# Purification of a Protein from Culture Filtrates of *Fusarium oxysporum* that Induces Ethylene and Necrosis in Leaves of *Erythroxylum coca*

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## ABSTRACT

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Culture filtrates of *Fusarium oxysporum* elicited ethylene production and necrosis when applied to leaves of *Erythroxylum coca*. A protein that induces ethylene production and necrosis in leaves of *E. coca* was purified from culture filtrates of an isolate of *F. oxysporum* pathogenic to *E. coca*. The protein was first concentrated using ultrafiltration where it was retained by a 1-kDa filter. The protein was purified by fast protein liquid chromatography using cation exchange chromatography followed by hydrophobic interaction chromatography. Gel filtration was used as the final purification step and to exchange salts. The protein migrated as a single band on sodium dodecyl sulfate-polyacrylamide gels with an estimated molecular mass of 22.5 kDa. The exact mass was determined by mass spectroscopy to be 23,996.1 Da. The mobility of the protein was not affected by reducing agents, but the 24-kDa protein broke down and was inactivated by excessive heat. The protein constituted a major component of the extracellular proteins produced in cultures three or more days old and reached a maximum concentration between 6 and 12 days. Biological activity of the protein could be detected by induction of ethylene down to a concentration of 50 ng per leaf (approximately 500 ng/g fresh weight) when applied as a hanging drop to the petiole of an excised *E. coca* leaf. The 24-kDa protein induced ethylene biosynthesis and necrosis in a wide variety of Dicotyledoneae, but we were unable to demonstrate activity in members of the Monocotyledoneae tested. It remains to be determined if the 24-kDa protein plays a role in disease development in the *F. oxysporum-E. coca* interaction.

*Erythroxylum coca* Lam. and *E. novogranatense* (Morris) Hieron., the principal sources of cocaine, are grown on large acreages in several South American countries (28). The measures currently used to control production include physical removal and/or use of broad-spectrum chemical herbicides. An ongoing epidemic of Fusarium wilt of coca caused by *F. oxysporum* Schlechtend.:Fr. in the Huallaga Valley of Peru may ultimately limit the production of coca leaves in this region (22,28), and it raises the possibility of using mycoherbicides as a means of limiting coca production. The high degree of host specificity of many mycoherbicides (21) makes this method a promising alternative to other control measures in the remote, ecologically sensitive, and diverse areas where coca is grown. Before mycoherbicides can be used as a control measure, the many variables that influence disease development must be studied. One of the most important aspects of any mycoherbicide is host range. The limited host range (2) of many *F. oxysporum* formae speciales makes them good candidates for use as mycoherbicides.

*F. oxysporum* as a species includes many saprophytic forms, some of which function as antagonists of pathogenic forms (22). Pathogenic isolates of *F. oxysporum* cause wilt (6) of specific plant species that serve as their hosts. The factors that determine the host range of the pathogenic forms of *F. oxysporum* are not known. It has been known for many years, however, that *F. oxysporum* can produce toxins (13), such as fusaric acid (16) and lycomarasmin (14). These toxins can elicit disease symptoms in plants, but their importance in disease development and host-range determination is unclear. In addition to these toxins, *F. oxysporum* produces many different types of enzymes capable of degrading cell walls (8). Recent studies have indicated that plants resistant to Fusarium wilt can be selected from cell-culture lines resistant to toxic components found in *F. oxysporum* culture filtrates (7,18,19). The culture filtrates contain several different toxic compounds, including carbohydrate components of cell wall hydrolysis (18) and glycoproteins (7).

Sutherland and Pegg (26) found that protoplasts of tomato lines susceptible to specific isolates of *F. oxysporum* were more sensitive to proteinaceous toxins present in culture filtrates of those same isolates. Nonhost plants were much less sensitive to toxins produced by isolates pathogenic to tomato than were susceptible tomatoes. These proteinaceous toxins induced responses in plants commonly observed after treatment with proteinaceous elicitors, including biosynthesis of ethylene (25,27), as well as many other host responses, including cell death (12,20,23,25,29), and may share similar mechanisms of action.

This report describes the purification of a protein from culture filtrates of an isolate of *F. oxysporum* pathogenic on *E. coca*. The protein was purified based on its ability to induce ethylene production and necrosis in leaves of *E. coca*.

