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Assessment of fungitoxicity of phylloplane fungi against *Alternaria brassicae* causing leaf spot of mustard

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In phylloplane, varieties of fungi are presented on the leaf surface, among these fungi were selected for the assessment of their fungitoxicity against *Alternaria brassicae* causing leaf spot disease of mustard. Colony interaction showed that *Trichoderma viride and Aspergillus flavus* possesed maximum per cent growth inhibition of *A. brassicae.* When the effect of volatile and non-volatile metabolites released by phylloplane fungi were observed, it was found that *T. viride* maximum arrested the hyphal growth of the pathogen. With increasing period of incubation, inhibition of the pathogen decreased while stimulation increased. Effect of foliar spray of metabolites from the test phylloplane fungi on the lesion development was also studied. It was noted that foliar spray before two days of inoculation of the pathogen was more effective. Use of composite mycoflora showed maximum percent inhibition of the lesion development on the leaf surface of host plant.

Key Words: Phylloplane fungi, fungitoxicity, volatile and non-volatile metabolites, foliar spray

Introduction

Brassica campestris is an important Rabi crop with about 3,560 thousand hectares under cultivation and annual production of 1,475 thousand tonnes. The 80% of total world production comes from India. Uttar Pradesh tops the list in rapeseed production. Leaf spot on *B. compestris* is one of the most wide spread and destructive disease of this plant in India. In northern India, the disease appears usually in December and the attack reaches its peak point towards the end of January.The disease is caused by *Alternaria brassicae* (Berk.) Sacc. Attempt to control the disease have been carried out by different physical, chemical and biological ways. A large number of synthetic inorganic and organic fungicides have been developed to control plant diseases. However, the use of many of such fungicides has now been cautioned due to their

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carcinogenicity, teratogencitiy and other residual toxicities (Lingk, 1991 and Mani, 2002).

To overcome all these problems, the concept of biological control was put forward. Advantages of a biological approach to disease control include reduced environmental damage, reduced human health risks and improved soil conditions and agricultural sustainability (Nakkeeran *et al.,* 2002). The leaf surface is the important substrate for the growth of microorganisms as it easily provides essential nutrients required for their life and growth. Phylloplane fungi have been poorly studied as compared to endophytes, saprobes and pathogenic fungi. Most phylloplane fungal studies have been concerned with pathogens or non-parasitic fungi of crops or economically important trees (Mishra and Dickinson, 1981). Antagonistic interaction on the leaf surface of vegetable crops has got a great promising for biological control of pathogens. Antagonistic effects of saprophytic microorganisms were reported by a number of researchers (Perello *et al.,* 2006; Goswami and Islam, 2002).

The aim of the present investigation was to find out effective phylloplane microfungi and their metabolites, against *Alternaria brassicae* causing leaf blight of mustard in place of pollutive synthetic fungicides.

Material and methods

In vitro fungitoxicity of some dominant phylloplane fungi against the pathogen *A. brassicae*

Colony interaction

In vitro screening of phylloplane fungi for their antagonistic potential was done by inoculating 5 mm agar block of selected species against *A. brassicae* on PDA medium, 3 cm apart from each other. The inoculated plates were incubated at 25 ± 2^0C and the observations were made after 6 days. The assessment of the colony interaction in dual culture was done by following the method described by Upadhyay and Rai (1987). The parameter used for the assessment was percent inhibition of radial growth of the pathogens by using the formula: $100 \times (R1 - R2)/R1$ where R1 denotes the radial growth of the pathogen towards the opposite side and R2 denotes the radial growth of the pathogen towards the antagonist (Fokkema, 1976).

Effect of non- volatile metabolites of some dominant phylloplane microfungi on growth behaviour of the test pathogen

