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Evaluation of antifungal metabolites produced by Pseudomonas fluorescens (PS02 and PS14) isolates for biocontrol of Fusarium culmorum

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ABSTRACT

Antifungal metabolites, inhibitory to Fusarium culmorum, were isolated from the antagonistic bacteria Pseudomonas fluorescens grown in King's B liquid medium. The cell-free filtrates obtained, were tested using an agar diffusion assay for their ability to inhibit Fusarium culmorum. The results showed that the cell-free filtrates of P. fluorescens PS02 and PS14 isolates inhibited mycelial growth and conidial germination of Fusarium culmorum. However, only extracts obtained by the solvent diethylether showed clear inhibition zones compared to those extracts obtained by dichloromethane. The Inhibition zones of mycelial growth and conidial germination of Fusarium culmorum were only observed for all the acidic extracts of both bacterial isolates.

INTRODUCTION

Production of diffusible or volatile antifungal antibiotics by certain strains of bacteria has been described as a powerful mode of action in disease suppression caused by pathogenic fungi on crop plants, and the bacteria that produce them are therefore of considerable interest as a practical means for plant disease biological control [Leifert et al., 1995; Thomashow and Weller, 1995; Podile and Prakash, 1996; Thomashow et al. 1997].

P. fluorescens has been shown to produce secondary metabolites active against many pathogenic fungi including Fusarium species (Loginov et al. 2003; Thomashow et al., 1990; O'Sullivan and O'Gara, 1992; Landa et al., 2003; Dwivedi and Johri, 2003 and Validov et al. 2005).

Most of the data on broad-spectrum activity of antibiotics produced by

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bacterial biocontrol agents are derived from assays performed in vitro as rapid and easy way to test the implication of antibiosis in the inhibition of plant pathogens. The antibiotics produced in vitro were generally assumed to be the compounds responsible for biocontrol in planta. Several metabolites including antibiotics, enzymes, and volatiles compounds produced by antagonistic bacteria have been studied extensively in the control of different plant pathogens [Kloepper et al., 1980; Weller, 1988; Whipps, 1997].

Microbial products may be so complex in their chemical structure that one compound may has two or more totally different chemical moieties which can interact with different receptors [Lange and Lopez, 1996]. The complexity in their structure may make natural products chemically and biologically diverse.

A procedure suitable for the extraction of many of the antibiotics produced by fluorescent Pseudomonas spp. isolated from the rhizosphere of wheat had been published [Bonsall et al., 1997], and can be adapted for other substances by adjusting the amount of sample required and selecting the appropriate solvents. This method can recover phenazine-1-carboxylic acid, its hydroxyphenazine derivatives, pyrrolnitrin, pyoluteorin, and 2,4-diacetylphloroglucinol (DAPG), but not the phenazine compound pyocyanin, which has different solubility properties. In general, samples are dispersed in an extractant (either diethylether or dichloromethane). Such extractions, primarily intended to remove unwanted chemicals, which are referred to as washes. Several types of washes are commonly used: aqueous acids such as Hydrochloric acid (HCl) or Acetic acid (CH_3CO_2H) for extracting basic compounds; and aqueous bases such as Sodium hydroxide (NaOH), Sodium carbonate (Na_2CO_3) or Sodium bicarbonate ($NaHCO_3$) for extracting acid compounds (Thomashow, et. al., 1990).

MATERIALS AND METHODS

Preparation of culture supernatants

For screening of the antifungal activity of bacteria, 200 ml of cell-free supernatant were used. The bacterial isolates used were P. fluorescens PS02 and PS14. These isolates have achieved significant practical impacts and successes for biological control of seedling blight diseases in the field (Elagael, 2005).

The inoculum for production of antifungal metabolites was prepared by growing each isolate in 5 ml of nutrient broth for 24 h at 28°C. A 2 ml inoculum of each P. fluorescens (PS02 and PS14) isolate was transferred into 100 ml of modified King's medium B (KMB) (30 g glycerol, 10 g of proteose

peptone, 0.5 g Dipotassium phosphate (K2HPO4), 5 g Magnesium sulfate (MgSO4) · 7H2O, and 1l ml distilled water) in a 250ml conical flask (Lee *et al*., 2003). Five replicate flasks (for each isolate) were incubated at 28° C in a reciprocal shaker at 150 rpm for 6 days. The supernatants were obtained by centrifugation of the culture at 8,000 rpm for 20 min, and the supernatants were filtered through sterile 0.1μm membrane bacterial filters to ensure the removal all bacterial cells.

Evaluation of antifungal activity of culture supernatants

Two different in vitro methods were used.

Method 1 (Supernatant incorporated into agar medium) as described by Elagael, (2005).