## MATERIALS AND METHODS

**Bioassays.** The *Erythroxylum* species were obtained from the collection of the Weed Science Laboratory in Beltsville, MD. The remaining plant species were obtained from commercial sources or from local plantings. The majority of plants were grown under greenhouse conditions from seed prior to use in bioassays. The bioassays were carried out using leaves that were fully expanded for less than 2 weeks. Each leaf was removed from the plant, and the petiole was freshly cut with a razor blade prior to placing the

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leaf in a jar (58 ml) with the petiole in assay buffer (10 mM 2-[*N*-morpholino]ethanesulfonic acid [MES], 250 mM sorbitol, pH 6.0). Up to 25 µl of sample (culture filtrates containing various concentrations of protein) was added to the assay buffer to reach a final volume of 150 µl. Alternatively, the sample was applied as a hanging drop to the end of the leaf's freshly cut petiole (hanging drop method). The leaf was placed in a jar with the petiole in 150 µl of assay buffer after the drop had been taken up by the leaf (usually within 15 min). The controls were treated with an equal volume of the uninoculated growth media (Czapek-Dox broth plus 1% casamino acids) or sample buffer (200 mM KCl, 20 mM MES, pH 5.0). The jars were sealed with rubber stoppers containing ports capped with rubber septa. The jars remained sealed for 48 h and were maintained on the lab bench at room temperature (approximately 25°C). Gas samples were taken from the head space 24 h after sealing the jars. Ethylene was quantitated by gas chromatography with a 150-cm alumina column and a flame ionization detector. Necrosis measurements were made 48 h after sealing the jars and are reported using a 0 to 3 scale where 0 = no damage, 1 = <10% necrosis, 2 = 10 to 50% necrosis, and 3 = 50 to 100% necrosis.

**Fungal culture conditions.** An isolate (EN-4) of *F. oxysporum* pathogenic to *E. coca* was provided by D. Sands (Montana State University, Bozeman). The coca pathogen was grown for 2 to 22 days in Czapek-Dox broth plus 1% casamino acids at room temperature (approximately 25°C).

**Purification of ethylene-inducing activity.** Mycelia and microconidia were removed from 100-ml volumes of fungal cultures by vacuum filtration across 0.45-µm filters. For larger volumes (generally 4 liters), the fungal cultures were filtered through four layers of cheesecloth to remove the mycelial mass and then filtered by tangential flow ultrafiltration across a 1-MDa polyethersulfone membrane in a Filtron Minisette system (Filtron Technology Corporation, Northborough, MA). The large molecular weight components in the filtrate were concentrated by ultra filtration across a 1-kDa filter to a volume of less than 200 ml. The retentate was diluted by the addition of 1 liter of 20 mM MES, pH 5.0, and reconcentrated to less than 200 ml to reduce the concentration of salts and exchange buffers. The retentate (>1 kDa) was diluted a second time, 1:1, with 20 mM MES, pH 5.0, before further purification by fast protein liquid chromatography (Pharmacia Biotech, Uppsala, Sweden).

The diluted >1-kDa fraction (400 ml) was applied to a Resource S column (preparative cation exchange). The Resource S column (6 ml) was equilibrated with 20 mM MES, pH 5.0, and eluted with a 240-ml gradient (8 ml/min, 0 to 300 mM KCl) that was linear between 0 to 45 mM KCL, 45 to 135 mM KCL, and 135 to 300 mM KCl. The fraction size was 5.0 ml. Active fractions were pooled and brought to 2 M ammonium sulfate prior to application to a hydrophobic phenyl Superose (2 ml) column equilibrated with 2 M $(NH_4)_2SO_4$ and 20 mM MES, pH 5.0. The protein was eluted with a linear gradient (50 ml, 0.3 ml/min) from 2.0 to 0 M $(NH_4)_2SO_4$ (20 mM MES, pH 5.0). The active fractions were collected and applied in 200-µl volumes to a 24-ml Superdex 75 10/30 column equilibrated with 200 mM KCl and 20 mM MES, pH 5.0, and 0.5-ml fractions were collected. The active fractions were pooled to constitute the purified protein. Protein determinations were made using Bradford analysis (Bio-Rad protein assay, Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as a standard.

