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DETECTION OF ANTIFUNGAL COMPOUNDS FROM ANTAGONISTIC MICROORGANISMS INHIBITING THE GROWTH OF *FUSARIUM OXYSPORUM* F. SP. *CICERIS*

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Abstract

Culture filtrate of the *Cladosporium* sp. (1C isolate) was found to inhibit 55% growth of *Fusarium oxysporum* f. sp. *ciceris* on Czapek dox medium (Oxoid). Another isolate (2C) reduced 24% growth and a strain of *Bacillus subtilus* inhibited 28% of the growth. The antifungal activity was found more in Czapek dox medium containing nitrite ($NaNO_2$ @ 2g\l) as nitrogen source as compared to nitrate ($NaNO_3$ @ 3g\l). The antifungal activity of the culture filtrate was best recovered into ethyl acetate at pH 3.00. Thin layer chromatography of the ethyl acetate and chloroform phases were done by using different solvent systems and the TLC plates were bioautographed by spraying test fungus which showed five inhibition zones produced by 1C isolate, representing five antifungal compounds and one by *Trichoderma harzianum*. Same inhibition zones were observed when the TLC plates were bioautographed against *F. oxysporum* f. sp. *ciceris*.

Introduction

Chickpea wilt caused by *Fusarium oxysporum* f. sp. *ciceris* (FOC) is a major problem in Thal area where most of the crop is cultivated. The disease is reported to cause 10% annual yield losses to the crop (Halila *et al.,* 1984). The fungus is soil borne as well as seed borne in nature. It can survive in the soil for more than five years; moreover it has symptomless carriers like lentil and peas (Sexana and Singh, 1987). It is impracticable to control the disease by crop rotation and by using fungicides (Haware and Nene, 1982). The absence of true resistance in chickpea against wilt disease and a continuous problem of the occurrence\development of new pathogenic races has aggravated the problem. Biocontrol agents can effectively be used for the control of diseases because these are cheaper, safer for the environment and long lasting. A variety of the soil microorganisms have demonstrated activity in the various soil borne plant pathogens, including Fusarium wilt pathogens (Larkin and Fravel, 1998). Fusarium wilt suppressive soils are known to occur in many regions of the world, and suppression has generally been shown to be biological in nature. Antagonists recovered from the wilt suppressive soils, especially nonpathogenic *F. oxysporum* have been used to reduce the fusarium wilt diseases of many crops (Larkin *et al.*, 1996 and Minuto *et al.*, 1995). Other biocontrol fungi, such as, *Stillbella Trichoderma, Gliocladium* and *Cladorrhinum* spp. have been used to control the variety of fungal pathogens (Lewis and Papavizas, 1993, and Lewis *et al.*, 1995, 1996). The biological control might have the problems of inconsistency, failure under certain conditions of applications, due to the lack of wide spectrum action or to the limited growth of biological control agents because of the presence of inhibitory levels of some pesticides residues. The potential antifungal metabolites produced by the antagonistic microorganisms could be identified and possibly be used as fungicides. Usually these biochemicals are less

hazardous to human beings and environments as compared to the synthetic fungicides.

The purpose of the study was to identify the potential antifungal metabolites produced by the different antagonistic microorganisms for their possible use as fungicides.

Materials and methods

In vivo studies of antagonistic activities: *Cladosporium* species (1C and 2C), a *Trichoderma harzianum* isolate were isolated from wilt suppressive soil. The *B. subtilus* was obtained from medium in which *Ascochyta rabiei* was not allowed to grow because it appeared as contaminant. The fungal isolates were grown on amended Czapek dox medium and bacterial isolate on liquid nutrient broth medium. The *Cladosporium* sp. (2C) was also grown on amended Czapek dox medium (amended by replacing $NaNO_3$ with $NaNO_2$ @ 2g\l). The flasks were incubated at 25±2 °C in dark for 14 days. The culture filtrates were centrifuged at 5000*g for 5 minutes, supernatants were removed and designated as 1N solutions. These solutions (50ml) were diluted to 100 ml with distilled water and were designated as 0.5N solutions. These 1N and 0.5N solutions were used to prepare Czapek dox agar medium (Oxoid). The media were autoclaved at 120°C; 15 pounds psi for 15 minutes and then 15 ml of the medium was poured in each petri plate (6 cm diameter plates). Two replicates were used for each treatment, for control distilled water was used. These plates were inoculated in the center with a disc (2mm diameter) of 7 days old culture of FOC and then incubated at 25±2°C. After 7 days the colony diameter were measured.

