Characterization of a Phytotoxic Glycoprotein Produced by *Phoma eupyrena* – A Pathogen on Water Lettuce

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Phoma eupyrena, the causal agent of leaf blight disease of water lettuce, when purified by affinity and ion exchange chromatography produced an extracellular glycoprotein (Pe 65) in concentrations of ~ 8 μg ml^{-1} in the stationary culture. Coomassie-blue stained SDS-PAGE analysis of culture filtrates and purified Pe 65 showed its molecular mass to be 65 kDa. The blighting and necrosis of leaf tissues were observed within 4–6 days when 1–5 μg of Pe 65 was injected into the mesophyll of water lettuce. These symptoms closely resembled those caused by foliar inoculation with the pathogen. Recognition of Pe 65 by N-glycosidase F treatment and by polyclonal antibodies raised in rabbit against the whole glycoprotein, strongly indicated that the protein is a highly glycosylated protein (50% carbohydrate) and that it is strongly enclosed by the antigenic glycosidic moiety.
KEY WORDS: *Pistia stratiotes*; *Phoma eupyrena;* phytotoxic glycoprotein; toxin; water lettuce.

INTRODUCTION

Pistia stratiotes (L.) Fam. Araceae, called water lettuce, tropical duck weed or pistia, is a free floating aquatic weed of waterways in tropical and subtropical areas (3,15). The aquatic tender weed found floating in stagnant shallow ponds has an immense range throughout the tropics. Vegetative reproduction is mainly by true buds and stolons. About 4 million dollars is spent annually in the State of Florida (USA) to control this aquatic weed (Don Schmitz, Florida Department of Natural Resources, personal comm.). Water lettuce increases water loss through transpiration and interferes with the waterways for recreation and irrigation. An additional problem is that larvae of *Mansonia* spp. mosquitoes use water lettuce roots as a source of oxygen and for protective cover (18). These mosquitoes are major pests and potential transmitters of several diseases in humans.

All these points highlight water lettuce as a threat to agriculture and the environment, thereby emphasizing the urgent need to take steps to manage this weed, in order to maintain ecological integrity. Widely adopted conventional methods, like cutting and burning have many limitations and are uneconomical due to the perennial nature and resprouting capacity of the plant. Although chemical control methods are practiced, they have their own

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limitations. Use of chemical herbicides leads to environmental pollution besides having its own inherent ill effects (16). These have, in part, fueled the current upsurge of interest in biological control of weeds, as an environmentally benign but potentially effective method of weed control (6,27,34). There is considerable current interest in the use of plant pathogenic microbes as agents for the biological control of weeds (28). One potential approach to control weeds is to use phytotoxins or their derivatives for direct application to the noxious plant (1,4,20,21). Many of the fungal pathogens of weeds among the known genera produce toxins, *i.e.*, *Alternaria, Helminthosporium, Fusarium*, etc.; some of these toxins are host-specific and others are not (7). Blighting of leaves, caused by *Phoma eupyrena*, is an important disease of water lettuce (*Pistia stratiotes* L.) in the tropics. No record of this disease is found in India (or) elsewhere in the world.

It has been suggested that the toxin produced by *Alternariae ichhorniae* is known to play an important role in the pathogenesis of the blight disease of water hyacinth (17,19,28,29). However, little practical work has been done on the characterization of *P. eupyrena* on *P. stratiotes*. In the present study, we attempted to characterize the phytotoxic compound (designated Pe 65) responsible for causing blighting of leaves on *P. stratiotes*, which ultimately leads to the mortality of the weed.

MATERIALS AND METHODS

Isolation of pathogen *Phoma eupyrena* isolated from naturally infected water lettuce (*Pistia stratiotes*) near Madurai pond, Tamil Nadu, India was used for this study. Diseased leaf tissue was cut into small bits (3 mm diam) with a sterilized scalpel. These bits were surface-sterilized in 96% ethanol (v/v) for 30 sec, then in 14% sodium hypochlorite (v/v) for 30 sec, followed by another 30 sec in 96% ethanol and washed further in repeated changes of sterile distilled water. The leaf bits were aseptically transferred to 20 ml of cool potato dextrose agar (PDA) (3.9% w/v, Difco, Detroit, MI, USA) in plastic petri dishes (90 mm diam), which were sealed with parafilm (American Standard Can, Greenwich, CT, USA). The stock cultures of the fungus on potato dextrose agar slants were stored at 4°C before being used for toxin production. The fungus was identified based on the morphological characteristics and further confirmed as *Phoma eupyrena* (Accession No. 4890.01) by P.N. Chowdhry (Indian Type Culture Collection, Division of Mycology and Plant Pathology, New Delhi, India).

