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Screening of chilli microflora and other biocontrol agents for their antagonistic effects on *Colletotrichum* spp. infecting chillies

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A B S T R A C T

Forty-five leaf and nine fruit samples of chilli were collected from different regions of Karnataka for isolation of chilli microflora. From these samples, 94 fungal and 89 bacterial isolates were derived, which included 50 isolates of fungi and 44 isolates of bacteria from phylloplane and pomoplane, 44 isolates of endophytic fungi and 45 of endophytic bacteria from inside the leaf and fruit tissues, respectively. Among them, 70 fungal isolates belonged to plant pathogenic genera like, *Alternaria, Cercospora, Colletotrichum, Curvularia, Glomerella, Mycosphaerella, Phoma* and *Stemphylium* and the other 24 isolates of fungi belonged to *Aspergillus, Acremonium, Chaetomella, Cunninghamella, Geotrichum, Gliocladium, Monodictys, Mucor, Myrothecium, Penicillium, Periconia* and *Pithomyces.* One-hundred and thirteen isolates of chilli microflora and 49 isolates of *Trichoderma*, 19 isolates of *Bacillus* sp., 34 isolates of *Pseudomonas fluorescens* and 29 isolates of yeasts from NBAII germplasm collection of biocontrol agents were tested for their antagonistic effect on *Colletotrichum gloeosporioides* and *C. capsici* by dual culture test. Among the isolates of chilli microflora tested, *Aspergillus flavus* showed 70.2% inhibition of *C. gloeosporioides* and 54.9% inhibition of *C. capsici.* Among the isolates of *Trichoderma* sp. tested, *T. virens* (Tvs-KSD isolate) and *T. pseudokoningii* (Tpk-1) showed highest percent inhibition of *C. gloeosporioides* (69.7%) and *T. viride* (Tv-5 isolate) showed highest percent inhibition of *C. capsici* (51.9%). Among the bacterial isolates tested, *Bacillus* S-15 showed highest percent inhibition of *C. gloeosporioides* (30%) and S-9 isolates showed highest percent inhibition of *C. capsici* (51.3%).

Introduction

Chilli (*Capsicum annuum* L) is one of the major spice crops grown in India. Among the various diseases of chilli, anthracnose, ripe fruit rot and dieback of chilli caused by *Colletotrichum capsici* and *C. gloeosporioides* are the most devastating in several chilli growing areas of the country (Than *et al*. 2008). These diseases cause severe damage to fruits both in field and storage. Heavily infected fruits may lose their normal red colour and turn straw /pale white. The pathogen also causes necrosis of tender twigs and the entire branch (Mesta *et al*. 2007). The disease is very severe in humid weather and spreads rapidly causing extensive losses (Pandey & Pandey, 2006). Although, fungicides like carbendazim, mancozeb and captan are known to be effective against this

disease (Arasumallaiah & Rangaswamy, 2008) continuous and extensive use of these chemical fungicides may be uneconomical, may lead to residue problems and also cause elimination of beneficial microflora. Studies conducted earlier indicated very little resistance in chilli germplasm against the diverse population of the pathogen. Hence, in order to minimize the fungicidal application, biological control strategies are to be developed. The antagonistic organisms offer great potential for effective management of diseases of vegetable crops without any adverse effect on the environment. Although there are several reports on the biological control of the diseases caused by *Colletotrichum* sp. in chillies (Hegde & Kulkarni 2001; Tamilvanan *et al.* 2006) efficient strains of antagonists have not been identified for management of these pathogens so far. This communication aims at isolation of natural microflora from chilli leaves and fruits, screening them for the antagonistic effects against *C. capsici* and *C. gloeosporioides* along with other antagonistic organisms to identify promising isolates for further field experiments.

Materials and Methods

Isolation of phylloplane and pomoplane fungi and bacteria from chilli

Forty-five chilli leaf samples of all ages and nine fruit samples (both green and ripe) from twenty-six varieties from different villages in Bangalore Rural, Chikkaballapur and Kolar districts of Karnataka were collected during July-September, 2009 for isolation of exophytic and endophytic fungi and bacteria. Isolation of phylloplane/pomoplane fungi and bacteria from chilli was carried out by plating leaf/fruit washings on Potato Dextrose Agar (PDA) and Nutrient Agar (NA) media, respectively using the procedure described by Ramanujam (2008). One gram leaves from each sample were cut into discs of 6-mm diameter, transferred to 100-ml sterile water blank and stirred for 20 min using magnetic stirrer. From these washings, dilutions of $10^{-3,}$ 10^{-4} and 10^{5} were prepared and one ml aliquots of these dilutions were plated on PDA and NA. The plates were incubated for 5 days at 25°C in BOD. The fungal/ bacterial colonies obtained on the Petriplates were purified and maintained on PDA/NA slants in a refrigerator. In case of isolation of pomoplane microflora, 10gm of fruits from each sample were used and the isolations were carried out as described above.

