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Full Length Research Paper

In vitro control of *Alternaria citri* using antifungal potentials of *Trichoderma* species

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The antifungal potential of five species of *Trichoderma* viz., *Trichoderma viride*, *Trichoderma aureoviride, Trichoderma reesei, Trichoderma koningii* and *Trichoderma harzianum* was investigated *in vitro* against *Alternaria citri*, the causal agent of the black rot disease on a broad range of citrus cultivars. Cultural filtrates of *Trichoderma* species were obtained by growing them on different media. The effect of different filtrate concentrations revealed that aqueous extracts of all *Trichoderma* species significantly reduced the fungal biomass of the target fungal pathogen. Generally, 100% culture filtrate of the test *Trichoderma* species significantly reduced the growth of fungus. On each medium, a different response was observed. Culture filtrate of *T. harzianum* was found highly effective in suppressing growth (up to 93%) of the test fungal species grown on malt extract medium. *T. harzianum* and malt extract medium were therefore selected for fraction analysis. There was 68% reduction in growth of the *A. citri* due to 1% concentration of ethyl acetate fraction of cultural filtrate of *T. harzianum* when grown in malt extract broth.

Key words: Citrus, *Alternaria citri*, ethyl acetate fraction, *Trichoderma harzianum, viride*, *aureoviride, reesei, koningii.*

INTRODUCTION

Plant diseases need to be controlled to maintain the quality and abundance of food, feed and fiber produced by growers around the world. Different approaches may be used to prevent, mitigate or control plant diseases (Chandler et al., 2008). Overdose usage of chemicals as pesticides and fertilizers is common in farming in most parts of the world, which threatens food safety of individuals. Some pesticides can be hardly cleaned from nature and have a potential capability to have adverse effect or destroy useful microorganisms which have positive effects in fertility of soil and growth of plants. To minimize or avoid side effects, biological control is an alternative and proper choice in pest management (Baniasadi et al., 2009). Many species of fungi and bacteria are potential biocontrol agents (Harman 2000; Harman et al., 2004). Fungal and bacterial antagonists have been found to be effective for the control of various post-harvest diseases (Janisiewicz et al., 2011), especially in fruits.

Trichoderma strains are among the most studied fungal biocontrol agents (Vinale et al., 2005). *Trichoderma* species are ubiquitous free-living fungi that are highly interactive in root, soil and foliar environments (Sivasithamparam and Ghisalberti, 1998). So far, most of the studies on *Trichoderma* species have been conducted with respect to their activity as biological control agents against fungal pathogens (Sahebani and Hadavi, 2008; Hanada et al., 2009; Nallathambi et al., 2009). They are commercially applied as biopesticides, thus limiting the abuse of chemical fungicides (Punja and Utkhede, 2003; Benítez et al., 2004). The antagonistic activity of *Trichoderma* depends on multiple synergistic mechanisms (Howell, 2003; Harman et al., 2004). The various mechanisms include antibiosis, parasitism, inducing host-plant resistance, competition, secretion of chitinolytic enzymes, mycoparasitism and production of inhibitory compounds (Harman, 2006). *Trichoderma*

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produces a number of secondary metabolites with biological activity (Ghisalberti and Sivasithamparam, 1991; Sivasithamparam and Ghisalberti, 1998).

Ghisalberti and Rowland (1993) identified five bioactive metabolites from liquid cultures of *Trichoderma harzianum*. Two of these, cyclonerodiol and the octaketide keto diol, were previously reported from a strain of *Trichoderma koningii*, whereas other three octaketide-derived compounds were reported for the first time. Senthilkumar et al. (2011) reported six antifungal compounds, including diethyl phthalate, tetradecanoic acid 9,12-octadecadienoic acid, oleic acid, 1,2-benzenedicarbooxylic acid, diisooctyl ester and squalene from local isolates of *T. harzianum*. Literature provides several reports of antagonism between *Trichoderma* species and other fungi and its potential exploitation as a bio-control agent (Benitez et al., 2004; Hermosa et al., 2004; Hanhong, 2011).