Supernatant (4 ml) at 4°C was added to 16 ml molten PDA prewarmed to 50°C in a universal bottle, mixed and poured into a Petri dish and allowed to set. Four mycelial plugs of actively growing cultures of F. culmorum were placed on the edge of the plate at 90° right angles to each other. Control plates contained no supernatants. Three replicate plates were used for each isolate and incubated at 25° C for 6 days. Antifungal activity was assessed qualitatively by comparing mycelial growth on test plates with control plates. Reduction in the radius of fungal colony indicates antifungal activity.

Method 2 (Well diffusion) as described by Loginov et al. (2003)

The fungus F. culmorum was grown on PDA at 25°C under continuous fluorescent light for 6 days. A conidial suspension was obtained by adding 10 ml of sterile distilled water to each plate and suspending spores using a sterile glass rod. The suspension was filtered into a sterile universal tube using a sterile funnel lined with sterile lens tissue; spores counted using a haemocytometer and adjusted to 10^5 conidia ml^{-1}. The conidial suspension (0.1ml) was used to prepare a lawn on a PDA plate. Agar plugs (5 or 10 mm diameter) were removed from these inoculated PDA plates with a sterile cork borer and 40 or 80μl of the cell-free supernatants were added to wells. Four replicate plates were used for each isolate and plates were incubated at 15°C for 7 days. Antifungal activity will be indicated by forming a clear zone of inhibition. Central mycelial plug inocula of F. culmorum were also used to determine inhibition of mycelial growth.

Partial purification of antifungal metabolites using solvent extraction

The supernatants (125 ml) for each isolate were mixed with 200 ml of saturated aqueous sodium bicarbonate solution in a dry conical flask and

divided into three aliquots; each aliquot was added to a separating funnel and dissolved using 150 ml of either diethylether ($C_4H_{10}O$) or dichloromethane (CH_2Cl_2). The solution was separated into two layers (organic and aqueous layers) and the aqueous layer was discarded. HCl (2N) was added to one fraction of the filtered organic phase until the pH was 2. To the second fraction, HCl (2N) was added and the pH was neutralized to 7. For the third fraction NaOH (2M) was added until the pH was alkaline (pH 11).

100 ml of either diethylether or dichloromethane were again added to each fraction, the aqueous phase was disposed off and the upper organic phase for each sample was retained as acidic, neutral and alkaline products. Each fraction was dried over a little anhydrous magnesium sulphate in order to remove any aqueous residue and then filtered through Whatman filter papers, 180mm, (Whatman Ltd). England) to discard the magnesium sulphate before being rotary evaporated to dryness by removing the solvent (either diethylether or dichloromethane). The residue (generally had a sludge-like consistency) was collected in small sterile bottle after dissolving in a small volume of diethylether and dried using the air from a fume cupboard until the diethylether was evaporated off.

The residues from the acidic, basic and neutral extractions were resuspended in 10% (v/v) dimethylsulfoxide (DMSO) in sterile distilled water and were evaluated for antifungal activity using method 2. The solvent was used as a control.

RESULTS

Screening of culture supernatants for antifungal activity against F. culmorum

Cell-free supernatants of the rhizobacterial isolates showed clear inhibitory activity against F. culmorum. Bacterial isolates (PS02 and PS14) used in this study produced antifungal metabolites which inhibited mycelial growth and conidial germination of F. culmorum.

Extraction and partial purification of antifungal metabolites from cell-free supernatant

Some extracts produced different colours when were compared to the original bacterial broth supernatants. The colours of the extracts ranged from orange, cream to colourless, most being the colourless.

Only extracts from diethylether solvent gave clear inhibition zones compared to the extracts from dichloromethane. A summary of the results is shown in Table 1.

Inhibition zones of mycelial growth (Fig. 1) and conidial germination (Fig. 2) of F. culmorum were observed for all the acidic extracts of all isolates. No

antifungal activities were observed with any of the basic or neutral fractions.

Table 1. The antifungal activity of acid, base and neutral fractions extracted from cell-free supernatants of bacterial isolates (PS02 and PS14) against F. culmorum based on in vitro bioassays.

Isolates	Diethyl ether ($C_4H_{10}O$)			Dichloromethane (CH_2Cl_2)		
	acidic	basic	neutral	Acidic	basic	neutral
PS02	+	-	-	-	-	-
PS14	+	-	-	-	-	-

(+) = inhibition and (-) = no inhibition.

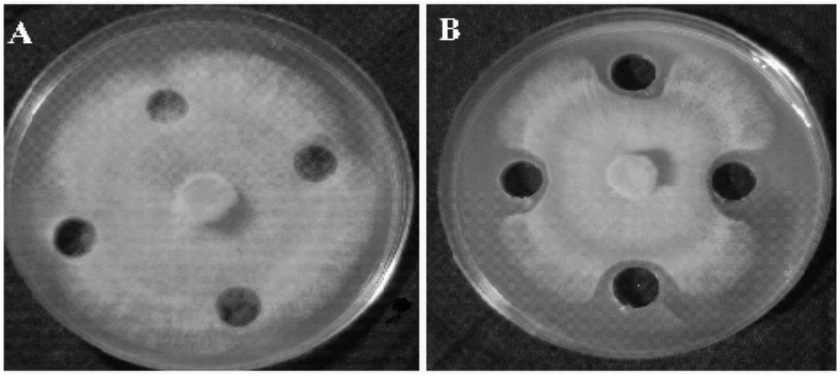

Fig. 1 Presence of inhibition (B) of mycelial growth of F. culmorum by acidic fractions extracted from a cell-free supernatant compared to the control (A) dimethylsulfoxide (DMSO) ”Solvent“.