Isolation of endophytic fungi from chilli leaves and fruits

Endophytic fungi from chilli leaves/fruits were isolated according to the procedure described by Kunihiko *et al.* (2002). Samples of leaves/fruits were dipped in 70% ethanol for one min to wet the surface, surface sterilized for 15 min. in a solution of 15% H_2O_2, dipped again for one min in 70% ethanol and then rinsed in sterile distilled water. From the surface sterilized leaves/fruits, segments of 2mm x 2mm were aseptically cut with a sterile scalpel and placed on 2% malt extract agar medium. The plates were incubated for 6 days at 25°C in BOD. The fungi growing out of the leaf/fruit segments were purified and recorded as endophytic fungi.

Isolation of endophytic bacteria from chilli leaves and fruits

Endophytic bacteria from chilli leaves/fruits

were isolated according to the procedure described by Mclnroy & Kloepper (1995). One gram of leaf/fruit sample was surface sterilized with 20% H_2O_2 and washed with 0.02M potassium phosphate buffer (pH 7) four times. Then the sample was macerated in 9 ml potassium phosphate buffer (0.02M, pH 7) and diluted to 10^{-2},10^{-3},10^{-4} and 10^{-5} dilutions. One ml of aliquots of each of these dilutions were plated on Tryptic soya agar (TSA) medium. The plates were incubated for 4 days at 25°C in BOD. The bacterial colonies obtained thereby on the Petri plates were purified and maintained on TSA slants in a refrigerator.

Screening of phylloplane and endophytic micrflora of chillies against Colletotrichum gloeosporioides and C. capsici by dual culture test

Antagonistic effect of 24 isolates of phylloplane, pomoplane and endophytic fungi of chillies and 89 isolates of phylloplane, pomoplane and endophytic bacteria were tested on *C. gloeosporioides* (Cg-1 isolate) and *C. capsici* (Cc-1 isolate) by dual culture test on PDA (Webber & Hedger 1986). After six/eight days of incubation, radial growths of C. *gloeosporioides/C. capsici* were measured towards the test isolate side and compared with the growths in the control plates. The measurements were used to calculate the percentage of inhibition of *C. gloeosporioides /C. capsici*.

Screening of other antagonistic organisms against C. gloeosporioides and C. capsici by dual culture test

Antagonistic effect of 49 isolates of *Trichoderma* spp. belonging to *T. viride* (18 isolates), *T. harzianum* (20 isolates), *T. virens* (7 isolates), *T. pseudokoningii* (2 isolates), *T. koningii* (1 isolate) and *T. hamatum* (1 isolate) from PDBC culture collection was tested on *C. gloeosporioides* (Cg-1) and *C. capsici* (Cc-1) by dual culture test as describes above. Similarly, 19 isolates of *Bacillus* spp. 34 isolates of *Pseudomonas fluorescens* and 29 isolates of yeasts from PDBC culture collection was tested on *C. gloeosporioides* and *C. capsici*.

The experiments were laid out maintaining three replications for each treatment. Data were statistically analysed using ANOVA.

Results and Discussion

Isolation of phylloplane and pomoplane fungi and bacteria from chillies

From 45 leaf samples and 9 fruit samples, 50 isolates of phylloplane fungi and 44 isolates of endophytic fungi were isolated. Of the 94 isolates of fungi isolated from chillies, 70 isolates have been identified to belong to plant pathogenic genera like *Alternaria, Cercospora, Colletotrichum, Curvularia, Glomerella, Mycosphaerella, Phoma* and *Stemphylium*. The other 24 isolates belong to saprophytic fungi like *Aspergillus flavus, Aspergillus oryzae, Acremonium implicatum, Chaetomella raphigera, Cunninghamella echinulata, Fusarium pallidoroseum Geotrichum candidum, Gliocladium catenulatum, Monodictys castanae, Mucor hiemalis, Myrothecium cinctum, Myrothecium verrucaria, Penicillium citrinum, Periconia lateralis, Periconia byssoides* and *Pithomyces*

flavus. Six saprophytic fungi are yet to be identified. The results indicated that majority (75%) of the phylloplane and pomoplane fungi isolated from chillies are pathogenic in nature and only 25% of these fungi are of saprophytic nature. Among the fungi isolated from chilli, *Alternaria* spp. were predominant. *Aspergillus flavus* was the most predominant among saprophytic fungi on the phylloplane and pomoplane of chillies. Forty-four isolates of phylloplane/pomoplane bacteria and 45 isolates of endophytic bacteria from leaf/fruit tissues of chillies were isolated from 45 leaf samples and 9 fruit samples. The identities of the 89 isolates of bacteria are yet to be established.