Pathogenicity tests The pathogenicity of *P. eupyrena* was assayed on *P. stratiotes* at the 5–7 leaf stage in pot culture under greenhouse conditions. The fungal pathogen isolated from *P. stratiotes* was multiplied on PDA medium in petri dishes for 7 days. Conidia were harvested from 7-day-old PDA culture by flooding the culture plates each with 10 ml of sterile distilled water and gently and superficially scraping the surface of the culture with a glass slide. The suspension was filtered through a layer of absorbent cotton wool. The conidial suspension was prepared so as to contain 10^7 conidia ml^{-1}, using a haemocytometer. The leaves were inoculated with a hypodermic syringe, placing 50 μl of conidial suspension (2×10^{-6} ml^{-1} conidia) on a freshly wounded central area of the lamina. Sterile water-sprayed plants were kept as control. Both inoculated and uninoculated control plants were covered with polythene bags and thus high relative humidity was maintained. Observations of the typical symptoms were recorded 7 days after inoculation, on a 0–6 scale: 0 = no symptoms; 1 = yellowish green blighting (<1%) confined to leaf margin alone; 2 = blighting covering 1–5% of the leaf; 3 = blighting

covering 6–25% of the leaf; 4 = blighting covering 26–50% of the leaf; 5 = Blighting and necrosis covering 51–75% of the leaf; 6 = more than 75% of the leaf showed necrosis and finally shredded and became submerged in the water. The fungus was re-isolated from the artificially infected water lettuce plant and the culture thus obtained was compared with the original culture.

Preparation of culture filtrates A 20-day-old culture of the fungus grown on PDA slants was transferred to petri dishes containing Richards's medium (50 g sucrose, 10 g potassium nitrate, 5 g potassium dihydrogen phosphate, 2.5 g magnesium sulphate, 0.02 g ferric chloride and 20 g agar, with the volume made up to 1 liter by the addition of distilled water). The plates were incubated at laboratory temperature (28±2°C) for 15 days under continuous illumination (20 μE $m^{-2}s^{-1}$) from white fluorescent lamps. For liquid cultures, an 8-mm-diam disc of a fungal colony was transferred to a 1 *l* Roux bottle containing 150 ml of Richards's medium adjusted to pH 7.0. Still cultures were grown at 28±2°C under continuous illumination (as above) for different time courses, *viz*., 7, 14, 21 and 30 days of culture. The mycelial mat was removed by filtration and the culture filtrate was freed of conidia and mycelial fragments by passing it through a 0.45-μm Millipore filter. Before storage, the culture filtrate was sterilized by passing it through a 0.22 μm Nalgene filter, which has low protein binding activity. The experiments were run in triplicate and the data were analyzed statistically according to Duncan's Multiple Range Test (DMRT) (12).

Bioassay of toxin of *Phoma eupyrena* on *Pistia stratiotes* Water lettuce plants were grown in 30-cm earthen pots each containing 6 *l* of water. When the plants were at the 5–7-leaf stage, the toxicity tests were performed by injecting a 50-μl drop of sterile crude culture filtrate using a hypodermic syringe. The plants were then incubated in a growth chamber at 28±2°C, 70% r.h. and 100 μE $m^{-2}s^{-1}$ illumination, 12:12 L:D. The corresponding controls (sterile water or uninoculated culture medium) were injected in other leaves of the same plant. The leaves without injection served as another control. The above experiment was replicated three times. The severity of blighting and necrotic lesions, which developed around the points of injection, was scored at 7 days on the 0–6 scale described above.

Toxin isolation, protein digestion, and peptide purification The toxin isolation, protein digestion and peptide purification of *P. eupyrena* were carried out by closely following the methodology and approach described by Fogliano *et al.* (11). However, the resulting peptides were purified by 5-μm reverse phase high performance liquid chromatography (HPLC) using an ODS column (25×4.6 mm I.D.) obtained from YMC Inc. (Kyoto, Japan). HPLC analyses were performed with a Shimadzu system (Kyoto, Japan) consisting of HPLC (LC-10AT) series with a programmable solvent module, a Shimadzu (SIL-WA) autoinjector with a sampling syringe speed of 5 μl sec^{-1} and a rinsing syringe speed of 100 μl sec^{-1} with a syringe volume of 500 μl equipped with a 5-μl sample loop and a Shimadzu (SPD-M10AVP) photodiode array detector set at 190 to 600 nm with a degasser (Shimadzu, DGO-14A) and a temperature specification (Shimadzu, CTO-10A) monitored by a COMPAQ, DESKPRO Computer system (Shimadzu).

