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Purification of antifungal protein against blister bark pathogen of *Casuarina equisetifolia* J. R. Forster et G. Forster

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A protein extract from the leaves of *Andrographis paniculata* (Acanthaceae) was found to inhibit the spore germination and hyphal extension of *Trichosporium vesiculosum*, the blister bark pathogen of *Casuarina equisetifolia*. The antifungal protein component was further purified from the crude extract and the molecular mass of the toxic protein was estimated to be 39.5 kDa.

Key words: *Andrographis paniculata, Casuarina equisetifolia, Trichosporium vesiculosum*, antifungal protein, spore, germination, hyphae.

Introduction

Casuarina equisetifolia J. R. Forster et G. Forster has a wide range of natural distribution occurring in sub-tropical and tropical coastlines from Northern Australia, throughout Malaysia, Melanesia and Polynesia and Northwards to the Kra Isthmus in South Thailand (PINYOPUSARERK and HOUSE 1993). It is a multipurpose species with utilities ranging from fodder, fuel wood, lumber for inexpensive housing and paper pulp. It also fits the agrarian ecosystem. The wilt or the 'blister bark' disease caused by the hyphomycetous fungus, *Trichosporium vesiculosum* Butler is the most destructive disease of *C. equisetifolia* (TITZE and VAN DER PENNE 1983, MOHANAN and SHARMA 1993). The disease has been reported from India, China, Vietnam, Thailand, Mauritius and Srilanka (MOHANAN and SHARMA 1993, NARAYANAN et al. 1996, PONGPANICH et al. 1996, CHONGLU 2000). It is reported mostly from regions to which the species has been introduced. The pathogen attacks trees of different ages causing large-scale mortalities, particularly in monoculture plantations. Mortality rates as high as 90% have been reported from India and Vietnam (SHARMA 1994). A few attempts have been made to identify potential fungicides to control the pathogen

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(JAMALUDDIN 1998, NARAYANAN et al. 1996, NARAYANAN 2000). However, they were not effective for curative treatment of the disease under field conditions (NARAYANAN and SHARMA 1996, NARAYANAN 2000). Hence, breeding for disease resistance is a viable option as a long-term strategy for management of the disease (SHARMA 1995, PINYOPUSARERK 1996). The use of molecular techniques like genetic transformation is an alternative strategy to generate disease resistant trees. The first step in this approach is identification of a gene whose product would be toxic to spore germination and hyphal growth of the pathogen. This study aims at identifying a protein toxic to *T. vesiculosum*.

Materials and Methods

Plant material

Young leaves of *Andrographis paniculata* (Burm. F.) Wall. Ex Nees (Acanthaceae) were collected from the germplasm collection of the botanical garden at the Institute of Forest Genetics and Tree Breeding, Coimbatore, India.

Fungal cultures: Two isolates of the fungus *Trichosporium vesiculosum* Butler, designated as Tv1 (collected from Panampally, Kerala, India) and Tv2 (collected from Coimbatore, Tamil Nadu, India) were maintained in potato dextrose agar medium.

Isolation and purification of the antifungal protein

Twenty-five grams of young leaves were extracted in 75 mL of ice-cold buffer containing 50 mM sodium acetate (pH 5.2), 1 mM ascorbic acid, 0.5 mM phenylmethylsulfonyl fluoride and 0.5% polyvinyl polypyrrolidone. Subsequent to centrifugation, the supernatant was precipitated with 30% ammonium sulfate, at 4 °C, overnight. The pellet was discarded and the supernatant was desalted using a Sephadex G-50 column. The crude protein was further purified using cation exchange chromatography.

Activation of the sorbent: 10 grams of carboxymethyl (CM) cellulose (Bangalore Genei Ltd.) was suspended in 15 volumes of 500 mM NaOH with continuous stirring and allowed to settle for 30 minutes. The upper liquid phase was decanted and the swollen sorbent was suspended in distilled water. The sorbent was washed in distilled water until the pH was 8.0. This was followed by suspension in 15 volumes of 500 mM HCl and subsequent washing with distilled water until the pH was neutral. The sorbent was finally equilibrated with 50 mM ammonium acetate, pH 3.7.

The swollen sorbent was then packed into a column (55 cm × 1 cm) and was washed with ten bed volumes of 50 mM ammonium acetate, pH 3.7. The crude protein was loaded on to the packed column and the proteins were eluted using a gradient of 50 mM to 500 mM ammonium acetate, pH 3.7. Fractions of 3 ml were collected and their absorbance was monitored at 280 nm. The fractions corresponding to the absorbance peaks were pooled and freeze-dried. The freeze-dried samples were suspended in 500 µL of sterile water. The samples were further desalted and concentrated by passing through a microcon (Millipore) column with a membrane nominal molecular weight limit NMWL of 3000 Daltons. The samples obtained were immediately stored at –20 °C until further use. The fractions were subsequently assayed for their antifungal properties.