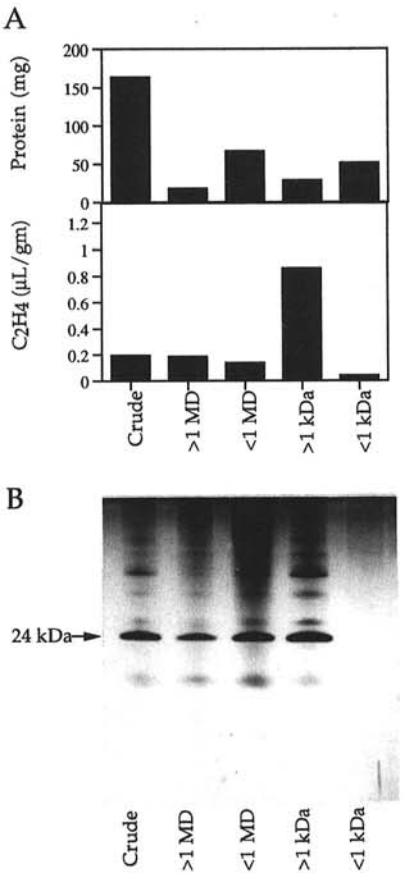


**Fig. 1.** Protein content and elicitor activity in 7-day-old culture filtrates of *Fusarium oxysporum* (EN-4). The fungal cultures were filtered through four layers of cheesecloth to remove the mycelial mass and then sequentially filtered by tangential flow ultrafiltration across 1-MDa and 1-kDa membranes. **A,** Protein content (total protein per fraction) and elicitor activity (ethylene per gram of leaf per 25 µl of each fraction). The protein concentrations and fraction volumes were as follows: crude = 41 µg/ml for 4 liters; >1 MDa = 47 µg/ml for 399 ml; <1 MDa = 18 µg/ml for 3.61 liters; >1 kDa = 190 µg/ml for 177 ml; <1 kDa = 12 µg/ml for 4.43 liters. **B,** Proteins (2 µg per lane) from each fraction were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver stained.

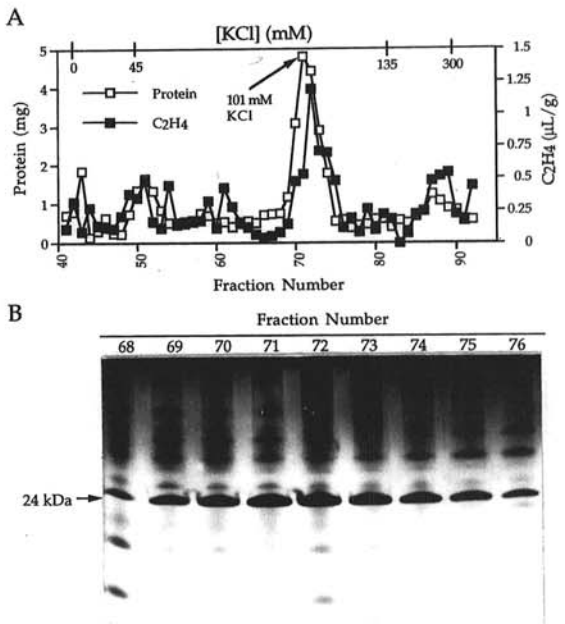


**Fig. 2.** Fractionation of elicitor activity in 7-day-old culture filtrates of *Fusarium oxysporum* (EN-4) using cation exchange (Resource S) column chromatography. The >1-kDa fraction (200 ml) was applied to a Resource S column (6 ml) equilibrated with 20 mM MES (pH 5.0) and eluted with a 240-ml nonlinear gradient (8 ml/min, 0 to 300 mM KCl) and collected in 5.0-ml fractions. **A,** Protein content (total protein per fraction) and elicitor activity (ethylene per gram of leaf per 20 µl of each fraction, ≤5 µg per fraction). **B,** Proteins (10 µl per lane, ≤2 µg per fraction) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver stained.

**Protein electrophoresis.** Protein patterns and purification were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Tris/Tricine system (24) with a Bio-Rad Mini-PROTEAN II. Protein was concentrated when necessary for gels by trichloroacetic acid precipitation. The stability of the purified protein's structure and biological activity was monitored by heating (95°C) in the presence or absence of 2% 2-mercaptoethanol. Usually protein samples were loaded onto gels without the addition of 2-mercaptoethanol or heating.

## RESULTS AND DISCUSSION

Treatment of coca leaves with culture filtrates of the coca wilt pathogen, *F. oxysporum* isolate EN-4, resulted in enhanced ethylene production that was measured 24 h after treatment (Fig. 1A). Ethylene is produced by tomato plants infected with *F. oxysporum* f. sp. *lycopersici*, and exogenous ethylene stimulates responses such as changes in peroxidase and polyphenoloxidase activities, epinasty, and leaf abscission similar to symptoms found in diseased tomato plants (17). Exogenous ethylene also accentuates the levels of necrosis formed in response to elicitors (4,9) and activates many plant defense genes (15). The bioassays of EN-4 culture filtrates concentrated by ultrafiltration indicated that a majority of the biological activity was found in the fraction that passed through the 1-MDa filter but was retained by the 1-kDa filter (Fig. 1A). Much of the protein component estimated to be in the crude fraction passed through the 1-kDa filter and did not show up on SDS gels (Fig. 1B), suggesting components other than protein were interfering with the Bradford analysis.

The ethylene-inducing activity eluted from the Resource S column (cation exchange) in one major peak at 101 mM KCl (Fig. 2A). The peak contained one major protein with an estimated molecular mass of 22.5 kDa and several minor proteins (Fig. 2B). The exact mass of the major protein was later determined by mass spectroscopy to be 23,996.1 Da (henceforth referred to as the 24-kDa protein). At least two other minor activity peaks were observed (fractions 50 to 52 and fractions 86 to 89), each of which was made up of several major proteins (data not shown). The 24-kDa protein eluted in a tailing peak that included the second minor activity peak (fractions 86 to 89). The 24-kDa protein of the main activity peak was similar in size to a major protein observed in the crude protein preps for EN-4. The smaller activity peaks were stored for future study.