Several pathogens are responsible for the spoilage of our agricultural output. The fruit industry is also affected, especially from the post-harvest fungal decay of fruit and vegetables. *Alternaria citri*, a post-harvest fungus belonging to phylum Ascomycota, infects citrus fruit and makes it inconsumable. Citrus stands as the second most important fruit worldwide after grapes in terms of area and production. The present study was carried out to investigate the potential of metabolites released by various *Trichoderma* species grown on different nutritional media to control selected plant pathogen.

MATERIALS AND METHODS

Procurement of fungal cultures

Pure cultures of different *Trichoderma* species viz., *Trichoderma viride* (FCBP-142), *Trichoderma reesei* (FCBP-271), *Trichoderma aureoviride* (FCBP-234), *T. koningii* (FCBP-765) *T. harzianum* (FCBP-140) and test pathogen *A. citri* (FCBP-970) were obtained from First Fungal Culture Bank of Pakistan (FCBP) and Institute of Plant Pathology (IPP), University of the Punjab, Lahore, Pakistan. These cultures has been isolated from air, cultivated soil of IPP experimental station, rhizospheric soil of *Litchi chinensis*, *Mangifera indica, Helianthus annuus* and *Rosa indica*, respectively. These isolates were revived and maintained on 2% malt extract agar (MEA) and preserved at 4°C for further use and reference.

Growth of *Trichoderma* for cultural filtrates

Cultural filtrates of test *Trichoderma* species were obtained by growing them on different media viz., malt extract medium (20.0 g malt extract and 1000 ml distilled water), yeast malt extract medium (3.0 g yeast extract, 3.0 g malt extract, 5.0 g peptone, 10.0 g glucose and 1000 ml distilled water), Sabouraud's dextrose medium (40.0 g glucose or maltose, 10.0 g peptone and 1000 ml distilled water), potato dextrose medium (20.0 g potato starch, 20.0 g dextrose and 1000 ml distilled water), double malt extract medium (40.0 g malt extract and 1000 ml distilled water), maize meal medium (40.0 g glucose, 1.5 g $KH_2PO_4.3H_2O$, 0.5 g KCl, 0.5 g $NaNO_3$, 0.5 g $MgSO_4.7H_2O$, 10.0 g maize meal and 1000 ml distilled water) and potato glucose medium (modified) (300 g potato puree, 20.0 g glucose and 1000 ml distilled water). All the chemicals were of analytical grade and were obtained from Merck (Germany) and ACROS (Belgium). Broth of each media was prepared and sterilized in autoclave at 121°C under 15 lb/inch^2.

Trichoderma species were inoculated by disc inoculation method. Each flask received one disc of 5 mm diameter and incubated on a shaker at the speed of 150 rpm at 25 ± 3°C for a week, receiving alternate periods of 12 h dark and light. After seven days, broth was filtered by using Whatman filter paper No.1. These cultural filtrates were taken as stock solution. Further dilutions viz., 25, 50, 75 and 100% were prepared by sterilizing the stock solution through 0.22 µM pore size filters and diluting it with calculated amount of freshly made respective autoclaved medium. Control (0%) for each medium was prepared in which the respective medium act as control. Each dilution was replicated three times. Subsequently, mycelial plugs from the edges of actively growing culture of *A. citri* were prepared using a cork borer of 5 mm diameter. One disc was transferred aseptically into each 250 ml flask containing 100 ml of prepared concentrations and control set. The flasks were incubated for seven days on an electric shaker with conditions as earlier described.

Estimation of growth inhibition

Growth inhibition, if any, on target fungus was assessed in terms of dry weight. Broth containing fungal biomass from replicate flasks was filtered on pre-weighed Whatman No. 1 filter papers. Biomass of *A. citri* was oven dried at 60°C for 6 h and reweighed to determine the oven dried weights. The rate of growth inhibition was determined from the oven dried fungal biomass to evaluate the relative effects of various concentrations of cultural filtrates of *Trichoderma* species on *A. citri*. The growth and antagonistic tests were performed for all tested *Trichoderma* species against the target fungus using all the seven media one-by-one. Through this analysis, the best *Trichoderma* species having maximum antagonistic effect on *A. citri* and the best medium enhancing the antagonistic effect was selected.