Protein concentrations were determined using the Lowry method (LOWRY et al. 1951). The molecular weight of the purified protein showing inhibition was determined in 12%

native polyacrylamide gel using standard molecular weight markers (Bangalore Genei Ltd.) and staining with Coomassie brilliant blue R250. The molecular weight of the protein was determined using the Kodak 1D version 3.5 software.

In vitro inhibition test by microtiter plate assay

The fractions separated by cation exchange chromatography were tested for their *in vitro* antifungal activity against fungal strains *Tv1 and Tv2*. To each well of a microtiter plate (12×8 wells) was added a suspension of spores (1.5×10^3) of one of the isolates, suspended in 80 μL of potato dextrose broth containing 0.1% Triton X 100. Serial dilutions were made individually for all the protein samples collected (1 μL, 5 μL, 10 μL, 15 μL and 20 μL of all protein fractions were made up to 20 μL with sterile water) and 20 μL of each fractionated protein at the above dilutions were added to the spore suspensions. A control containing 80 μl of spore suspension and 20 μL of sterile water was maintained. Another control was maintained with 80 μl of spore suspension and 20 μL of heat-treated protein (20 μL of the protein sample was heated at 100 °C for 15 minutes). The plate was incubated at 32 °C. Observations were made for spore germination in both treated and untreated wells after 24, 48 and 72 hours and microphotographs were taken using a Nikon UFX-DX camera.

In another study, the spores of the two isolates were allowed to germinate for 24 hours in potato dextrose broth and then the fractionated proteins were added in different dilutions as described in the previous experiment. In the control wells, 20 μL of sterile water or 20 μL of heat-treated protein was added 24 hours after spore germination. Observations were made for hyphal extension in all the wells (both control and treated) after 24, 48 and 72 hours. Microphotographs were taken using a Nikon UFX- DX camera.

Results

The crude protein was purified through a CM cellulose column and three peaks were collected (Fig. 1A). All the three freeze-dried, desalted fractions were tested for their antifungal activity against the two isolates of *T. vesiculosum* (Tv1 and Tv2). The first fraction completely inhibited the spore germination of both isolates even after 72 hours (Figs. 1 D, F, H). The optimal concentration of the protein component in fraction 1 showing total inhibition of spore germination was found to be approximately 10 μg mL^{-1} for both isolates (Fig. 2A). In the control wells (with sterile water), the spores germinated by 24 hours and, by 72 hours, profuse hyphal extension was observed for both pathogens (Figs. 1C, E, F). In the second control (with heat-treated fraction 1 protein), spore germination was observed (Fig. 2A). The other two protein fractions (2 and 3) showed no antifungal activity and growth was comparable to the control (data not shown).

In another experiment, the effect of the protein in fraction 1 on the hyphal extension of the pathogen isolates was determined. In both isolates, hyphal growth was inhibited after the protein was added. The optimal concentration inhibiting hyphal extension was found to be similar for both isolates and was approximately 10 μg mL^{-1}. In both isolates, the hyphae were found to have swollen and had increased vacuolation, 24 hours after protein addition (Figs. 2 C, E). No lysis of the tip was observed in both isolates. The hyphal extension was totally inhibited in the treated wells. In contrast, the control wells (with both sterile water