The effect of non-volatile metabolites was studied by culture filtrate method. The selected leaf surface fungi and *A. brassicae* were grown on PDA medium in Petri dishes at 25± 2^0C for 4 days. Two equal size blocks (5 mm each) of antagonistic species, cut from the actively growing margins of 4 day old cultures, were inoculated separately into 250 ml Erlenmeyer flasks each containing 100 ml sterilized Potato Dextrose broth in triplicates. After 10 days of incubation at 25± 2^0C, the static cultures were filtered firstly through Whatman filter paper number 44 and finally through Seitz filter (G 4) attached to vacuum pump to obtain cell free culture filtrates. Two, four and eight ml culture filtrates of each selected leaf surface fungi were poured into empty sterilized plates in three replicates separately which was immediately followed by pouring 18, 16 and 12 ml of autoclaved and cooled PDA medium, so as to make the final concentrations of the culture filtrates 10, 20 and 40 %, respectively. The control set was prepared by using sterilized distilled water mixed in the same ratio in the medium. Five mm agar blocks of actively growing colonies from 5 days old culture of *A. brassicae* were cut from the margin of the colony and inoculated at the center of Petri-plate separately containing PDA medium and the culture filtrate. The control set was made by pouring 20 ml PDA medium only in sterilized Petri-plates. The inoculated Petri-plates were incubated at 25± 2^0C and measurement of the radial colony growth was done after 4 days of incubation. The percent inhibition in the radial growth of the colony was calculated by the following formula:

Per cent growth inhibition = (C-T) / C×100, where C = Growth in control and T= Growth in treatment

Effect of volatile substances of some dominant phylloplane microfungi on growth behaviour of the test pathogen

The leaf surface fungi selected in earlier experiment were used in the present study. The method described by Dennis and Webster (1971) was followed to study *in vitro* effect of volatile metabolites of the leaf surface fungi on the test pathogen. Petri dishes of 9 cm diameter containing 20 ml of pda medium were inoculated centrally with 5mm disk of each fungus separately in five replicates and wrapped in polythene bags. The petri dishes were incubated at 25±2^0C for a week, after which the same size bottom plate containing 20ml PDA medium pre-inoculated with 5 mm agar block of the test pathogen replaced the lid of each dish. Both the dishes were taped together by cello tape

and re-incubated at 25±2^{0}C. The lids of uninoculated dishes of control were also replaced in the same way, which served as control. The colony diameter of the test pathogen was measured after 3, 4, 5 and 6 days of incubation. The percent inhibition of colony diameter was calculated.

In vivo fungitoxicity of selected dominant microfungi against the pathogen, *A. brassicae*

Interactions on leaf surface

The same fungal species were selected which taken from earlier experiments. The effect of non-volatile metabolites of the selected leaf surface fungi on the development of lesion size caused by the pathogen was studied. Experiments were performed after 45 days of the establishment of the plant.

Effect of metabolites of the selected leaf surface fungi on the disease development of pathogen was studied by preparing culture filtrates of the leaf surface fungi by the method described earlier. Each plant was sprayed with 5 ml of each metabolite using a sterile atomizer, 2 days before or after inoculation with the pathogenic fungus (1x10^3 spores/ml). The same quantity of sterile distilled water as well as liquid potato dextrose was sprayed for control. In all these experiments, the treated leaves were covered with wetted polythene bags in order to maintain high humidity. Old leaves were avoided because they tend to become moribund during the experiment. Size of lesions developed on treated leaves of the host plant was measured after 7 days and compared with the controls.

Percent inhibition of lesions development was calculated by the following formula:

$$\text{Percent inhibition} = \frac{\text{Lesion size on leaves inoculated with pathogen} - \text{Lesion size on leaves treated with fungal metabolites}}{\text{Lesion size on leaves inoculated with pathogen}}$$

Necrotic areas smaller than 2mm diameter, formed due to hypersensitive reaction around the wound, were not counted as successful infection.

Results

Result of colony interaction showed that out of the 9 selected phylloplane fungi, *Alternaria alternata*, *Aspergillus flavus, Aspergillus terreus,*

Cladosporum cladosporioides, Cladosporum herbarum, Curvularia pallescens, Fusarium sp., *Penicillium citrinum and Trichoderma viride*, maximum inhibition of the pathogen was recorded with *T. viride*, and followed by *A. flavus* (upto 70%). Other antagonists also inhibited the growth of pathogen at varied degree. *C. pallescens* showed the least inhibition against the pathogen (Table 1).

Table 1. Screening of phylloplane fungi against *A. brassicae* on agar plates by dual culture technique

Test fungi	Interaction score* for *A. brassicae*	Percent inhibition of radial growth
Alternaria alternata	2	45
Aspergillus flavus	1	70
Aspergillus terreus	5	58
Cladosporium cladosporioides	2	54
Cladosporium herbarum	4	49
Curvularia pallescens	2	35
Fusarium sp.	4	42
Penicillium citrinum	4	40
Trichoderma viride	3	74

*= Scores are according to Skidmore and Dickinson (1976)

It is evident from the results that the growth of the test pathogen was suppressed with volatile and non-volatile metabolites emanated from different leaf surface fungi. Metabolites of *T. viride* is more pronounced effect on the growth of the pathogen and followed by *A. flavus* in comparison to other test antagonists (Fig 1 and 2).