Antiserum production, antibody purification, and protein analysis Samples were prepared as described previously by Fogliano *et al.* (11) by injecting 0.1 mg of *P. eupyrena* (phytotoxic compound) to a 5-month-old immunized white rabbit in 500 ml Freund's incomplete adjuvant (Sigma Chemical Co., St. Louis, IL, USA). The serum was collected and stored at -20°C and the antibody titer was determined by the procedure described by Harlow

and Lane (14) (data not shown). Antibodies specifically recognizing the glycoprotein were affinity-purified (14) by incubating 2-ml fractions of 1:10 diluted antiserum in Tris-buffered saline with 2% gelatin and 0.1% Tween 20 for 2 h with a polyvinylidene-difluoride (PVDF) strip containing Pe 65 band (obtained from SDS-PAGE) and pre-blocked with 3% bovine serum albumin (BSA) (Sigma) and 3% gelatin. The immunoblotting procedure followed the protocol described by Fogliano *et al.* (11). Detection was performed with 4-chloro-1-napthol according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). Molecular weight of the compound was determined using prestained kaleidoscope protein markers (Bio-Rad).

Quantification of the peptide component of the glycoprotein was performed according to Bradford (5) using BSA as a standard. The glycosidic moiety was quantified using mannose as a standard as described by Dubois *et al.* (10). After SDS-PAGE and blotting, detection of the carbohydrate moiety was performed by a Glycotrack kit (Oxford GlycoSystem, Abington, Oxon, UK) following the manufacturer's instructions. Removal of N-linked oligosaccharides was performed by an N-glycosidase F (Boehringer Mannheim GmbH, Germany) treatment according to the method described by Tarentino *et al.* (33). After digestion, the sample was neutralized with HCl, dialyzed, and analyzed by SDS-PAGE.

RESULTS

The phytotoxic glycoprotein production of *P. eupyrena* was significant when the cultures were approximately 7 days old, reached a maximum by the 21st day, and remained roughly unchanged for up to 30 days after inoculation. However, the concentration of extracellular total proteins increased up to 78 μg ml^{-1} and the average yield of Pe 65 was 8 μg ml^{-1} of culture, which was statistically highly significant when analyzed according to DMRT; (least significant difference between any two means at P=0.05 is 1.02 (Fig. 1).

Blighting and necrosis of water lettuce leaf tissues were induced by samples of both the high- and the low-molecular-weight fractions obtained from the concentrated culture filtrates of *P. eupyrena* after passing through the tangential ultrafiltration. However, the low-molecular-weight toxin produced by *P. eupyrena* was not considered in this work. The high-molecular-weight fraction was loaded onto an affinity chromatography Concanavalin A- Sepharose 4B column. Active fractions were pooled, dialyzed, and further purified by anion exchange chromatography. The elution profile and the biological activity of each fraction are shown in Figure 2.

The Coomassie blue staining of Pe 65 showed a 65 kDa banding pattern, suggestive of a glycoprotein, in 14-, 21- and 30-day-old cultures (Fig. 3, lanes a, b, c). A similar 65 kDa band appeared in the purified Pe 65 when subjected to ultra filtration, Concanavalin A and anion exchange chromatography (Fig. 3, lane d). To confirm the results, the samples of Pe 65 were blotted on a PVDF membrane, and carbohydrates were detected by specific periodate oxidation of the sugar residue followed by biotin hydrazide labeling. To detect the carbohydrate moiety of purified Pe 65, the proteins were subjected to periodate treatment resulting in a strong positive reaction, indicating the presence of a carbohydrate moiety (Fig. 3, lane e), while there was a negative reaction in the absence of periodate treatment (Fig. 3, lane f), indicating the absence of carbohydrate moiety. Following quantification of the glycosidic and peptide moieties of Pe 65 (5,10), the dry weight of the moieties was 50% and 30%, respectively.