Fractionation of selected media

The *Trichoderma* species and the medium that was selected in the first assay were used for further analysis. Two liters of malt extract (selected medium) was prepared and autoclaved. A disc of 5 mm diameter of *T. harzianum* (selected *Trichoderma* species) from actively growing margins was inoculated in each 250 ml flask containing 100 ml broth. Inoculated flasks were incubated on shaker for four weeks under conditions as earlier described. After four weeks, filtration was done through sterilized muslin cloth followed by Whatman filter paper No.1. This filtrate was preserved at 4°C in refrigerator. The cultural filtrate was used within one week of filtration to avoid contamination or chemical alteration.

The extraction procedure was carried out with reference to Lazarovits et al. (1979). A volume of 400 ml of filtrate was taken in a 1000 ml separating funnel. Four organic solvents were used for extraction. First, a volume of 200 ml of butanol was added to the filtrate in a separating funnel, shaken well and kept until the two phases get separated. The upper butanol layer was separated and was vacuum-dried in rotary evaporator. The remaining filtrate was extracted similarly in succession with *n*-hexane, chloroform and ethyl acetate. All the organic fractions were collected separately and dried in rotary evaporator at 45°C. Finally, all the fractions were subjected to desiccation in an electric oven with a continuous current of air at 45°C to remove any traces of solvents and to obtain the final residues.

Aqueous solutions of butanol, chloroform and ethyl acetate residues were prepared in sterilized distilled water to make the final concentration of 6 mg ml^{-1}. Residues of *n*-hexane were first

dissolved in dimethoxy sulfoxide (DMSO) and then sterilized distilled water was added to obtain the final concentration of 6 mg ml^{-1}. To check the bioactivity of fractions, 2, 1, 0.5 and 0.25% dilutions of the organic solvent residues solutions were formed by adding specific quantity of residue solution and distilled water to 2 ml malt extract (ME) broth. The experiment was repeated twice with three replications for each treatment. Control set contained only ME and water, while in case of *n*-hexane control, a dilute solution of DMSO in water was mixed in malt extract broth. A conidial suspension of *A. citri* was formed and 0.2 ml of inoculum was given to all treatments which contain 320,000 conidia/0.2 ml as determined through hemocytometer. Test tubes were incubated at 25°C for seven days. After the incubation period, data was recorded by taking the dry biomass weights of *A. citri*. Biomass analysis predicted the growth inhibition of *A. citri* caused by the application of different fractions of *T. harzianum*.

Statistical analysis

Standard errors of means of three replicates of each treatment were computed using computer software Microsoft Excel. All the data were analyzed by analysis of variance (ANOVA) followed by Duncan's multiple range test to separate mean differences (Steel and Torrie, 1980) using computer software SPSS and COSTAT.

RESULTS

Effect of cultural filtrates of *Trichoderma* species on *A. citri* biomass production grown on different culture media

Effect of filtrate concentrations of five *Trichoderma* species on the biomass production of *A. citri* was checked on seven different media (Table 1). The data reveals that the aqueous extracts of all *Trichoderma* species significantly reduced the fungal biomass of the target fungal pathogen *A. citri.* However, variability among the species and culture media was evident.

Effect of cultural filtrates of *Trichoderma* species on growth of *A. citri* grown on malt extract

Cultural filtrates of all the five *Trichoderma* species grown on malt extract medium significantly decreased biomass of test fungi. A gradual decrease in biomass was observed as the concentration of extract of each species was increased except that of *T. koningii*. Cultural filtrate of *T. harzianum* remarkably suppressed the biomass of the test fungal pathogen. The highest tested concentration of *T. harzianum* filtrate caused approximately 93% inhibition on growth of *A. citri*. This trend was followed by *T. aureoviride, T. koningii, T. reesei* and *T. viride* as suppression in fungal growth, due to their aqueous extracts having values of 92, 81, 74 and 68 at 100%, respectively. A slight contrasting behavior was observed in case of *T. koningii* as *A. citri* displayed the highest growth on 75% while maximum growth inhibition of *A. citri* was noticed at 50% extract concentration.