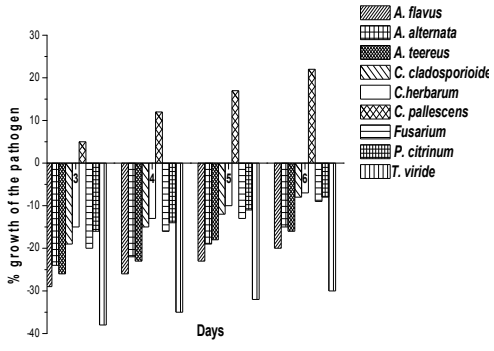

Fig 1. Effect of Volatile substances of some dominant phylloplane micro fungi on growth behaviour of the test pathogen after 3, 4, 5 and 6 days of incubation

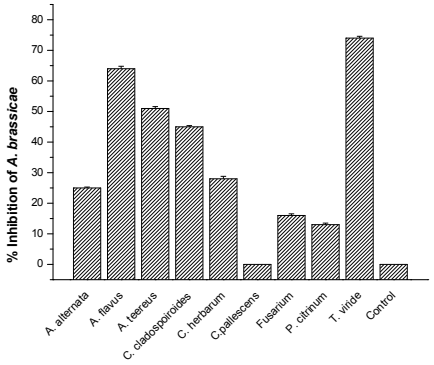

Fig 2. Effect of Non-Volatile substances of some dominant phylloplane micro fungi on percent inhibition of test pathogen

It is clearly shown that the spores of all the test microorganisms in each concentration, either collectively or individually, reduced the development of lesion caused by the pathogen on leaves. There was significantly increased in inhibition of lesion development with increasing concentrations of spores of individual test saprophyte. Maximum inhibition was caused by composite mycoflora, and followed by *T. viride*, *A. flavus, A. terreus, C. cladosporioides* and *A. alternata* while *C. pallescens* was found less effective (Fig 3). Significant reduction in inhibition of lesion development was recorded with spray of spore suspension 2 days before the pathogen inoculation.

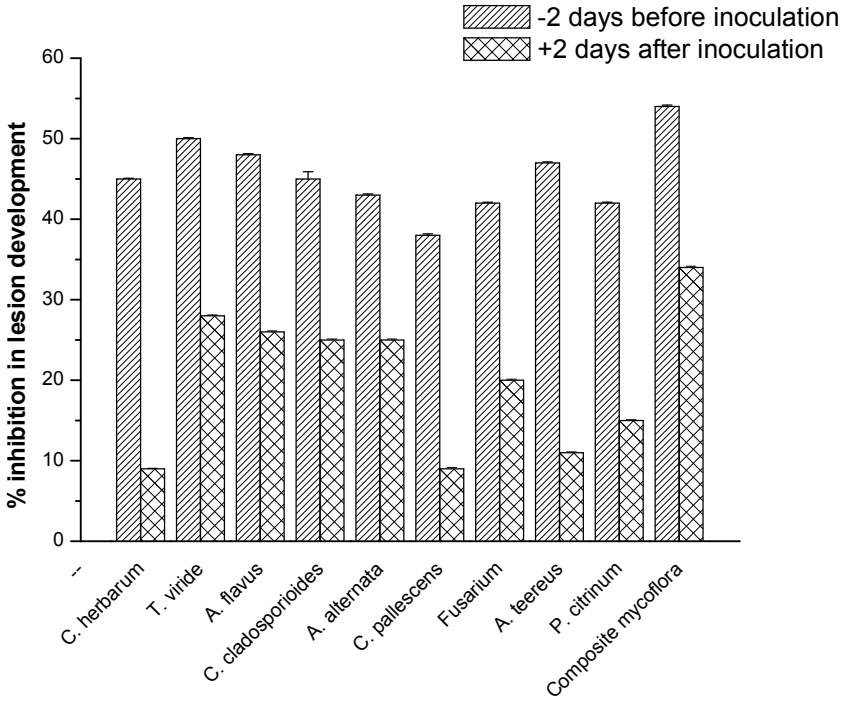

Fig 3. Effect of metabolites of some phylloplane fungi sprayed on leaves 2 day before (-) or 2 day after (+) inoculation with *A. brassicae* (spore concentration / ml 1×10^3) on percent inhibition of the lesions development on mustard leaves.

