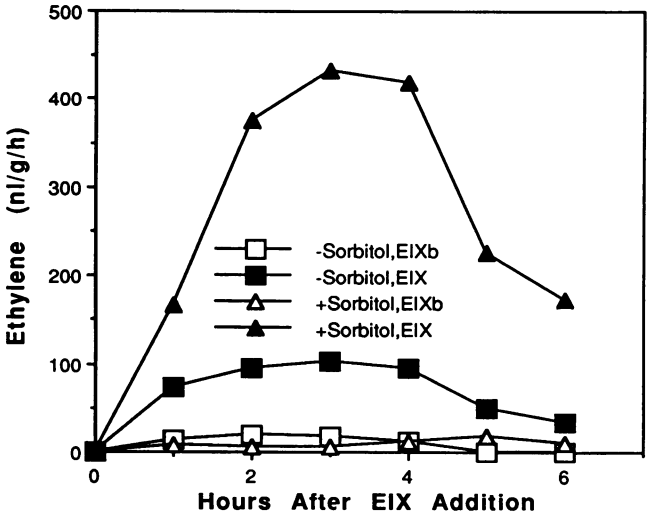


**Figure 4.** Osmotic effects on ethylene production by EIX-treated detached tobacco leaves. Five micrograms of native EIX or boiled EIX (EIXb) was applied to the petioles of ethylene-pretreated leaves (120 μL/L, 14 h) and the tobacco leaves were placed in jars containing basal media plus or minus 250 mM sorbitol. The jars were sealed for 15 min at hourly intervals, after which ethylene production was determined.

that the enhancement of EIX-induced ethylene biosynthesis in ethylene-pretreated leaves was a response to ethylene only. This may be attributed to an increased capacity to convert ACC to ethylene by the ethylene forming enzyme (5).

Addition of the ACC synthase inhibitor AVG to discs from EIX-treated leaves resulted in effective inhibition of ethylene biosynthesis (Fig. 3). Cellulysin was previously shown to stimulate ACC synthase activity as evidenced by increased ACC content and ethylene production in treated tobacco leaf discs (1). The present data suggest that EIX is inducing ethylene biosynthesis in detached leaves in a similar manner. The ability of AVG to inhibit ethylene production in discs taken from EIX-treated leaves also indicates that ACC is being synthesized directly in the discs, rather than being transported into the discs from other sites of synthesis in the leaf such as the petiole or midrib.

The removal of sorbitol from the assay media resulted a fourfold reduction of ethylene production in response to EIX treatment (Fig. 4). The level of osmoticum used does not induce plasmolysis and leaves placed in the sorbitol containing assay media remain turgid for more than 48 h. It is unclear if the osmoticum is enhancing ethylene production by preventing osmotic shock of the tissue or perhaps directly effecting the interaction of EIX with the leaf tissue.

### Tissue Necrosis

Extensive necrotic lesions developed 6 to 8 h after EIX was applied to ethylene-pretreated detached leaves (Fig. 5). The degree of necrosis varied in proportion to the level of EIX applied. Necrotic lesions also formed after EIX treatment of fresh leaves or detached leaves incubated in an ethylene-depleted atmosphere. However, in the absence of ethylene-