Effect of cultural filtrates of *Trichoderma* species on growth of *A. citri* grown on yeast malt extract

Growth response of *A. citri* on yeast malt extract with different concentrations of filtrates obtained from *Trichoderma* species portrayed an erratic pattern of biomass production. Growth rate of fungus showed variable response to different concentrations of aqueous extracts of *Trichoderma* species. Different concentrations of filtrate of *T. viride* showed controlled growth of pathogen as the concentration gradient increased from 25 to 75% with an exception of 100% whose inhibition rate was noticed at 4% less than that of 75%. In case of *T. aureoviride* and *T. reesei*, a normal trend of low growth rate at high concentration was followed; however, lower filtrates concentration of 25% increased growth of *A. citri* up to 14.74 and 4.91% when compared to control.

Moreover, filtrate concentrations of *T. koningii* revealed inconsistent results as statistically significant increase of 36.84% with respect to control was recorded in 50% concentration. Growth suppression of the test fungus was supported, particularly in 25 and 75% concentrations with insignificant differences among each other, whereas 32.63% inhibition of pathogen was noticed at 100% filtrate concentration. Aqueous extract of *T. harzianum* remarkably suppressed the biomass of the pathogen *A. citri* by following the general trend of decreased growth rate as the concentrations increased and suppression in fungal biomass due to 25, 50, 75 and 100% concentrations was 53, 55, 62 and 63%, respectively.

Effect of cultural filtrates of *Trichoderma* species on growth of *A. citri* grown on Sabouraud's dextrose

A variable effect of various filtrate concentrations of the extract acquired from different *Trichoderma* species grown on Sabouraud's dextrose medium was recorded. Growth pattern of *A. citri* exhibited variable and erratic mode of inhibition by filtrate concentrations of each species. The highest decrease in fungal biomass was exhibited by 100% filtrate concentration of *T. viride* which was 52%. Arrested biomass production was observed in 50 and 75% filtrate concentration that caused 13 to 16% inhibition, while 1.80% higher growth than control was recorded for *T. viride* at 25% filtrate concentration. Effect of cultural filtrates of remaining *Trichoderma* species did not show a significant reduction in biomass production of fungal pathogen, due to the fact that the antagonistic effect was largely overcome by the active growth of the fungus, more prominently in case of lower concentrations of *T. koningii* filtrates.

Although the growth rate in 100% filtrates concentration of *T. aureoviride*, *T. reesei, T. koningii* and *T. harzianum*

Table 1. Effect of different filtrate concentrations of five *Trichoderma* species grown on seven different media on the growth of *Alternaria citri*.