Bioautography: The culture filtrates of 1C isolate (100ml each) were partitioned separately into half the volumes of chloroform and ethyl acetate three times each by adjusting the pH of the culture filtrates to 3.0. The culture filtrates of *T. harzianum* were also partitioned into ethyl acetate at pH 3.0. These organic phases were dried over anhydrous sodium sulphate, rotavapoured to dryness and then dissolved into 2ml of methanol. Thin layer chromatography of these phases were done on self coated silica gel 60 GF254 plates (0.5 mm thickness) separately in each of the solvent system: n- Hexane: ethyl acetate (9:1), benzene: acetone: acetic acid (35:5:1), chloroform: methanol (48:2) and n-pentane: diethyl ether: acetic acid (75:25:1). The developed TLC plates were sprayed by the spore suspension of test fungus (*Cladosporium cucumerinum*) in sterilized Czapek dox medium. The plates were placed in humidity chamber at 25°C in dark for 4 days. The TLC plates were also bioautographed against FOC.

Results and discussions

Culture filtrates of all the three microorganisms reduced the growth of chickpea wilt pathogen. The culture filtrate of 1C isolate reduced the colony diameter of *F. oxysporum* f.sp. *ciceris* up to 55% at 1N concentration. While at 0.5N concentration 36% reduction occurred respectively (Table). The culture filtrate of 2C isolate inhibited 24% colony diameter at 1N concentration, while at 0.5N concentration 9.4% reduction was observed (Table). The culture filtrate of *B. subtilus* reduced 28% colony diameter at 1N concentration (Table). This indicated

that all the three microorganisms were producing antifungal metabolites in the culture filtrates. These metabolites were also stable after autoclaving at 120°C on 15 pounds psi for 15 minutes. None of the culture filtrates completely inhibited the growth of chickpea pathogen; it might be due to the presence of sublethal concentration of these metabolites at 0.5N and 1N concentrations. These antifungal metabolites might exhibit more lethality in purified form. The 1C isolate was found to be the most potent of all the isolates and could be used for the control of fusarium wilt disease of chickpea.

The *Cladosporium* isolate (2C) produced more antifungal activity on amended Czapek dox containing sodium nitrite as compared to sodium nitrate, indicating that nitrite was the better nitrogen source for the production of antifungal activity. The utilization of the nitrite ions is relatively easy for the different fungi as compared to the nitrate ions because the nitrate ions are converted into nitrite, then into ammonium and ultimately into amino acids. Bioautography of the TLC plates showed five inhibition zones representing five antifungal compounds extracted by ethyl acetate from the culture filtrate of 1C isolate, at the Rf values 0.08, 0.16, 0.38, 0.56, 0.83 and one by *T. harzianum* at Rf value 0.92. The solvent system n-hexane: ethyl acetate (9:1) gave best resolution of the antifungal compounds of 1C isolate on silica gel TLC plates and for *T. harzianum* solvent system benzene: acetone acetic acid (35:5:1) was used. The chloroform extract of 1C isolate yielded one inhibition zone at Rf value 0.83. The ethyl acetate extracted more antifungal activity from the culture filtrate as compared to chloroform because it extracted more antifungal compounds from the culture filtrates. The inhibition zones were observed at the similar Rf values when the TLC plates were bioautographed by using FOC, showing the activity of these compounds against the chickpea wilt pathogen. The potential antifungal compounds produced by these antagonistic microorganisms could be purified and identified to use as the fungicides for the control of the notorious vascular wilt pathogen (*F. oxysporum*).

Table. Inhibition of the colony diameter of FOC by the culture filtrates of *Cladosporium* sp. and *B. subtilus* through poisoning food method.

S.No.	Isolate/ Nitrogen Source/ Concentration	Ave. Colony Diameter (Cm)	Percentage Inhibition Than control
1	1C/NO_3/1.0N	1.075	55%
2	1C/NO_3/0.5N	1.525	36.45%
3	2C/NO_3/1.0N	1.825	24%
4	2C/NO_3/0.5N	2.175	9.4%
5	2C/NO_2/1.0N	1.300	43%
6	2C/NO_2/0.5N	2.050	14.58%
7	*B.subtillus*/1.0N	1.725	28%
8	Control	2.375	-----

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