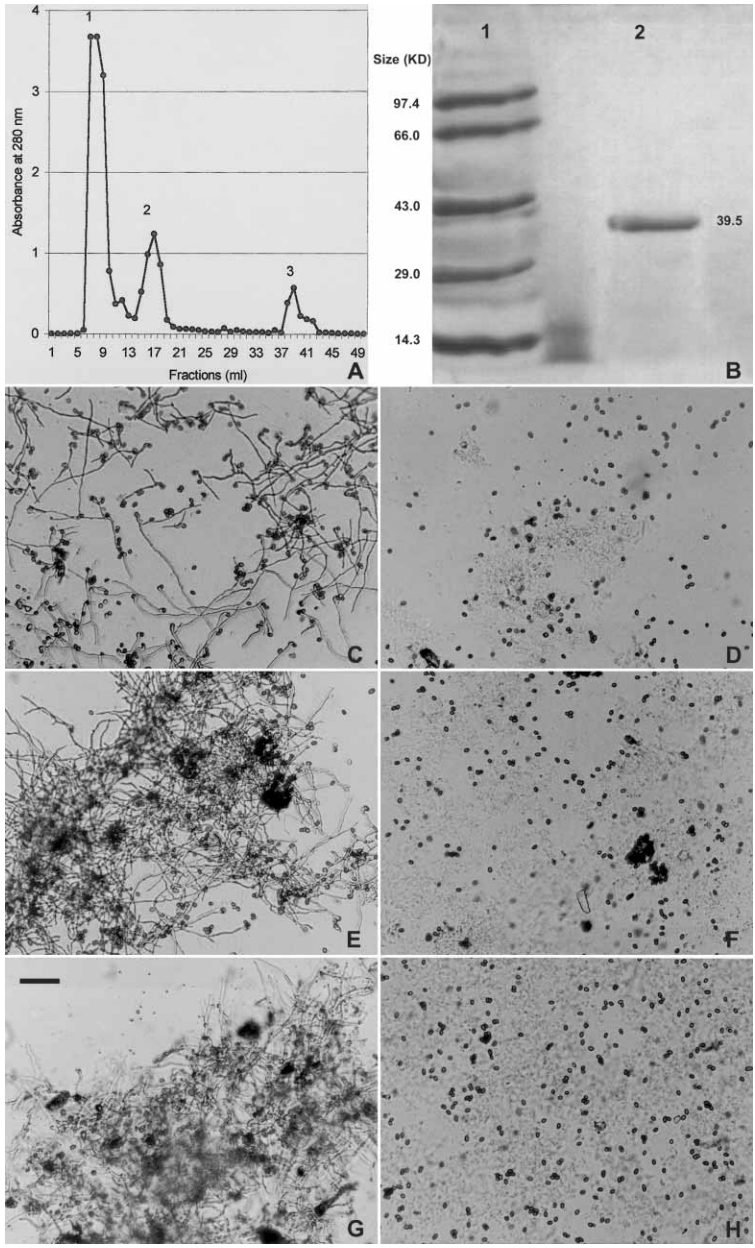

Fig. 1. **A.** Cation exchange chromatogram of the crude leaf protein of *Andrographis paniculata*; **B.** Native polyacrylamide gel electrophoresis of the purified protein Lane 1: Standard molecular weight markers Lane 2: 39.5 kDa antifungal protein; **C.** Spore germination of *Trichosporium vesiculosum* (*Tv1*) in control well (after 24 hours); **D.** Inhibition of spore germination in treated well (after 24 hours); **E.** Spore germination of *Trichosporium vesiculosum* (*Tv1*) in control well (after 48 hours); **F.** Inhibition of spore germination in treated well (after 48 hours); **G.** Spore germination of *Trichosporium vesiculosum* (*Tv1*) in control well (after 72 hours); **H.** Inhibition of spore germination in treated well (after 72 hours) (Bar = 10 μm)