Media	*Trichoderma* species				
	T. viride	***T. aureoviride***	***T. reesei***	***T. koningii***	***T. harzianum***
Malt extract (%)					
0	0.482^{a}	0.482^{a}	0.482^{a}	0.482^{a}	0.482^{a}
25	0.232^{b}	0.119^{b}	0.267^{b}	0.102^{b}	0.137^{b}
50	0.227^{b}	0.114^{b}	0.206^{c}	0.071^{b}	0.095^{c}
75	0.179^{c}	0.068^{c}	0.16^{d}	0.13^{b}	0.057^{d}
100	0.152^{d}	0.04^{d}	0.126^{e}	0.094^{b}	0.036^{e}
Yeast malt extract (%)					
0	0.285^{a}	0.285^{ab}	0.285^{a}	0.285^{b}	0.285^{a}
25	0.187^{b}	0.327^{a}	0.299^{a}	0.118^{d}	0.133^{b}
50	0.174^{bc}	0.236^{bc}	0.178^{b}	0.39^{a}	0.128^{b}
75	0.134^{bc}	0.214^{c}	0.174^{b}	0.11^{d}	0.106^{c}
100	0.145^{c}	0.192^{c}	0.138^{b}	0.192^{c}	0.105^{c}
Sabouraud's dextrose (%)					
0	0.168^{a}	0.168^{abc}	0.168^{bc}	0.168^{b}	0.168^{ab}
25	0.171^{a}	0.13^{c}	0.237^{a}	0.364^{a}	0.205^{a}
50	0.147^{a}	0.187^{a}	0.183^{b}	0.33^{a}	0.211^{a}
75	0.142^{a}	0.176^{ab}	0.166^{bc}	0.176^{b}	0.189^{a}
100	0.081^{a}	0.134^{bc}	0.13^{c}	0.128^{b}	0.135^{b}
Potato dextrose (%)					
0	0.446^{a}	0.446^{a}	0.446^{a}	0.446^{a}	0.446^{a}
25	0.28^{ab}	0.257^{ab}	0.268^{ab}	0.257^{ab}	0.361^{a}
50	0.364^{a}	0.241^{ab}	0.218^{ab}	0.191^{b}	0.203^{ab}
75	0.282^{ab}	0.193^{b}	0.197^{b}	0.144^{b}	0.084^{b}
100	0.11^{b}	0.161^{b}	0.183^{b}	0.113^{b}	0.047^{b}
Double malt extract (%)					
0	0.162^{c}	0.162^{b}	0.162^{ab}	0.162^{ab}	0.162^{c}
25	0.288^{a}	0.264^{a}	0.175^{ab}	0.183^{a}	0.315^{a}
50	0.226^{b}	0.143^{bc}	0.226^{a}	0.108^{abc}	0.229^{b}
75	0.118^{d}	0.149^{bc}	0.193^{a}	0.103^{bc}	0.156^{c}
100	0.079^{e}	0.1^{c}	0.072^{b}	0.063^{c}	0.07^{d}
Maize meal (%)					
0	0.879^{a}	0.879^{a}	0.879^{a}	0.879^{a}	0.879^{a}
25	0.406^{b}	0.513^{ab}	0.752^{a}	0.293^{b}	0.412^{b}
50	0.357^{b}	0.359^{b}	0.454^{ab}	0.183^{b}	0.214^{b}
75	0.314^{b}	0.374^{b}	0.4^{ab}	0.106^{b}	0.137^{b}
100	0.167^{b}	0.18^{b}	0.169^{b}	0.079^{b}	0.065^{b}
Potato glucose (%)					
0	1.122^{a}	1.122^{a}	1.122^{a}	1.122^{a}	1.122^{a}
25	0.604^{b}	0.706^{b}	0.955^{b}	0.482^{b}	0.74^{b}
50	0.581^{b}	0.617^{c}	1.083^{ab}	0.361^{c}	0.702^{b}
75	0.524^{c}	0.455^{d}	0.78^{c}	0.446^{bc}	0.737^{b}
100	0.338^{d}	0.401^{e}	0.525^{d}	0.465^{b}	0.299^{c}

In a column, values with different letters show significant difference (P≤0.05) as determined by Duncan's multiple range (DMR) test.

was still lower than control, whereas, in case of 50 and 75% concentrations of *T. aureoviride,* a significant increase in growth of *A. citri* was recorded. While for 25, 50 and 75% concentrations of *T. reesei*, a statistically significant growth promoting effect was evident and a stimulus in biomass production of 41, 9 and 1.2%, respectively with respect to control was recorded. In the same way a boost in biomass production was recorded for *T. koningii* up to 117, 96 and 5% and for *T. harzianum* was 22, 26 and 13%, respectively.

Effect of cultural filtrates of *Trichoderma* species on growth of *A. citri* grown on potato dextrose

It is evident from the results that culture filtrates of all the five *Trichoderma* species grown on potato dextrose medium significantly reduced the growth of *A. citri.* A pronounced reduction in biomass was noticed on 100% filtrate concentration by all species. The adverse effect of filtrates of *T. harzianum, T. viride* and *T. koningii* was more pronounced than the effect of *T. aureoviride* and *T. reesei.* There was 89.46, 75.34, 74.66, 63.90 and 58.97% reduction in growth due to 100% culture filtrates of *T. harzianum, T. viride, T. koningii*, *T. aureoviride* and *T. reesei,* respectively, as compared to control. In case of *T. viride*, a dissimilar pattern was noticed in which an increase in growth of *A. citri* was observed at 50% filtrate concentration.

Effect of cultural filtrates of *Trichoderma* species on growth of *A. citri* grown on double malt extract

The dry biomass assay of *A. citri* treated with different concentrations of cultural filtrates of five *Trichoderma* species revealed a rather erratic pattern of growth. Except for 100% filtrates concentration of almost all *Trichoderma* species that caused a considerable increase in biomass production of the pathogen, a statistically significant increase in growth was evident in lower concentrations viz. 25 to 50% of *T. viride* (78 to 40%) and *T. harzianum* (9 to 41%) in comparison to the control. In case of *T. aureoviride* and *T. koningii*, 63 and 13% elevation in growth was noticed at 25% filtrate concentration with respect to the control. In higher concentrations, both species showed gradual decrease in growth. *T. reesei* exhibited an unpredictable growth response. The growth of the test fungal species was markedly supported by 25, 50 and 75% concentrations of *T. reesei* with a very sharp increment in growth, from 8 to 40% in comparison to the control.