In vitro screening of phylloplane and endophytic microflora of chillies against C. gloeosporioides and C. capsici.

In the dual culture test, 17 isolates of phylloplane saprophytic fungi, 5 isolates of pomoplane saprophytic fungi and 2 isolates of endophytic fungi from leaves were tested against *C. gloeosporioides* and *C. capsici* by dual culture test. Among these 24 isolates of fungi tested, *A. flavus* showed 70.2% inhibition of *C.gloeosporioides* and 54.9% inhibition of *C. capsici. P.citrinum* showed 61.6% inhibition of *C.gloeosporioides* and 47.9% inhibition of *C. capsici* (Table 2). An unidentified phylloplane fungus (EXF-48) showed 50% inhibition of *C. gloeosporioides* and 59.9% inhibition of *C. capsici*. Another phylloplane fungus, *Acremonium implicatum* showed 44.1% inhibition of *C. gloeosporioides* and 59.9% inhibition of *C. capsici*. The other fungi showed low level of inhibition of *C. gloeosporioides* (0.8-37.8%) and inhibition of *C. capsici (*8.4-55.4%). The two endophytic fungi tested showed much less inhibition of *C. gloeosporioides* and *C.capsici.*

Table 1.
Isolation of exophytic and endophytic fungal and bacterial isolates from chilli leaves and fruits

Sample	No. Samples	Fungi			Bacteria		
		Exophytic	Endophytic	Total	Exophytic	Endophytic	Total
Leaves	45	24	26	50	29	29	58
Fruit	9	26	18	44	15	16	31
Total	54	50	44	94	44	45	89

Table 2.
Inhibition of *Colletotrichum gloeosporioides* and *C. capsici* by phylloplane/ pomoplane/ endophytic fungi

Sl. No	Phylloplane/pomplane fungal isolate	% inhibition of *C. gloeosporioides*	% inhibition of *C. capsici*
1	*Aspergillus flavus* . (EXF14)*	70.2	54.9
2	*Penicillum citrinum* (EXF6)*	61.7	47.9
3	Unidentified (EXF48)*	50.0	59.9
4	*Acremonium implicatum* (EXF1)*	44.1	47.4
5	Unidentified (EXF39)*	37.8	55.4
6	*Periconia lateralis* (EXF2)**	34.3	48.0
7	Unidentified (EXF49) *	24.4	41.9
8	Unidentified (EXF50) *	22.3	53.1
	S.EM	0.2	0.2
	CD at 5%	0.7	0.5

*= Phylloplane fungi; ** = Pomoplane fungi

Eighty-nine isolates of phylloplane/pomoplane /endophytic bacteria showed 0.6-33.3% inhibition of *C. gloeosporioides* and 2.0-46.0% inhibition of *C. capsici* (Table-3). Among these isolates, ENB-29, ENB-30, ENB-25, EXB-16 and EXB-24 isolates showed higher percent inhibition of *C. gloeosporioides* while other

isolates like, ENB-24, ENB-30, ENB-45, EXB-22 and EXB-44 showed higher percent inhibition *C. capsici* (Table 3).

Table 3.
Inhibition of *Colletotrichum. gloeosporioides* and *C. capsici* by phylloplane/pomoplane /endophytic bacteria

Sl. No.	Bacterial isolate	% inhibition of *C. gloeosporioides*	% inhibition of *C. capsici*
1	ENB29***	33.3	23.0
2	ENB30***	31.1	45.2
3	ENB25***	29.7	28.6
4	EXB16*	28.2	25.2
5	ENB45****	22.1	33.3
6	ENB24***	11.32	46.0
7	EXB22*	15.7	32.0
8	EXB44**	6.7	30.8
	S.Em ±	0.2	0.2
	CD (P=0.05)	0.5	0.6

* = Phylloplane bacteria; ** = Pomoplane bacteria; *** = Endophytic bacteria from leaves; **** = Endophytic bacteria from fruits

Among the chilli microflora isolates tested against *C. gloeosporioides* and *C. capsici*, fungal isolates were found to be more antagonistic than bacterial isolates as indicated by the per cent inhibition. Although *Aspergillus flavus* (EXF14) and *Penicillum citrinum* (EXF6) showed higher inhibition of *Colletrichum* spp., their practical usage as biocontrol agents is limited because these genera are known to show allergic reactions to human beings and other animals (Peraica et al. 1999). Hence, the other promising fungal isolates may further be tested in field trials.