Fractions of the main activity peak were pooled and applied to a phenyl Superose column (hydrophobic interaction). The majority of the biological activity (Fig. 3A) bound to the phenyl Superose column and eluted as a single peak at 1.0 M $(NH_4)_2SO_4$. The biological activity eluted parallel with the 24-kDa protein observed in previous steps (Fig. 3B). When applied to the Superdex 75 10/30 column (gel filtration), the 24-kDa protein eluted as a single peak paralleling the biological activity (Fig. 4A and B), but its retention depended on the salt concentration of the equilibration buffer. In low-salt buffer (20 mM MES, pH 5.0), the 24-kDa protein was retained longer by the column than when applied in high-salt buffer (200 mM KCl, 20 mM MES, pH 5.0). The tendency of proteins to adhere to dextran matrices under low-salt conditions has been noted previously (23) and offers a unique purification step if necessary. The basic and hydrophobic character of the 24-kDa protein is similar to a subgroup of elicitors produced by fungi that includes the *Trichoderma viride* xylanase (10) and the elicitins from *Phytophthora* species (23). It is unclear whether these basic hydro-

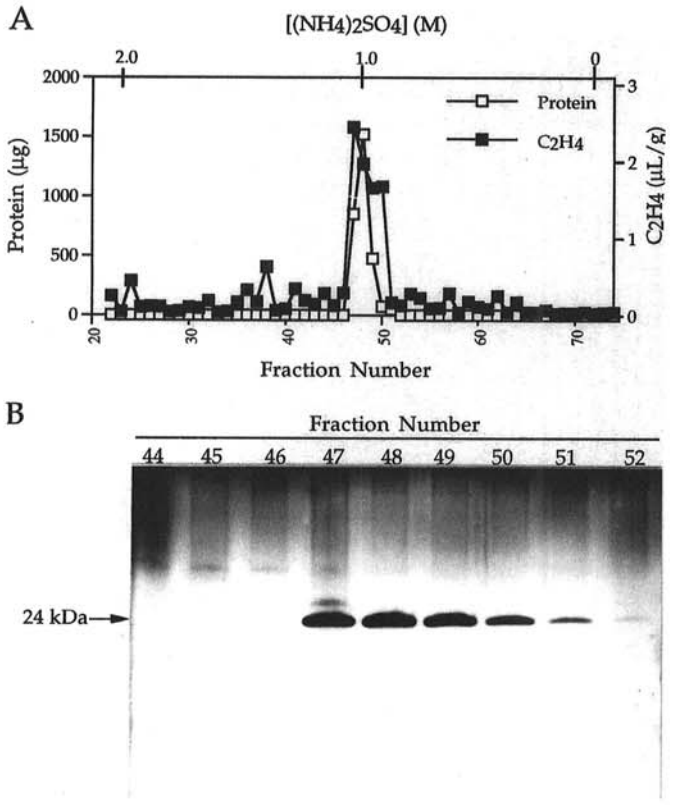


**Fig. 3.** Fractionation of elicitor activity in 7-day-old culture filtrates of *Fusarium oxysporum* (EN-4) using hydrophobic (Phenyl Superose) column chromatography. Active fractions from the cation exchange column (fractions 70 to 74) were pooled and brought to 2 M $(NH_4)_2SO_4$ prior to loading 21 ml onto a phenyl superose (2 ml) column equilibrated with 2 M $(NH_4)_2SO_4$, 20 mM MES, pH 5.0. The protein was eluted with a linear gradient (50 ml, 0.3 ml/min) from 2.0 to 0 M $(NH_4)_2SO_4$ (20 mM MES, pH 5.0). **A,** Protein content (total protein per fraction) and elicitor activity (ethylene per gram of leaf per 3 µl of each fraction, ≤4 µg per fraction). **B,** Proteins (1.5 µl per lane, ≤2 µg per fraction) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver stained.