Effect of cultural filtrates of *Trichoderma* species on growth of *A. citri* grown on maize meal

Antifungal assay of cultural filtrates of *Trichoderma* species grown on maize meal revealed that all the concentrations of *Trichoderma* species was significant against test fungal species except for 25% filtrate concentration of *T. reesei*. The relative intensity of this effect, however was found to vary with the species involved, as well as the concentrations of the filtrate employed. There was a gradual decrease in biomass as the concentration of filtrate was increased from 25 to 75% and a remarkable decrease in growth at 100% filtrate concentration. The highest adverse effect was recorded due to 100% cultural filtrate of *T. harzianum*, where 93% reduction in growth of *A. citri* was noticed. Followed by this, 91, 81, 80.77 and 79.52% growth reductions were exhibited by *T. koningii, T. reesei* and *T. aureoviride,* respectively. A slight variation from regular trend was observed for 75% filtrate concentration of *T. aureoviride* which instead of exhibiting decreased growth showed elevated growth.

Effect of cultural filtrates of *Trichoderma* species on growth of *A. citri* grown on potato glucose

Generally, filtrates of all species of antagonist fungus grown on potato glucose also significantly reduced the fungal biomass of the target pathogen. However, the variability in effect was also evident. Filtrate concentrations of all the tested *Trichoderma* species extensively checked the growth of *A. citri.* About 73, 70, 64, 59 and 53% reduction in fungal growth was exhibited by 100% concentration of *T. harzianum, T. viride, T. aureoviride*, *T. koningii,* and *T. reesei,* respectively. A gradual decrease in biomass was observed as filtrate concentration increased in case of *T. viride* and *T. aureoviride*, whereas this trend was slightly disturbed in case of *T. reesei, T. koningii* and *T. harzianum*. Filtrate concentration of *T. reesei* exhibited minimum inhibition in growth of *A. citri*. In case of *T. koningii*, 50% filtrate concentration displayed 68% inhibition, while at higher concentrations of 75 to 100%, inhibition rate decreased.

Antifungal effect of fractions of *T. harzianum*

Analysis of variance showed that the antifungal effect of butanol, chloroform and n-hexane filtrate fractions of *T. harzianum* grown on malt extract medium failed to reduce the growth of tested fungi significantly (Figures 1 and 2). Only ethyl acetate fraction of the filtrate significantly checked the fungal growth in this bioassay. Ethyl acetate fraction of filtrate obtained from *T. harzianum* significantly reduced the growth of *A. citri.* However, variability among different concentrations of extract was also observed (Figure 1). The highest retardation among all the concentrations of four fractions was executed by 1% ethyl acetate fraction extracts and the reduction in fungal biomass was 68%. In contrast, the higher concentration (2%) depicted less toxicity against *A. citri* where only 23% reduction was noticed. The retardation in fungal biomass