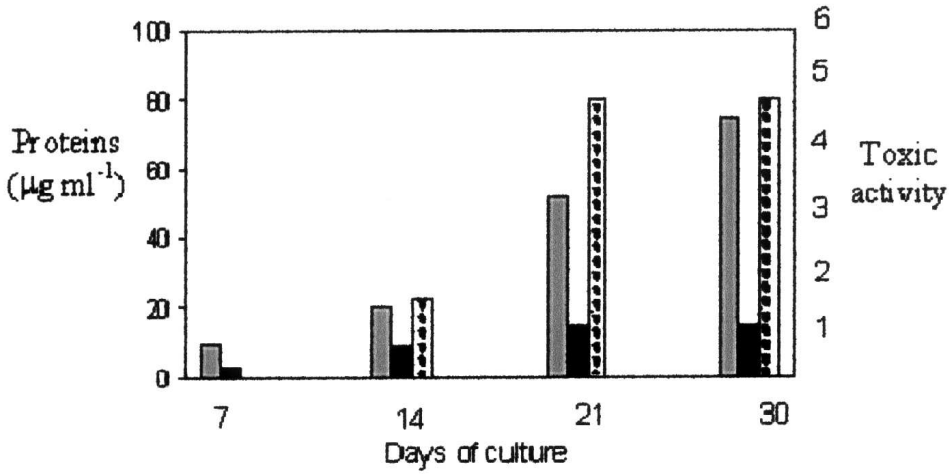

Fig. 1. Time course production of Pe 65 by *Phoma eupyrena*. Data represent the means of three independent experiments. Least significant difference between any two means at P=0.05 is 1.02. Gray bars, total proteins; black bars, amount of Pe 65; dotted bars, phytotoxic activity.

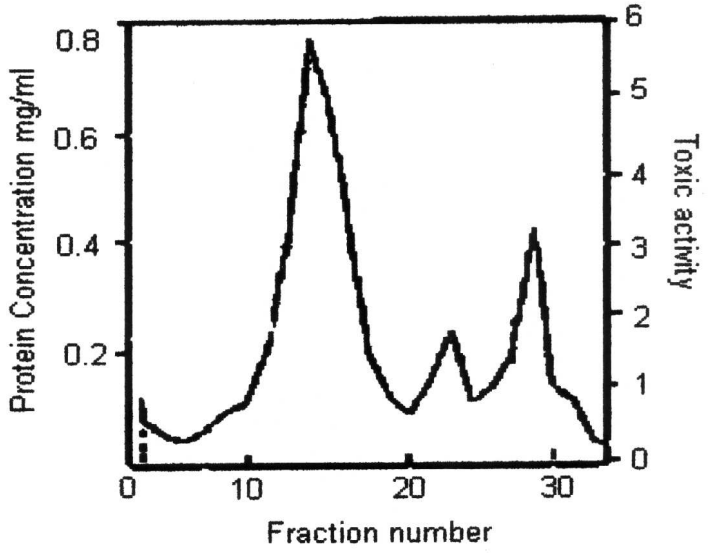

Fig. 2. Elution profile and biological activity of the active fractions after anion exchange chromatography. Total proteins and phytotoxic activity of each fraction were determined.

The purified Pe 65 toxin of *P. eupyrena* of water lettuce at 5, 25 and 50 μg ml^{-1} induced the typical blighting and necrotic symptoms within 4–6 days, extending to the whole sector of the leaf where the injection was made (Fig. 4).

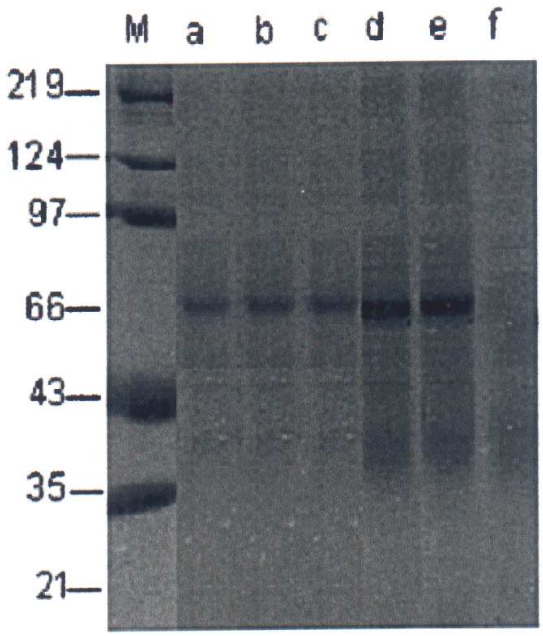

Fig. 3. Coomassie blue staining of culture filtrates of *Phoma eupyrena* and purified Pe 65. Culture filtrates from lane a, 14-day-old culture; lane b, 21-day-old culture; lane c, 30-day-old culture; lane d, Pe 65 purified by ultra filtration, concanavalin A and anion exchange chromatography; lane e, proteins subjected to periodate oxidation and glycotrack detection to detect the carbohydrate moiety of purified Pe 65; lane f, as in lane e, without the periodate oxidation step; lane M, sizes of the marker protein.