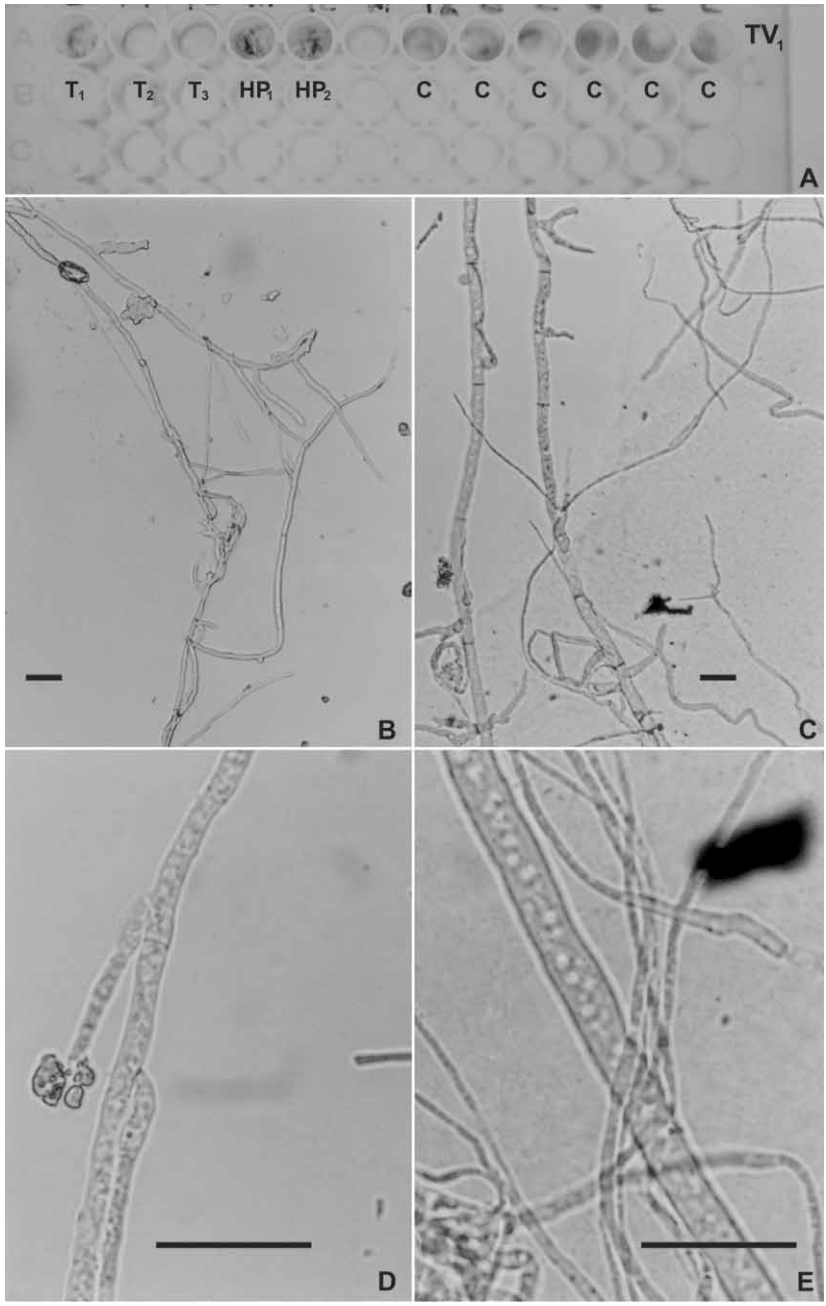

Fig. 2. **A.** Microtitre plate bioassay of *Trichosporium vesiculosum* (*Tv1*) C: Control; T_1-5 μg mL^{-1} of protein; T_2- 10 μg mL^{-1}; T_3- 20 μg mL^{-1}; HP_1- 10 μg mL^{-1} of heat-treated protein; HP_2-20 μg mL^{-1} of heat- treated protein; **B.** Hyphal structure of *Trichosporium vesiculosum* (*Tv1*) in control well; **C.** Hyphal structure of *Trichosporium vesiculosum* (*Tv1*) in protein-treated well **D.** Hyphal structure of *Trichosporium vesiculosum* (*Tv1*) in control well **E.** Hyphal structure of *Trichosporium vesiculosum* (*Tv1*) in protein-treated well (Bar = 5 μm)

and heat-treated protein) showed complete hyphal extension and no swelling of the hyphae was observed (Figs. 2 B, D).

The molecular mass of the protein component in the first fraction was determined, in 12% native PAGE using standard markers, to be approximately 39.5 kDa (Fig. 1B).

Discussion

Disease management continues to be a major challenge ever since the domestication of plants started. This is because narrowing down the genetic base of populations selected for optimal productivity makes them vulnerable to pathogens. Hence, production strategies including protection against serious pathogens are extremely important for sustainability.

Genetic screening for disease resistance is a widely employed strategy for many plant diseases. Although the blister bark disease has long been reported in India, there has been no systematic screening of casuarinas for disease resistance (SHARMA 1995). The incorporation of genetic transformation strategy for the generation of disease resistant casuarinas could be an alternative approach. This strategy requires genes the products of which are toxic to germination and growth of the pathogen. In the present study, a protein toxic to the pathogen was isolated from the leaves of *A. paniculata*. Such studies are very limited for *T. vesiculosum*. We earlier reported the inhibition of this pathogen by crude protein extracts of several plants, such as *Plumbago capensis*, *Piper longum*, *Rauwolfia tetraphylla* and partial inhibition by *Plumbago zeylanica*, *Terminalia arjuna* and *Terminalia cattapa* (GHOSH et al. 2000, THAPLIYAL et al. 2000). Several reports are available on the use of antifungal genes for the generation of disease resistant transgenics (e. g. YOSHIKAWA et al. 1993, JACH et al. 1995, LIANG et al. 1998, TERRAS et al. 1995). But these studies are exclusively limited to crop species. The identification of antifungal proteins, which are not race-specific and single-gene encoded, is the first step to incorporate tolerance into the genome by genetic modification.

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