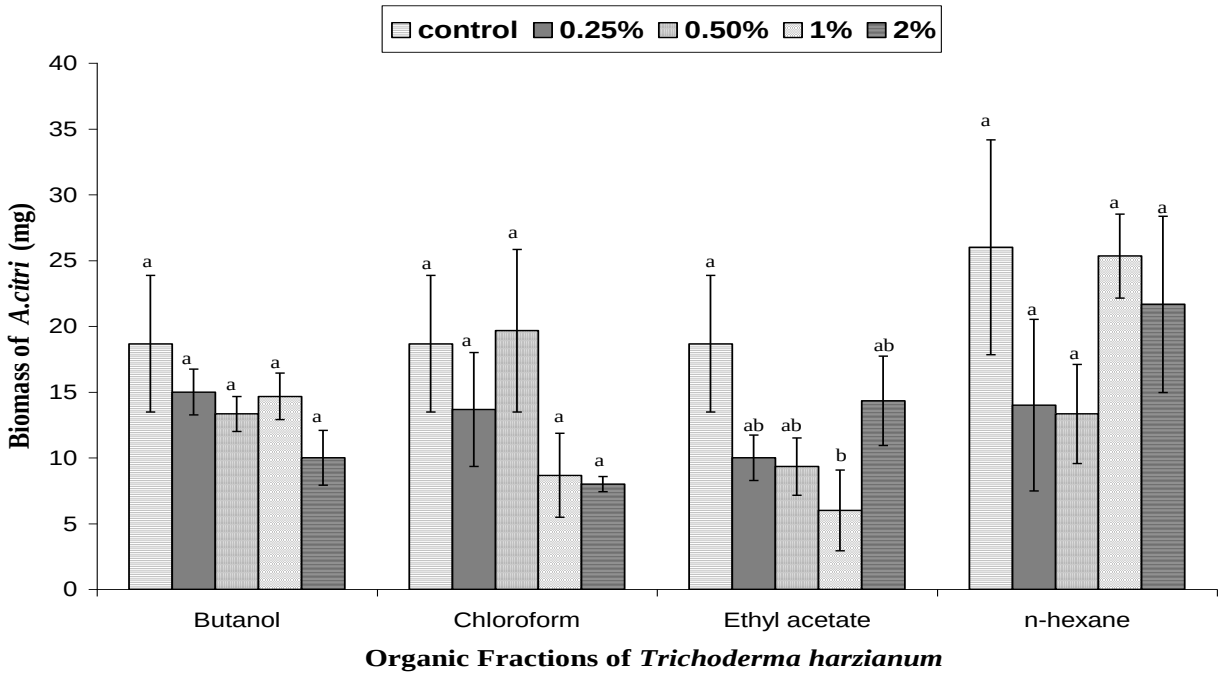

Figure 1. Effect of different concentrations of organic fractions of *Trichoderma harzianum* on the growth of *Alternaria citri*. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference (P≤0.05) as determined by Duncan's multiple range (DMR) test.

due to 0.25 and 0.5% concentrations was 46 and 50%, respectively (Figure 2).

DISCUSSION

The results of this conceptual study clearly reflect that *Trichoderma* species had inherent ability to induce antagonistic effects on fungal pathogen. The relative intensity of this effect however varied with the species involved, as well as the particular concentrations of the extract employed and the type of media. Aqueous extracts of all *Trichoderma* species significantly reduced the fungal biomass of the target fungal pathogen. *Trichoderma* species are known to produce a range of bioactive secondary metabolites, including gliovirin, gliotoxin, viridine, pyrones and viridiol, (Jones and Hancock, 1987; Jones et al., 1988). However, no particular trend of growth inhibition of pathogen was observed in this study; on each medium, a different response was evident.

Generally, 100% culture filtrate of the test *Trichoderma* species significantly reduced the growth of fungus. It is also reported from a recent study that the highest exudate concentration of *T. viride* and *Cercospora salina* (90%) showed a good potency as antifungal agent (El-Kassas and Khairy, 2009). Presently, culture filtrate of *T. harzianum* was found highly effective in suppressing growth (up to 93%) of the tested fungal species grown on malt extract medium, which increased the biocontrol potential of selected strain (Table 1). Hence this species and medium was selected for subsequent investigations. Also on yeast malt extract, potato dextrose, maize meal and potato glucose media, *T. harzianum* exhibited adverse effect on *A. citri* growth. The variation in fungicidal activity among the five *Trichoderma* species could be attributed to the presence of different types of chemical constituents in different species (Wang et al., 2003; Zhou et al., 2008; Eneyskaya et al., 2009; Yang et al., 2009).

Different culture media also play their role in increasing or decreasing the biocontrol potential of species and influence the fungicidal activity of each species. The results of the present study reveal that metabolites of *T. harzianum* exhibited pronounced fungicidal activity against *A. citri*. Production of toxins belonging to the trichothecene class has been reported earlier from *T. harzianum* in 1998 by Sivasithamparam and Ghisalberti. Trichothecenes have been proven to be phytotoxic (Harris et al., 1999). These are sesquiterpenoid epoxides and represent a large family of toxic secondary metabolites produced by a variety of filamentous fungi (Ueno, 1980). Many of the toxic properties of the trichothecenes are attributed to their ability to inhibit protein and DNA synthesis (McLaughlin et al., 1977) and to induce apoptosis in eukaryotic cells (Okumwai et al., 1999).