Other fungal isolates like *Gliocdium catenulatum, Pithomyces flavus, Cunninghamella echinulata, Mucor hiemalis, Aspergillus oryzae, Geotrichum candidum, Monodictys castanae, Myrothecium verrucaria, Helicostylum fresenii, Fusarium pallidoroseum, Periconia byssoides, Myrothecium cinctum, Chaetomella raphigera, Acrodictys fimicola*, Unidentified (EXF7 and EXF25) showed 0.8 to 36.0 % inhibition of *C. gloeosporioides and 8.4 to 46.5 %* inhibition of *C. capsici.*

Other bacterial isolates (ENB14, 15, 23, 26,27, 28,31,39,40, 41,42) showed 4.2 to 27.5% inhibition of *C. gloeosporioides and 30.6 to 41.3 %* inhibition of *C. capsici*

Screening of other antagonistic organisms against C. gloeosporioides and C. capsici by dual culture test

Among the 49 isolates of *Trichoderma* spp. tested, *T. virens* (Tvs-ksd) isolate and *T. pseudokoningii* (Tpk-1) showed highest percent inhibition (69.7%) of *C. gloeosporioides* followed by *T. viride* isolates (Tv-NGP, Tv-11, Tv-4. Tv-23) which showed 62.7-66.2% inhibition (Table 4). Among *T. harzianum* isolates, Th-4 isolate showed 60.4% inhibition of *C. gloeosporioides*. With regard to percent inhibition of *C. capsici*, *T.viride* (Tv-5 isolate) showed highest percent inhibition (51.9%) followed by *T. viride* (Tv-4 isolate), which showed 50.4% inhibition. *T. viride* and *T. hamatum* isolates have been found to be inhibitory to *C. capsici in vitro* (Pathania *et al.* 2004; Chirame and Padule 2005; Mandeep *et al*. 2006; Priya *et al.* 2008). The present studies indicate the inhibitory effect of *T. pseudokoningii* and *T. virens* on *C. gloeosporioides* and *T. viride* isolates (Tv-5, Tv-4) on *C. capsici*.

Table 4.
Inhibition of *C. gloeosporioides* and *C. capsici* by different isolates of *Trichoderma* sp.

Sl. No	*Trichoderma* isolate	% inhibition of *C. gloeosporioides*	% inhibition of *C. capsici*
1	*T. viride* -Tv-NGP	66.1	27.8
2	Tv-11	65.4	35.3
3	Tv-4	63.8	50.4
4	Tv-23	62.7	30.8
5	Tv-31	56.1	26.3
6	Tv-5	55.1	51.8
7	Tv-CBE	52.4	26.3
8	Tv-18	50.5	36.8
9	*T. harzianum* -Th-4	60.4	29.3
10	Th-21	56.7	30.8
11	Th-3	56.1	39.1
12	Th-8	56.1	23.3
13	Th-7	55.3	29.3
14	Th-v2	50.0	19.5
15	Th-NGP	50.0	28.6
16	*T. virens* Tvs-KSD	69.7	25.6
17	Tvs-12	58.0	45.1
18	*T. pseudokoningii* Tpk-1	69.7	29.3
19	*T. koningii* Tk-1	52.1	30.1
20	*T. hamatum* 138	51.1	24.0
	S.Em $\pm$	6.0	0.1
	CD (P=0.05)	17.0	0.4

Among 53 isolates of bacteria tested, *Bacillus* S-15 showed highest percent inhibition of *C. gloeosporioides* (30%) and S-9 isolates showed highest percent inhibition of *C. capsici* (51.3%). Among *Pseudomonas* isolates, PBA6(2) isolates showed 25.6% inhibition of *C. gloeosporioides* and *PBA-5 and PBA-14(1)* isolates showed 41.3% inhibition of *C. capsici* . Among 29 isolates of yeasts tested, GRWY4 isolate showed highest percent inhibition of *C. gloeosporioides* (39.6%) and of *C. capsici* (35.8%) .

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