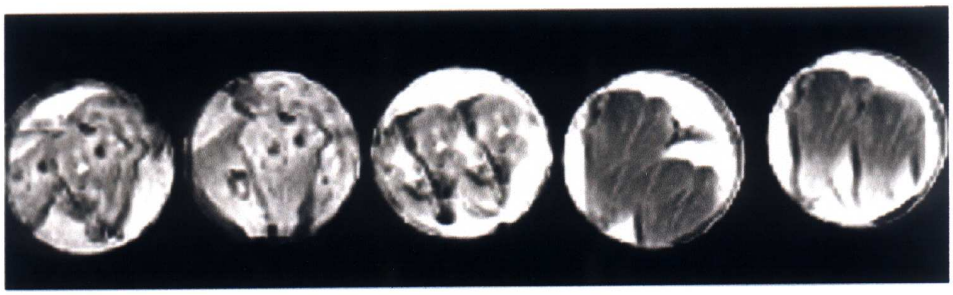

Fig. 4. Blighting and necrosis development on water lettuce leaves within 4–6 days after injection of (from left to right): 5, 25, and 50 μg of Pe 65; control without injection, and with control injection of sterile water alone.

Western blot analysis of purified Pe 65 showed a smeared band when probed with crude antiserum (Fig. 5, lane a). However, a sharp 65 kDa-banding pattern was observed in the affinity-purified antiserum (Fig. 5, lane b) and at different ages of the culture filtrates of *P. eupyrena* (Fig. 5, lanes c, d, e).

The effect of N-glycosidase F treatment on Pe 65 in Western blot analysis revealed

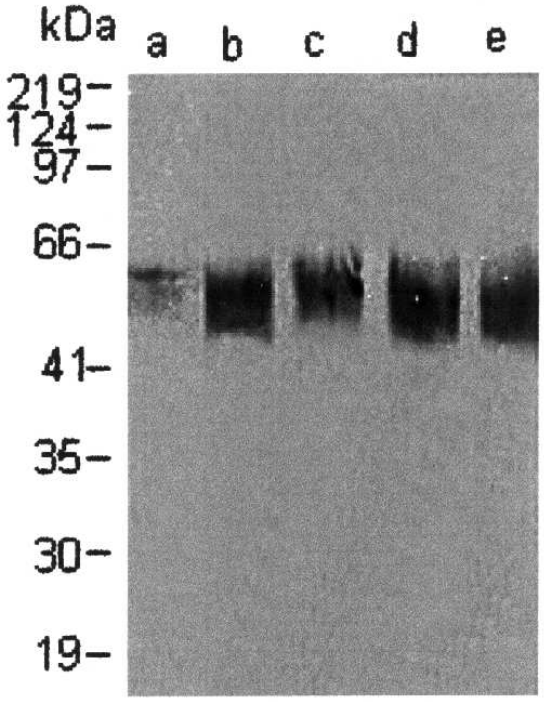

Fig. 5. Western blot analysis with crude and affinity purified polyclonal antibodies against Pe 65. Lane a, purified Pe 65 cross reacting with crude antiserum; lane b, as lane a, but with affinity purified antiserum; lanes c, d, e: 14-, 21-, and 30-day-old culture filtrates of *Phoma eupyrena*. Molecular markers are reported on the left side.

that the partially deglycosylated pattern of a 65 kDa-purified Pe 65 was recognized by the purified antiserum. Interestingly, there was an incubation-time-dependent increase of the electrophoretic mobility of deglycosylated Pe 65 when observed at 30, 60 and 120 min after treatment, suggesting that the antibodies recognize mainly the glycosidic part of the toxin (Fig. 6).

