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RESEARCH ARTICLE

Management of chilli (*Capsicum annuum*) anthracnose using fungicides and biocontrol agents

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ABSTRACT: An antagonistic *Burkholderia* sp. strain TNAU-1 and the fungicide, Cabrio Top were evaluated for their efficacy in suppressing mycelial growth of chilli anthracnose pathogen isolates of *Colletotrichum capsici*, *C. gloeosporioides* and *Alternaria alternata in vitro*. Antagonistic bacterium inhibited the growth of all the aforementioned pathogenic isolates in dual culture on PDA medium and the inhibition zone ranged from 10.7 mm (CBE1) to 16.0 mm (TEN10). The test fungicide Cabrio Top was highly effective in suppressing the radial growth of all the test fungi with minimal inhibitory concentration at 250 ppm. In the field trial, Cabrio Top at @ 1750 g/ ha was found to be most effective in controlling anthracnose and increasing yield. Control plots recorded the lowest yield of 2132 kg/ha and 2507 kg/ha in Trial I and Trial II, respectively. Cabrio Top @1750g/ha recorded the highest yield of 3091kg/ha and 3304 kg/ha in Trial-I and Trial-II, respectively. Foliar application of powder formulation of *Burkholderia* sp. strain TNAU-1 showed a comparable level of disease control and increased the yield to that of foliar application with Cabrio Top under field conditions.

Key words: *Burkholderia*, cabrio top, *Colletotrichum*, control

Chilli is the fourth most important vegetable crop in the world and first in Asia, with world production approximately 31.17 million tonnes of fresh chilli and 3.3 million tonnes of dry chilli in 2014 (FAO STAT, 2014). Several fungal, bacterial and viral diseases are reported to affect chilli (Nakkeeran *et al*., 2006). Anthracnose disease in chilli is one of the most important limiting factors of chilli production worldwide, especially in tropical and sub-tropical regions which causes both qualitative and quantitative yield loss (Than *et al*., 2008). Anthracnose causes yield loss up to 50% in India (Sharma *et al*., 2005). Cultural methods, biological control, application of chemical fungicides and use of resistant cultivars are amongst the effective disease control measures that have been employed to control chilli anthracnose (Than *et al*., 2008). Although the management of anthracnose disease is still being extensively researched, commercial cultivars of *C. annuum* that are resistant to anthracnose have not yet been developed. Use of fungicides appears to be the most practical measure for management of anthracnose disease. However, fungicide resistance often develops quickly, upon consistent usage of a single compound. The fungicide recommended for management of chilli anthracnose is manganese ethylenebisdithiocarbamate (Maneb), although it does not consistently control the severe form of the disease (Smith, 2000). The strobilurin fungicides azoxystrobin (Quadris), trifloxystrobin (Flint), and pyraclostrobin (Cabrio) have recently been recommended for the control of anthracnose of chilli, but only preliminary reports are available on efficacy of these fungicides against severe form of the disease. Hence, the present investigation was carried out to assess the effect and efficacy of bio control agents and fungicides against chilli anthracnose.

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MATERIALS AND METHODS

Fungal isolates and biocontrol agents

Fungal isolates *viz*., *Colletotrichum capsici*, *C. gloeosporioides* and *A. alternata* and bio-control agent *Burkholderia* sp. strain TNAU-1 were collected from culture collection centre of the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore.

In vitro screening of *Burkholderia* sp. strain TNAU-1 against *C. capsici*, *C. gloeosporioides* and *A. alternata*

In vitro inhibition of mycelial growth of *C. capsici*, *C. gloeosporioides* and *A. alternata* was assayed by dual culture technique. An actively growing mycelia plug of test fungi was placed in one side of the Petri plate containing PDA medium and allowed to grow for 24 hrs and then antagonist *Burkholderia* sp. strain TNAU-1 was streaked on Petri plate in a line at opposite side perpendicular to the pathogen. PDA medium inoculated with the fungus alone served as control. The plates were incubated at room temperature (28±2°C) for seven days

and the inhibition zone (mm) was recorded by measuring distance between edges of fungal mycelium and the antagonistic bacterium. Three replications were carried out for each treatment.