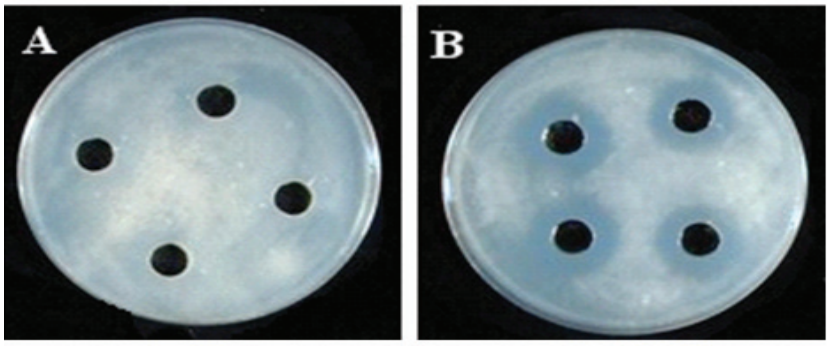

Fig. 2 Presence of inhibition (B) of conidial germination of F. culmorum by acidic fractions extracted from a cell-free supernatant compared to the control (A) dimethylsulfoxide (DMSO) “Solvent”.

DISCUSSION

In this study it has been shown that antifungal metabolites are produced in vitro by P. fluorescens isolates from the rhizosphere of wheat. Some metabolites inhibited conidial germination and mycelial growth of F. culmorum. Chan et al. (2003) reported that a cell-free filtrate of Bacillus subtilis (D1/2) was active against macroconidium germination and hyphal growth of F. graminearum and a range of common fungal pathogen phytopathogens, including eight Fusarium species, three other ascomycetes and one basidiomycete. This strongly suggests that antibiosis may be an important mechanism of biocontrol by P. fluorescens isolates (PS02 and PS14).

P. fluorescens (PS02 and PS14) successfully produced antifungal metabolites in a culture medium that was modified for optimum antibiotic production based on the study of Leifert et al. (1995) and Lee et al. (2003). However, proof that such mechanisms were active in biocontrol would require the development of methods which would allow the demonstration of the production and activity of antibiotics in planta. Leifert et al. (1994) found that antibiotic production was dependant on the growth substrate, bacterial strains and incubation time. In this present study, using glycerol and proteose peptone, as carbon and nitrogen sources in modified KMB for P. fluorescens isolates appeared to allow the production of antifungal metabolites.

Antifungal metabolites produced by P. fluorescens (PS02 and PS14) were successfully extracted from cell-free culture supernatants. Several reports of antifungal activity by Pseudomonas species and the literature were reviewed [Mazzola *et al*., 1995; Boer *et al*., 1998; De La Fuente *et al*., 2000; Chan et al., 2003; Landa *et al*., 2003; Lee *et al*., 2003]. So far Gram-negative bacteria, especially Pseudomonas strains, have been intensively investigated as biological control agents [Turner and Backman 1991].

Loginov et al. (2003) described active metabolites of Pseudomonas spp. grown in King's B liquid medium. Low-molecular-weight extracellular metabolites were isolated from the culture medium after the bacterial biomass had been removed by centrifugation and the resulting supernatant was ultra-filtered. The filtrate was tested using an agar diffusion assay for its ability to inhibit seven of Fusarium spp., including F. culmorum, F. gibbosum, F. oxysporum, F. solani, F. semitectum, F. avenaceum and F. moniliforme. Their results showed that the cell-free filtrate inhibited all seven Fusarium spp. and the authors described a new group of peptide metabolites (triglyceridepeptides), produced by Pseudomonas spp.

Our results showed that, the main metabolites produced by P. fluorescens

responsible for the inhibition F. culmorum were the acid fractions. However, the correlation between the ability to form metabolites that are effective against F. culmorum in vitro and any observed effects on the reduction of F. culmorum or other Fusarium disease in soil experiments has not been determined. Therefore it is not possible to conclude whether these metabolites play any part in the reduction of the incidence and severity of plant disease achieved by the application of P. fluorescens isolates in this study. It was not possible to confirm the formation of these metabolites in the rhizosphere or in planta. This is an area which requires further investigation.

In conclusion, the findings of the present study showed that naturally occurring P. fluorescens isolates PS02 and PS14 produced antifungal metabolites inhibitory to F. culmorum. Further work is required to identify all the specific antifungal metabolites in our study and each metabolite should be purified and evaluated in vitro for antifungal activity.

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