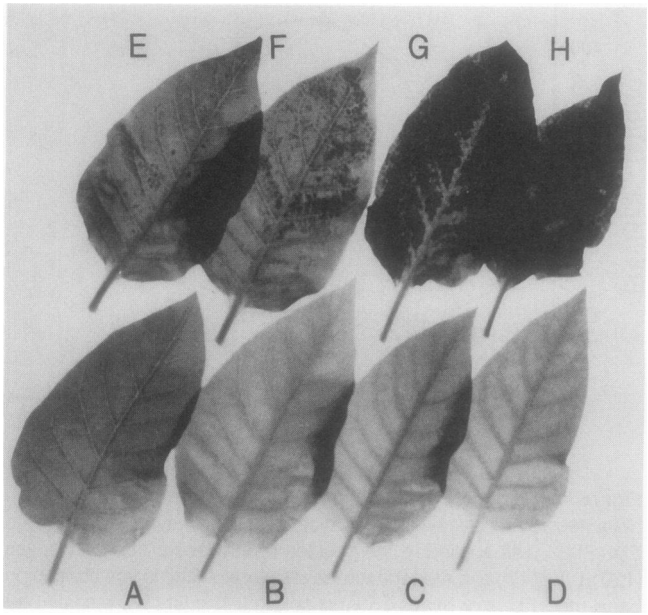


**Figure 5.** Necrotic lesion development in leaves treated with EIX. Ethylene-pretreated tobacco leaves (120 μL/L, 14 h), were treated with varying levels of EIX and the leaf petioles placed in media. Symptom development was photographed after 24 h. Leaves A through H were treated with 0, 25, 50, 100, 250, 500, 1000, 5000 ng of EIX, respectively.

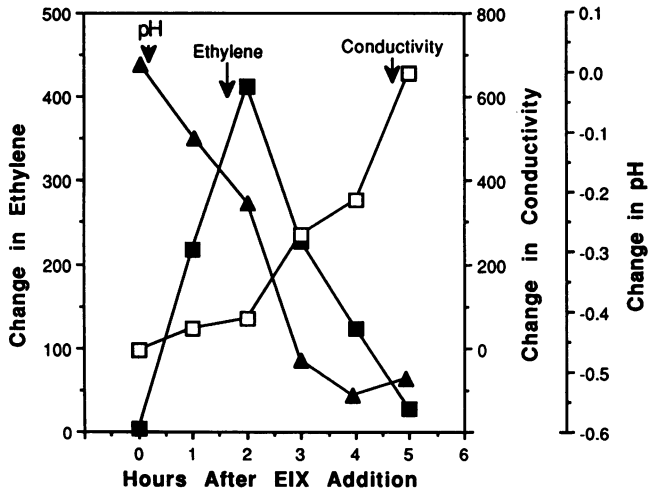


**Figure 6.** Effect of EIX on ethylene biosynthesis (nl/g/H), conductivity (μmhos) and pH. EIX (5 μg) was applied to freshly cut petioles of ethylene-pretreated (120 μL/L, 14 h) tobacco leaves. Leaf discs were cut at hourly intervals and floated on distilled water. The flasks were sealed for 1 h for ethylene accumulation, after which the conductivity and pH of the water was determined. The data are expressed as the difference between EIX-treated samples and boiled EIX-treated controls. The conductivity and pH of control samples remained nearly constant at 65 μmhos and 6.7, respectively. Ethylene production by control samples was low (12.4 nL/g/h) to nondetectible.

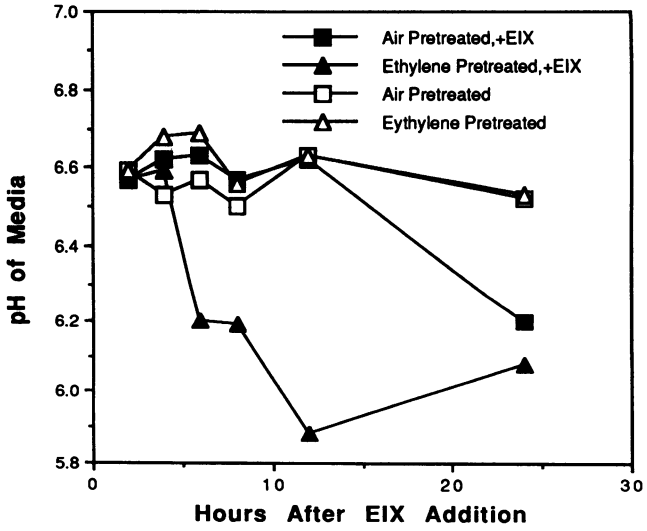


**Figure 7.** Effect of ethylene-pretreatment on changes in media pH in response to EIX treatment. Leaves were pretreated with ethylene (120 μL/L, 14 h) or an ethylene-purged atmosphere (14 h) prior to application of 5 μg native EIX. Leaf discs were cut at hourly intervals, placed in 1 mL deionized distilled water for 1 h, and the pH of the water was determined.