Development of talc-based powder formulation of *Burkholderia* sp. strain TNAU-1

Talc-based powder formulation of *Burkholderia* sp. strain TNAU-1 was developed as described by Vidhyasekaran and Muthamilan (1995). All the preparations were carried out under aseptic conditions i.e., the formulation was prepared in the pre-fumigated room and then floor of the room also wiped with disinfectant. Ten gram of carboxy methyl cellulose was mixed with 1 kg of talc (hydrated magnesium silicate) and the pH was adjusted to 7.0 by adding calcium carbonate. The mixture was then autoclaved for 30 min on each of two consecutive days. *Burkholderia* sp. strain TNAU-1 was grown in potato dextrose broth for 48h at 28±2°C on a rotary shaker. Four hundred milliliters of bacterial suspension containing 9×10^8 CFU ml^{-1} was added to 1 kg of the sterilized talc mixture and thoroughly mixed. The formulation was packed with 35% moisture content in polythene bags, sealed and stored at room temperature (28±2°C). The survival of *Burkholderia* sp. strain TNAU-1 in the prepared formulation was evaluated at different time intervals by dilution plating.

In vitro* evaluation of fungicides against *Colletotrichum capsici*, *C. gloeosporioides* and *A. alternata

A new fungicide molecule, Cabrio Top [Metiram (ethylene bis dithiocarmate) + Pyraclostrobin (Strobilurin); BASF-Pvt.Ltd.] was evaluated *in vitro* for its fungitoxicity against mycelial growth of *C. capsici*, *C. gloeosporioides* and *A. alternata* by poisoned food technique. The fungicide Mancozeb was used for comparison. The test fungicides were suspended in sterile distilled water and added to PDA medium to a final concentration of 5, 10, 50, 100 and 250 ppm. A 8-mm dia mycelial disc of each pathogen was cut from 7-day old culture and placed in the centre of Petri plates containing PDA medium amended with fungicides. PDA medium inoculated with each isolates served as control. The plates were incubated at room temperature for 10 days until the control plate was completely covered by test fungi. Each treatment was replicated thrice. The per cent inhibition of growth of test pathogens was calculated by using the formula:

$$I = \frac{C - T}{C} \times 100$$

Where, I= Per cent inhibition, C= Growth in control, T= Growth in treatment

Field trials

Two field trials were conducted simultaneously at Vellakaratur and Puthuvelamangalam villages of Salem district in Tamil Nadu where epidemics of chilli anthracnose occur annually, to determine the efficacy of powder formulation of *Burkholderia* sp. strain TNAU-1 and fungicides in controlling chilli anthracnose. Bio-control agent *P. fluorescens* Pf1 and fungicides Cabrio Top, Metiram, Pyraclostrobin, Mancozeb and Chlorothalonil were included in the treatment for comparison. The first foliar spray were given immediately after the first appearance of disease symptoms followed by two sprayings at 15 days interval. The susceptible cultivar, Roshini (Sygenta Pvt. Ltd) was used in the field trials. The trials were laid out in a randomized block design (RBD) with eleven treatments and three replications with a plot size of 20 m^2 and with a spacing of 90 × 60 cm. The recommended package of practices were followed to cultivate the chilli crop. The severity of anthracnose disease was recorded at fruiting stage of the crop on 10 plants (10 fruits/plant) selected at random in each replication of the treatment following the 0-9 scale, where 0 indicates no infection and 9 indicates infection greater than 25% (Montri *et al*., 2009) as described earlier. Percent Disease Index (PDI) of chilli anthracnose was calculated by using the formula:

$$\text{PDI} = \frac{\text{Sum of numerical ratings}}{\text{Total number of fruit observed}} x \frac{100}{\text{Maximum category value}}$$

Fruit yield and plant height were also recorded.