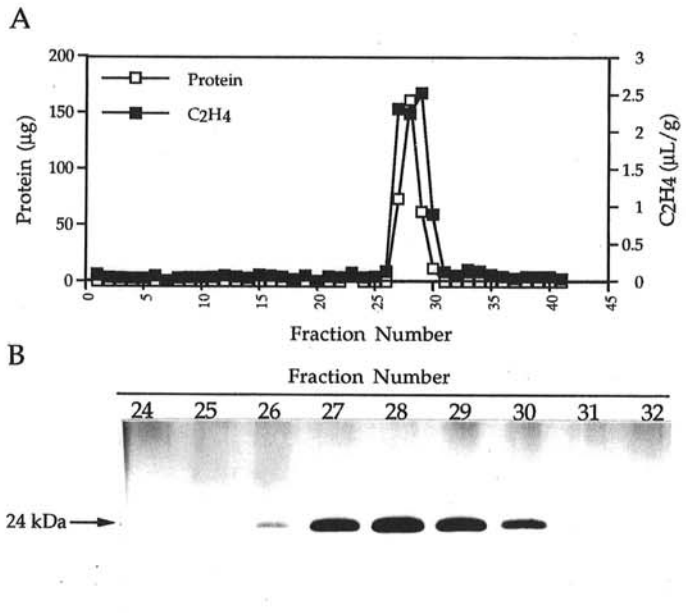


**Fig. 4.** Fractionation of elicitor activity in 7-day-old culture filtrates of *Fusarium oxysporum* (EN-4) using molecular sieve (Superdex 75 10/30) column chromatography. The active fractions from the hydrophobic column (fractions 47 to 49) were collected and applied in 200-µl volumes to a Superdex 75 10/30 column (24 ml) equilibrated with 200 mM KCl, 20 mM MES, pH 5.0. The protein was eluted (1 ml/min) and collected in 0.5-ml fractions. **A,** Protein content (total protein per fraction) and elicitor activity (ethylene per gram of leaf per 13 µl of each fraction, ≤4 µg per fraction). **B,** Proteins (6 µl per lane, ≤2 µg per fraction) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver stained.

phobic characteristics directly influence the protein-plant cell interactions or whether they simply result from their nature as extracellular proteins.

The purified protein was detectable on SDS-polyacrylamide gels in quantities of at least 10 ng (Fig. 5B). The purified protein (50 ng [2 pmol] or more) elicited measurable increases in ethylene when applied as a hanging drop to young coca leaves (Fig. 5A). Necrosis of coca leaves was often visible by 24 h after treatment and continued to develop until 48 h after treatment. Extensive necrosis was observed when 500 ng of the 24-kDa protein was applied to coca leaves as a hanging drop. Since the average leaf weight for these experiments was 0.093 g, from 20 to 30 pmol of 24-kDa protein per g of leaf was required to induce measurable levels of ethylene. Ten times more (200 to 300 pmol per g of leaf) of the 24-kDa protein was required to induce extensive necrosis in young coca leaves when applied as a hanging drop. Rapid and extensive induction of necrosis often limited ethylene production by coca leaves over the 24-h sampling period, suggesting that 1 µg of the purified protein applied as a hanging drop to a coca leaf saturated the system. Others have reported that the ethylene biosynthetic pathway is sensitive to elicitor-induced cellular disruption (3).

The hanging drop method is a much more sensitive assay than the method of diluting the protein sample into the assay buffer before allowing uptake. In the hanging drop method, the entire protein sample was taken up by the leaf in 15 min or less, whereas only 30 to 40 µl of the assay buffer containing protein was taken up over the length of the experiment. Significant variability in ethylene and necrosis occurred, even when high concentrations of the 24-kDa protein were applied as a hanging drops. Much of the variability was likely due to uneven uptake and spread of the protein within the leaf (11) as well as many other factors such as leaf age or nutritional status. In all cases, the biological activity (ethylene and necrosis) was distinguished from controls that were consistently near zero.

The biological activity of the 24-kDa protein was relatively insensitive to heat. Heating the protein for 10 min at 95°C, resulted in no measurable inactivation of activity (Fig. 6A). However, heating at 95°C for 1 h or more resulted in breakdown of the protein