pretreatment the development of lesions was delayed by more than 8 h, and was less severe. Necrotic lesion development was not observed when EIX was applied to tobacco leaf discs bathed in assay media (1, 10, 11). Osmotic agents have been shown to decrease necrotic symptom development in several plant interactions with necrosis inducing factors (2, 3, 21). As was the case with ethylene biosynthesis induction, we observed that osmoticum in the assay medium was required for optimum development of necrosis in the detached leaf system suggesting the osmoticum may function differently in the two systems.

## Cell Leakage

Discs taken from ethylene-pretreated detached leaves which had been EIX-treated showed extensive electrolyte leakage between 3 and 4 h after EIX application (Fig. 6). The increase in electrolyte leakage was associated with declines in ethylene production and media pH. Increases in electrolyte leakage have also been observed in plants in response to pathogens (14) and proteinacious elicitors (17). The drop in media pH in response to EIX of air-pretreated leaves was delayed by more than 6 h (Fig. 7) when compared with ethylene-pretreated leaves indicating ethylene-pretreatment influences cellular responses other than ethylene production.

These experiments did not address the question as to whether increased ethylene production in ethylene-primed tissue directly effects the degree of cellular leakage in EIX-treated tissue. However, preliminary data suggest that ethylene biosynthesis induction and cell leakage are independent responses.

## EIX Movement

EIX was easily detected in treated leaves by Western blot analysis of extracted leaf proteins (Fig. 8). After 2 h of treat-

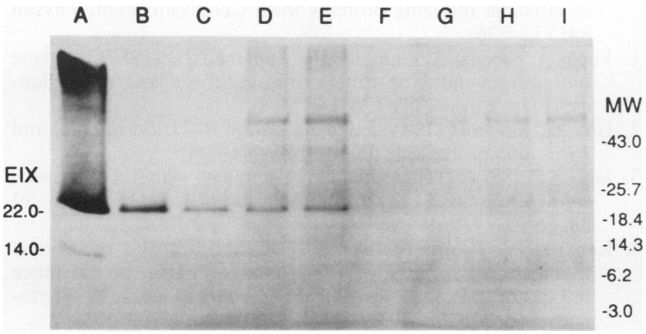


**Figure 8.** EIX movement in intact leaves. Ethylene-pretreated detached leaves were treated with EIX (0 or 5 μg) and petioles placed in media for 2 h. Discs were cut at 3 cm intervals along the leaf midribs and protein extracts were prepared for Western blot analysis. Lane A contains 2.5 μg EIX purified from xylan-induced *Trichoderma viride* cultures. Lanes B to E were loaded with disc extracts taken 0, 3, 6, and 9 cm from the point of EIX application. Lanes F to I were loaded with disc extracts taken 0, 3, 6, and 9 cm away from the petiole end of control leaves.

ment, EIX was observed in extracts from discs all along the leaf midrib. Other bands identified in control samples, as well as EIX-treated samples, probably result from nonspecific binding to plant proteins of high concentration. Western blots also show extracts of primarily mesophyll tissue from EIX-treated leaves to contain substantial EIX (BA Bailey, unpublished data). It appears that EIX moves freely through the leaf vascular system, but how or if EIX exits the vascular tissue is not clear.

The speed of EIX movement is evident when the time of earliest detectable ethylene induction in the detached leaf bioassay is compared to leaf disc assays (1). Localization of EIX solely to the petiole application site and subsequent induction of ethylene biosynthesis in distal mesophyll tissues would have supported the hypothesis that xylan fragments generated by EIX xylanase activity transmit the signal for ethylene induction. The rapid movement of the EIX protein throughout the leaf, while not supportive of this hypothesis, still does not preclude it.