In the second set of experiments, different fractions from the extract of *T. harzianum* were checked against selected pathogen. The extracts reduced the fungal

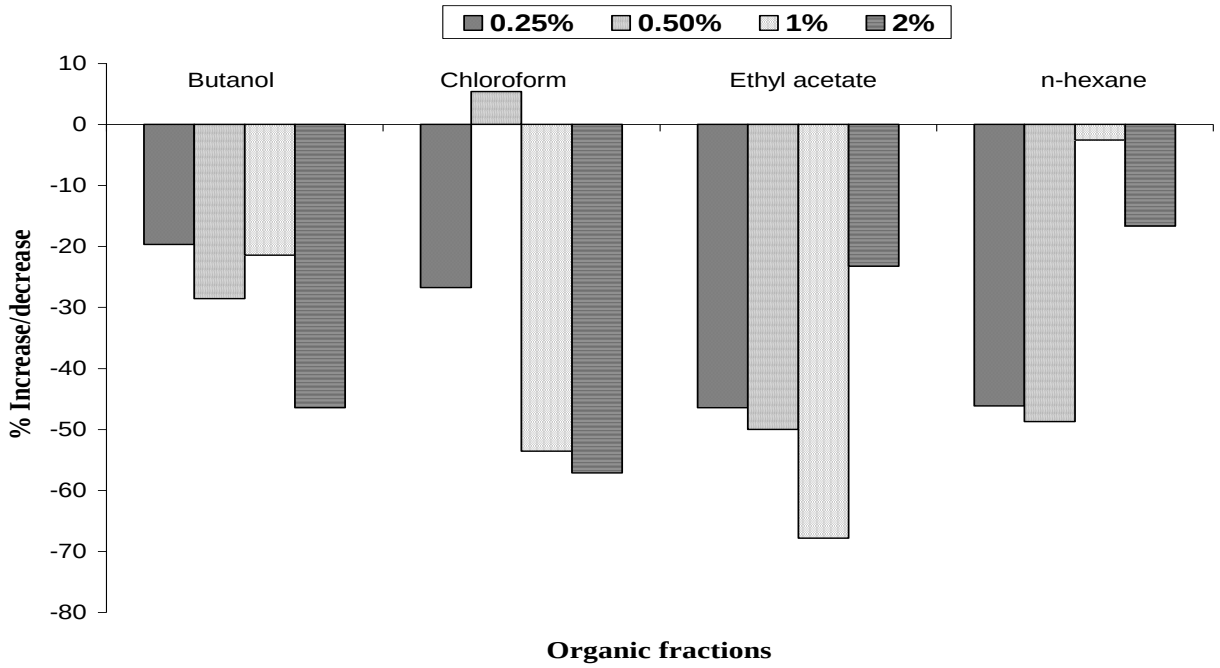

Figure 2. Effect of different concentrations of organic fractions of *Trichoderma harzianum* on percentage losses in dry biomass production of *Alternaria citri* growth.

growth, although variability among the influence of different concentrations was evident. No particular trend was observed in response to fractions of extracts filtrate. Significant results were exhibited by all the concentrations of ethyl acetate fraction which showed maximum fungistatic activity. Butanol fraction proved least toxic and did not inhibit the growth of *A. citri* statistically. This might be associated with the presence of nutritional compounds present in this extract that stimulated fungal growth and masked the inhibitory effect (Levin et al., 1988). Extract of same species in different solvents exhibit variable fungicidal activity against the target fungal species. This variation in fungicidal activity of the extracts in different solvents may be attributed to the different chemical nature of the four solvents. Water, butanol and ethyl acetate are polar, while n-hexane and chloroform are non-polar in nature. It is likely that different types of chemicals were extracted in different solvents, thus resulting in variable activity of the extracts of a species. Such fractionation guided bioassays thus can lead to the discovery of new bioactive compounds rather than a detection of a non-persuasive chemical. This investigation leads to the conclusion that ethyl acetate fraction of the selected filtrate possess most potent antifungal components of the filtrate that can be taken for further analyses and chemical identification.

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