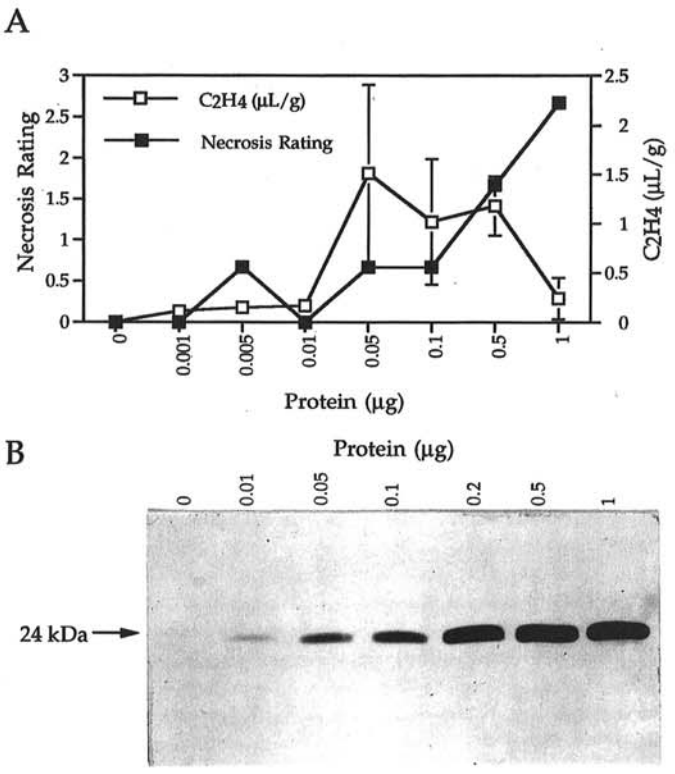


**Fig. 5.** Effect of 24-kDa protein concentration on ethylene production and necrosis in coca leaves and detection of the 24-kDa protein by silver staining. **A,** Varying protein concentrations (0 to 1 µg) were assayed for biological activity using the hanging drop method. Ethylene ($C_2H_4$ per gram of leaf) and necrosis rating (0 = no necrosis to 3 = complete necrosis). **B,** Protein (0 to 1 µg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver stained.

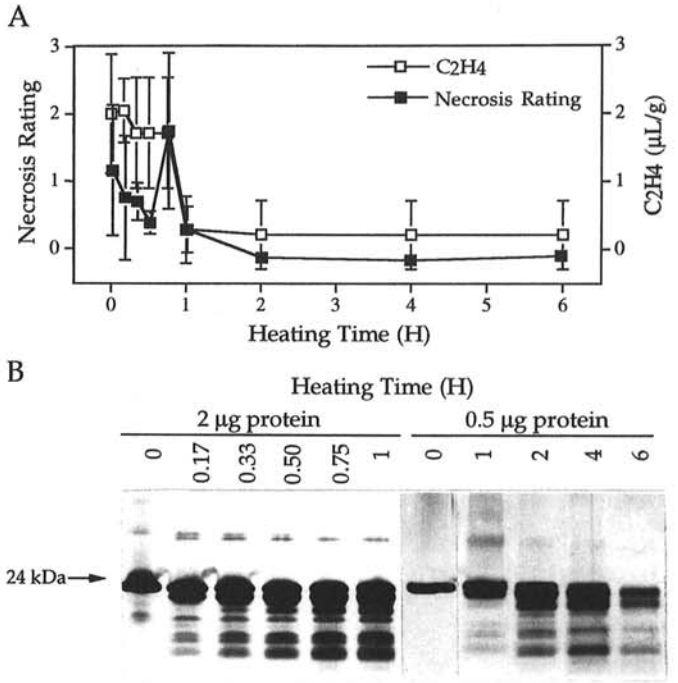


**Fig. 6.** Effect of heat on the biological activity of the 24-kDa protein. Protein was heated at 95°C for varying lengths of time up to 6 h. **A,** The protein (4 µg per assay) was assayed for biological activity using the hanging drop method. Ethylene ($C_2H_4$ per gram of leaf) and necrosis rating (0 = no necrosis to 3 = complete necrosis). **B,** The protein (2 or 0.5 µg of protein) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver stained.

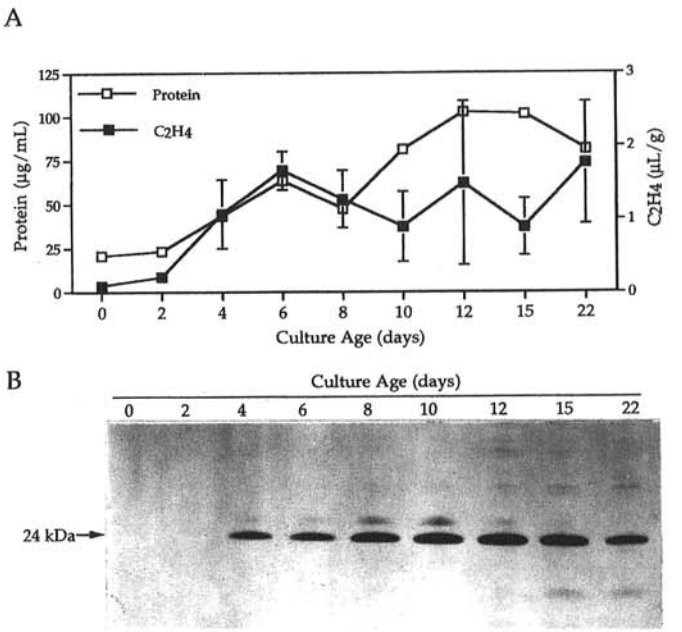


**Fig. 7.** Time course for the accumulation of protein and elicitor activity in crude culture filtrates of *Fusarium oxysporum* (EN-4). **A,** Protein content (µg of protein per milliliter) and elicitor activity (ethylene per gram of leaf per 20 µl of each crude filtrate, ≤2 µg per filtrate). **B,** The protein (3.9 µl per filtrate, ≤0.4 µg per filtrate) in the filtrates was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver stained.

into discrete smaller molecular weight components, as determined by SDS-PAGE (Fig. 6B), and was associated with loss of biological activity (Fig. 6A). At high concentrations, break down of the protein could be detected after only 5 min, but protein of the native molecular weight remained after more than 1 h of heating. Biological activity could be detected after 45 min of heating. Some other proteins that act as elicitors exhibit stability of biological activity in heat treatments similar to that of the 24-kDa protein. Glycoproteins are often heat stable and can be isolated from autoclaved mycelial walls (27). Harpin (29), a nonglycoprotein elicitor produced by *Erwinia amylovora*, is heat stable, which is useful in its purification.