### Effect of EIX on Intact Plants

When EIX was applied to ethylene-treated intact plants, necrotic lesions were observed on leaves above and below the point of application (Fig. 9) within 8 h. Leaves on the side opposite the treated leaf did not develop necrotic lesions. Lesions developed slower on partially expanded leaves near the apical meristem, and were not apparent until 24 h after EIX application. Thus, it appears that EIX or a secondary messenger produced in response to EIX treatment travels up and down the central axis of the plant via the vascular system. Apparently, there is only limited lateral movement. How the signal moves down the plant against the pull of transpiration has not been determined, but it is under investigation.

### Concluding Remarks

Electrolyte leakage (14, 17), ethylene production (5, 18, 20), and necrosis (14, 17) are symptoms associated with the hypersensitive response in plants responding to pathogen attack. Plant pathogens have been shown to produce xylanases similar to EIX in xylan-induced cultures (6), but to date EIX-like proteins have not been identified in extracts of diseased plants. However, Farmer and Helgeson (9) were able to identify a 46 kD proteinaceous elicitor from *Phytophthora parasitica* var nicotianae in infected tobacco plants which was associated with a β-(1–4) endoxylanase activity. The ready

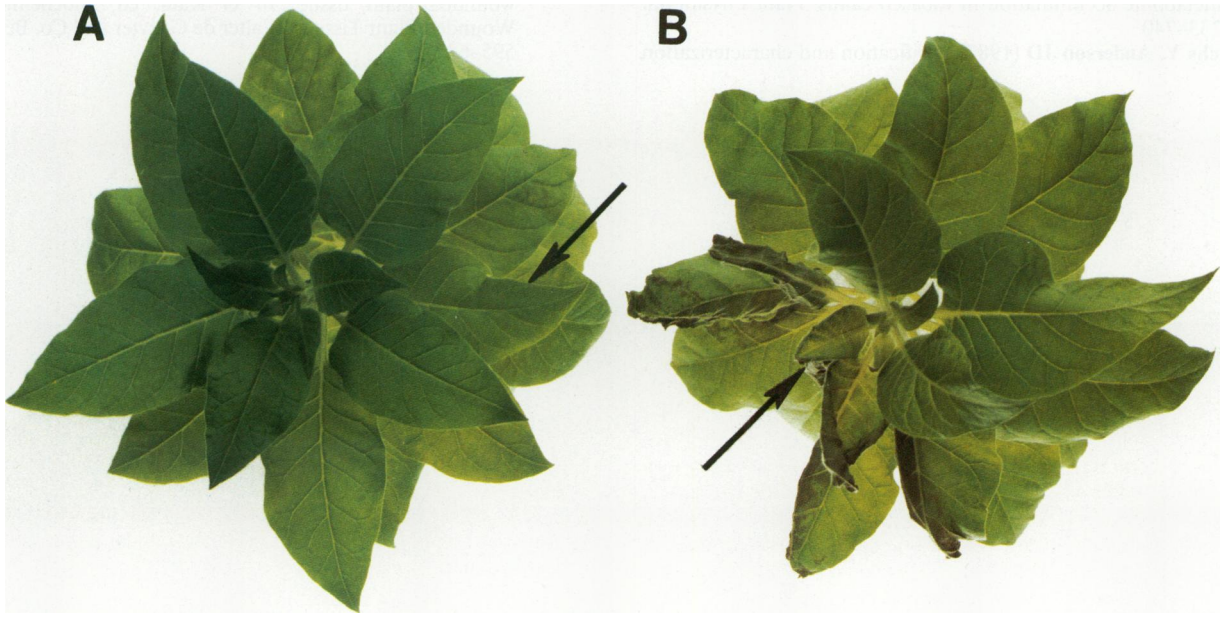


**Figure 9.** Necrotic lesion development in ethylene-pretreated (120 μL/L, 14 h) whole plants after EIX application. Boiled EIX (A) or EIX (B) (50 μg) was applied to a leaf petiole midway up the plant stem.

availability of a purified protein capable of eliciting a plant response resembling hypersensitivity will facilitate study of this defense mechanism. Additionally, the ability of this protein to be translocated in the vascular system of the plant suggests that EIX or similar proteins may be the first signal recognized by the plant in the course of infection.

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