Discussion

The surface of aerial plant parts provides a habitat for epiphytic microorganisms, many of which are capable of influencing the growth of

pathogens. These saprophytic organisms play an important part in reducing incidence of foliar disease of crops in the field (Evueh and Ogbebor, 2008).

Screening is a critical step in the development of biocontrol agents. The success of all subsequent stages depends on the ability of a screening procedure to identify an appropriate candidate (Macsppeden Gardener and Fravel, 2002). Dual culture screening is exclusively related to interaction studies and potential antagonists are typically ranked according to their ability to inhibit the growth of the pathogen expressed by an inhibition zone (Skidmore and Dkinson, 1976).

From the result of *in vitro* experiment, it was evident that nine different phylloplane fungi were screened against *A. brassicae*, possessed varied degree of inhibition (Table 1). Amongst nine species *T. viride* and *A. flavus* were seems to be strong antagonist against the pathogen. Antagonism of *Trichoderma* species against several pathogens has been reported (Elad, 2000; Howell, 2002; Eziashi *et al.,* 2006, Rajendiran *et al.,* 2010). The interaction of the antagonists and the pathogen and occurrence of inhibition zone on agar media could be commonly considered as a result of the production of antibiotics, cell wall degrading enzymes and competition for nutrients and space (Ghisalberti and Sivaithamparam, 1991; Faull *et al.,* 1994; Etabarian *et al.,* 2000). Doi and Mori (1994) showed that volatiles from *trichoderma* species were able to arrest the hyphal growth of different fungal pathogens on agar plate. These authors reported that *T. viride* produced volatiles which had potential to inhibit the hyphal growth of *Lentinus lepidus* and *Corious versicolor*. Antifungal metabolite from *Trichoderma virens* were also used for controlling leaf spots of Chinese-kale caused by *Alternaria brassicicola* (Intana *et al.,* 2005).

It is obvious form the result that high concentration of metabolites of all the test fungi reduced the redial growth of the test pathogen. Variation in extent of effect was noticed at different days of incubation at different concentrations of metabolites. The metabolites of *T. viride*, *A. flavus, A. terreus, A. alternata* and *C. cladosporioides* were noted to be the most effective against *A. brassicae* as more than 40% growth of the pathogen was inhibited by all these fungi. Maximum inhibition of *A. brassicae* was recorded with metabolites of *T. viride and A. flavus*. The inhibition increased with the prolongation of incubation period in most of the cases except *C. pallescens*. It was also observed that with increasing incubation period, the percent inhibition of mycelial growth decreased whereas percent stimulation increased. Variation in the degree of stimulation or inhibition of pathogenic fungus may be attributed to the concentration of volatile metabolites and the specific sensitivity of the responding fungus. The decreasing inhibition and increasing stimulation of growth might be explained in the view of above fact with laps of time, the

decreasing inhibition of growth of the pathogen may be attributed to the depletion of volatile inhibitory substances moving to evaporation at faster rate and increasing stimulation may be the result of additive effect of volatile metabolite at slow rate. In the present study *T. viride* came out as strong antagonist against the test pathogen *A. brassicae* followed by *A. flavus* which is in accordance with the reports of several earlier workers (Perello *et al.,* 2003, 2006). The dominance of these genera may be accounted for by the abundance of their spores in the atmosphere.

Significant reduction in lesion development was recorded with the metabolites of the test phylloplane microorganisms on leaves. Time of spray of filtrates of the pathogen also affected the development of lesion. The most pronounced effect was observed when metabolites of all the test fungi were mixed together followed by *A. flavus, A. terreus, A. alternata, C. cladosporioides, C. herbarum* and *fusrium* sp. The maximum inhibition of *A. brassicae* by composite mycoflora *in vivo* may be due to cumulative effect inhibitory substances produced by test saprophytes. Better protection from disease was achieved when the metabolites were sprayed 2 days before the inoculation of pathogen. Significant increase in inhibition of lesion development with increasing concentrations of spores of individual test saprophyte is in accordance with the reports of several other workers (Pandey, 1990; Pal and Sharma, 1992).

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