TABLE 1. Induction of ethylene biosynthesis and necrosis in leaves of various plant species by the 24-kDa elicitor protein from the plant pathogen *Fusarium oxysporum*

| Species<br>Common name | Family | Elicitor[a] | Ethylene (µl/20 h ± SD) | Necrosis (± SD)[b] |
|---|---|---|---|---|
| *Erythroxylum coca* | Erythroxylaceae | + | 0.76 ± 0.40 | 2.0 ± 0.8 |
| var. *coca* | | − | 0.03 ± 0.04 | 0 |
| *E. novogranatense* | | + | 1.19 ± 0.49 | 2.5 ± 0.6 |
| var. *novogranatense* | | − | 0.21 ± 0.14 | 0 |
| *Coriandrum sativum* | Apiaceae | + | 0.28 ± 0.03 | 2.0 ± 1.4 |
| Coriander | | − | 0.02 ± 0.02 | 0 |
| *Petroselinum crispum* | | + | 0.47 ± 0.55 | 2.0 |
| Parsley | | − | 0 | 0.5 ± 0.7 |
| *Ocimum basilicum* | Lamiaceae | + | 0.14 ± 0.07 | 2.0 ± 1.0 |
| Sweet basil | | − | 0.07 ± 0.04 | 0 |
| *Salvia officinalis* | | + | 0.18 ± 0.09 | 3.0 |
| Sage | | − | 0.07 ± 0.10 | 0 |
| *Origanum majorana* | | + | 1.19 ± 0.30 | 3.0 |
| Sweet margoram | | − | 0 | 0 |
| *Sesamum indicum* | Pedaliaceae | + | 0.04 ± 0.06 | 3.0 |
| Black sesame | | − | 0.11 ± 0.09 | 1.5 ± 0.7 |
| *Lycopersicon esculentum* | Solanaceae | + | 0.25 ± 0.21 | 2.0 |
| Tomato | | − | 0.03 ± 0.04 | 1.0 |
| *Capsium annuum* | | + | 0.38 ± 0.03 | 3.0 |
| Pepper | | − | 0.50 ± 0.41 | 1.0 |
| *Brassica chinensis* | Brassicaceae | + | 0.43 ± 0.09 | 2.0 |
| Pak-choi | | − | 0.06 ± 0.02 | 0 |
| *Luffa aegyptiaca* | Cucurbitaceae | + | 1.17 ± 1.12 | 0 |
| Luffa gourd | | − | 0.06 | 0 |
| *Cucumis sativus* | | + | 0.43 ± 0.25 | 0 |
| Cucumber | | − | 0.04 | 0 |
| *Phaseolus vulgaris* | Fabaceae | + | 0.44 ± 0.28 | 3.0 |
| Bean | | − | 0.11 ± 0.11 | 0.3 ± 0.5 |
| *Trifolium repens* | | + | 2.85 ± 1.08 | 3.0 |
| White clover[c] | | − | 0.06 ± 0.04 | 0 |
| *Albizia julibrissin* | | + | 0.34 ± 0.03 | 0.5 ± 0.7 |
| Mimosa | | − | 0.03 ± 0.04 | 0.5 ± 0.7 |
| *Cannabis sativa* | Cannabidaceae | + | 0.10 ± 0.07 | 0.8 ± 0.5 |
| Cannabis | | − | 0.05 ± 0.04 | 0 |
| *Acer saccharum* | Aceraceae | + | 1.78 ± 0.22 | 1.3 ± 0.6 |
| Sugar maple[c] | | − | 0.19 ± 0.13 | 0 |
| *Cornus florida* | Cornaceae | + | 1.39 ± 0.88 | 3 |
| Dogwood[c] | | − | 0.14 ± 0.09 | 0 |
| *Diospyros virginiana* | Ebenaceae | + | 2.45 ± 1.17 | 3 |
| Persimon[c] | | − | 0.13 ± 0.13 | 1 |
| *Quercus phellos* | Fagaceae | + | 0.21 ± 0.09 | 2 |
| Willow oak[c] | | − | 0.11 ± 0.10 | 0 |
| *Pyrus calleryana* | Rosaceae | + | 0.25 ± 0.26 | 2.7 ± 0.6 |
| Bradford pear[c] | | − | 0.11 ± 0.03 | 0 |
| *Malus floribunda* | | + | 1.01 ± 0.26 | 3 |
| Crab apple[c] | | − | 0.07 ± 0.01 | 0 |
| *Allium porrum* | Liliaceae | + | 0 | 0 |
| Leek | | − | 0 | 0 |
| *Zea mays* | Poaceae | + | 0.08 ± 0.02 | 0 |
| Maize | | − | 0.06 ± 0.06 | 0 |
| *Triticum aestivum* | | + | 0.05 ± 0.04 | 0 |
| Wheat[c] | | − | 0.05 ± 0.02 | 0 |
| *Stenotaphrum secundatum* | | + | 0 | 0 |
| St. Augustine grass | | − | 0 | 0 |
| *Phalaris arundinacea* | | + | 0.16 ± 0.09 | 0 |
| Canary reed grass[c] | | − | 0.19 ± 0.06 | 0 |