DISCUSSION

In this paper we describe the involvement of phytotoxic glycoprotein (Pe 65) secreted in culture by *P. eupyrena* causing blighting and necrosis in water lettuce. To prove the involvement of the toxin in disease development, the toxin should produce typical symptoms of the disease (18). Toxin studies have led to some practical applications in agriculture. *Helminthosporium* toxin can be used to eliminate susceptible seedlings in plant breeding programmes making mass screening against the disease rapid and efficient (35-37). The toxin should exhibit the same host specificity of the pathogen (31).

In the present investigation, when the phytotoxic Pe 65 is injected in water lettuce leaves at a dosage of 5 μg per leaf (0.1 ml of a 5 μg ml^{-1} solution), Pe 65 induced blighting followed by necrosis of tissues within 4–6 days. The glycosidic moiety of Pe 65 represents 50% of the glycoprotein. Removal of carbohydrates exposes the toxin to the action of

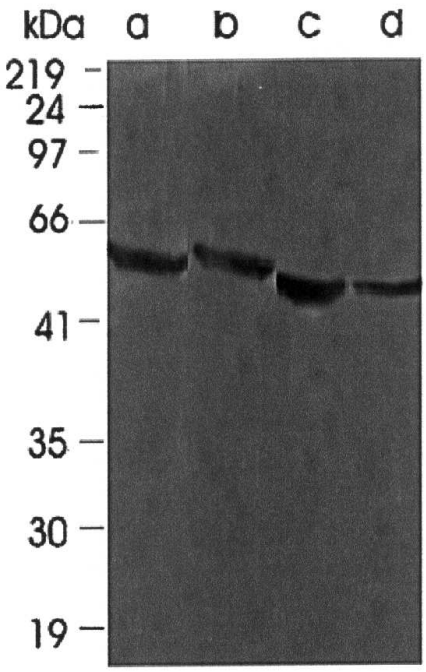

Fig. 6. Effect of N-glycosidase F treatment on Pe 65. Detection of the carbohydrate moiety in Western blotting with affinity purified antibodies. Lane a, purified Pe 65 (control); lanes b, c, d: 30-min, 60-min, and 120-min treatment, respectively. Molecular markers are reported on the left side.

proteolytic enzymes, which are ineffective on the entire molecule. This finding suggests that the protein moiety of Pe 65 is completely enveloped by the glycosidic part. The results imply that the toxin may be required for successful penetration by the pathogen to the weed host. Thus, the role of toxins in the pathogenesis may determine the disease severity as virulence factors (37) rather than being directly involved in the initial step of infection. These results are in agreement with the findings of Nishimura and colleagues (24), and of Scheffer (30). Vidhyasekaran *et al*. (35) reported that a host-selective toxin was produced by *Drechsleraoryzae*, which showed phytotoxicity to susceptible plants at 0.5 μg ml^{-1} and to resistant plants at 100 μg ml^{-1}. In the present study, toxin of *P. eupyrena* of water lettuce has been proved to be highly virulent. Vidhyasekaran *et al*. (35) and Haegi and Porta-Puglia (13) reported that the virulence of the pathogen was correlated with the ability to produce the toxin, and the toxin should have been isolated from susceptible host plants after infection. It was interesting to note that the immunoglobulins were able to recognize the particular glycosylation pattern of the 65 kDa glycoprotein and that the glycosylation pattern did not change during the course of the culture of *P. eupyrena*.

Nachmias *et al.* (22,23) reported many years ago a 93 kDa glycoprotein from the cultures of an Israeli strain of *Phoma tracheiphila* on mal secco disease of citrus. A toxin with a carbohydrate moiety was reported to be produced by *Pyrenophora graminea* infecting barley (13) and by *Septoria glycines* infecting soybean (31). Mannose and glycoprotein phytotoxins were isolated from culture filtrates of *Ceratocystis ulmi* (32)

and *Phoma tracheiphila* (2) and other plant pathogens (8,26,37). Glycoprotein toxins were implicated primarily in causing wilt symptoms (32), but a few, such as that from *P. tracheiphila* (2,11), reproduced necrotic symptoms of the diseases.

The glycoprotein nature of the toxin was initially inferred from chemical analysis for carbohydrate and protein and was supported by detached leaf method (37). Dow and Callow (9) also reported that components from *Cladosporium fulvum* culture filtrates causing permeability changes in isolated mesophyll cells were glycopeptides. In the present study, the data reveal that 65 kDa extracellular glycoprotein produced in culture by *P. eupyrena* is the most toxic component of those investigated from this organism.

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