[a] Elicitor (1 µg) was applied as a hanging drop to the petiole of detached leaves.
[b] Necrosis measurements were made 48 h after sealing the jars and are reported using a 0 to 3 scale, where 0 = no damage, 1 = <10% necrosis, 2 = 10 to 50% necrosis, and 3 = 50 to 100% necrosis.
[c] Leaves were collected from local plantings during the spring of 1995.

Protein with a molecular mass of 24 kDa began to accumulate in culture filtrates by 4 days and continued to accumulate until 10 days under the culturing conditions used (Fig. 7B). After 22 days, there was a slight reduction in the level of 24-kDa protein. Biological activity was correlated initially with the accumulation of protein in the culture filtrate of isolate EN-4 (Fig. 7A) until 8 days. The toxin lycomarasmin, which has not been isolated in infected plant tissue, accumulated to high levels in cultures only after 40 days or more (14). Concentrations of fusaric acid, which has been isolated from infected plant tissue, reached a maximum in 20-day-old cultures (16).

The 24-kDa protein induced responses in many plant species other than *E. coca* (Table 1). Both of the *Erythroxylum* species tested responded to the 24-kDa protein by producing ethylene and necrosis. Among the other plant species tested, some species failed to produce measurable ethylene but necrosed extensively (black sesame), whereas others produced ethylene but did not show necrosis (Cucurbitaceae). The limited number of members of the Monocotyledoneae tested did not respond to the 24-kDa protein by either ethylene biosynthesis or necrosis. The host-range comparisons carried out suggest that most dicots are sensitive to the 24-kDa protein, whereas the monocots tested were not sensitive. The elicitor Harpin induces necrosis in a broad range of dicots (29). This does not mean that all plant selections or cultivars within a species should be expected to respond in the same way. Variability between the response of various cultivars to elicitors is well established and ranges from sensitive to completely insensitive (5). The lack of measured response of monocots to the 24-kDa protein could have resulted from a lack of recognition of the protein, an inability of the protein to reach the site of action, or a different response system altogether that does not involve ethylene or necrosis. Finally, even among the dicots, the type and level of response varied greatly. Some plant species responded much more strongly than coca, producing much higher rates of ethylene and/or more rapid necrosis.

The pathogenic forms of *F. oxysporum* cause disease characterized as wilt in numerous hosts. The specialization of wilt-causing *F. oxysporum* formae speciales as invaders of xylem tissue within the vascular system of specific host plant species is associated with a similar symptomology of disease development characterized by loss of turgor, yellowing of infected plant parts, leaf drop, vascular discoloration, and plant death (6). The strong similarity of symptoms among divergent plant species supports the hypothesis that factors contributing to symptom development are shared between the different fungal-plant interactions. The fungus *F. oxysporum* is known to produce toxins with activity across a broad range of plant species (13,16). *F. oxysporum* also produces numerous enzymes capable of digesting plant cell walls and other cellular structures (8). The action of these enzymes may produce products capable of functioning as elicitors and/or toxins to plant cells.

Culture filtrates from both pathogens and nonpathogens have induced ethylene and/or necrosis in many different systems (20, 23,25,29). In at least one case, sensitivity to a proteinaceous elicitor from an apparent nonpathogen was genetically determined (5). Sutherland and Pegg (26) found that the proteins in 10-day-old shake cultures of *F. oxysporum* f. sp. *lycopersici* were more toxic to tomato protoplasts than to protoplasts of nonhost plants.

It is not known if the 24-kDa protein functions in any phase of disease development or if it is present in diseased tissue. The production of antibodies to the purified protein will facilitate its immunolocalization within infected plant tissue. Ultimately, we may be required to make protoplasts from both host and nonhost plant species to assess the specificity and function of the 24-kDa elicitor in the *F. oxysporum-E. coca* interaction.

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