Testing endophytic colonization of *Burkholderia* sp. strain TNAU-1

In order to detect endophytic colonization of *Burkholderia* sp. strain TNAU-1 in chilli plants, the plants sprayed with powder formulation of *Burkholderia* sp. strain TNAU-1 were uprooted 15 days after spraying and *Burkholderia* sp. strain TNAU-1. The endophytic colonization of *Burkholderia* sp. strain TNAU-1 in chillies after foliar application was studied by analyzing the chilli plants from treatments following the method of Rajendran and Samiyappan (2008). Whole plants from both treatment and control were manually uprooted and brought to laboratory. Root, stem, fruit and leaf sections (2-3 cm long) were made using a sterile scalpel. Plant samples were first weighed and surface sterilized with 1% sodium hypochlorite (NaOCl) in 0.05% tritonX 100 for 10 min and rinsed four times in 0.02 M sterile potassium phosphate buffer, pH 7.0. An aliquot (0.1 ml) was taken from the final buffer wash and transferred to 9.9 ml potato dextrose broth to serve as sterility check. Samples were discarded if growth was detected in the sterility check samples within 48 h. Each sample (0.5g) was triturated with a sterile mortar and pestle in 9.5 ml of the final buffer wash. Serial dilutions up to 10^8 of the triturate were made in 0.02 M sterile potassium phosphate buffer, pH 7.0.

Each dilution of every sample (0.1 ml) was placed on Petri plate containing PDA medium. The plates were incubated at 28 ± 2°C for 48 h. After incubation, the individual bacterial colonies developed in the plates were confirmed by colony PCR for the presence of *Burkholderia* sp. strain TNAU-1 using specific primers (forward primer 5'-CGAACGGGTGAGTAATAC-3' and reverse primer 5'- GCTGGCACGTAGTTAGC-3') (Vijayasamundeeswari *et al*., 2010).

Table 1. *In vitro* antagonistic activity of *Burkholderia* sp. strain TNAU-1 against *Colletotrichum capsici*, *C. gloeosporioides,* and *Alternaria alternata*

Isolate	Inhibition Zone (mm)
C. gloeosporioides isolate TEN1	11.0^{ef}
C. gloeosporioides isolate TEN2	14.0^{c}
C. capsici isolate TEN3	15.0^{b}
C. capsici isolate TEN4	11.0^{ef}
C. capsici isolate TEN5	12.1^{d}
C. capsici isolate TEN6	11.5^{de}
C. capsici isolate TEN7	11.3^{ef}
C. capsici isolate TEN8	11.0^{ef}
C. capsici isolate TEN9	13.3^{c}
C. capsici isolate TEN10	16.0^{a}
Gibberella sp. isolate MDU1	11.5^{de}
C. capsici isolate CBE1	10.7^{f}
C. capsici isolate DG1	14.0^{c}
C. capsici isolate TN1	11.0^{ef}
C. capsici isolate SLM1	11.2^{ef}
A. alternata isolate SLM2	14.0^{c}

Data are mean of three replications
Means followed by the same letter in a column are not significantly different (P=0.05) by LSD

RESULTS

Testing antagonistic activity of *Burkholderia* sp. strain TNAU-1 *in vitro* against causal agents of chilli anthracnose

The antagonistic *Burkholderia* sp. strain TNAU-1 was tested for its efficacy in suppressing mycelial growth of *C. capsici*, *C. gloeosporioides* and *A. alternata* isolates *in vitro*. *Burkholderia* sp. strain TNAU-1 inhibited the growth of all the isolates of *C. capsici*, *C. gloeosporioides* and *A. alternata* in dual culture on PDA medium and the inhibition zone ranged from 10.7 mm (CBE 1) to 16.0 mm (TEN 10) (Table 1). A talc-based powder formulation of the *Burkholderia* sp. strain TNAU-1 was developed for field application.

In vitro evaluation of fungicides against chilli anthracnose pathogens

The fungicides, cabrio top and mancozeb were evaluated for their comparative fungitoxicity against mycelial growth of *C. capsici, C. gloeosporioides* and *A. alternata in vitro* at the concentration levels of 5, 10, 50, 100 and 250 ppm. The results indicated that both the fungicides were highly effective in suppressing the radial growth of all the test fungi. Both the fungicides at 250 ppm concentration showed 100% inhibition against all the tested fungi (Table 2). At 100 ppm concentration, 100% inhibition of *C. capsici* and *A. alternata* and 79% inhibition of *C. gloeosporioides* was recorded in Cabrio Top. A progressive increase in percent inhibition of radial growth of pathogens was observed with increase in the concentration of the fungicides.

Field trials

The field trial results revealed that all the test fungicides and biocontrol agent significantly reduced the incidence of anthracnose and increased the yield when compared with the untreated control. Among the fungicides under investigation, Cabrio Top @ 1750 g/ha was found to be most effective in controlling anthracnose and increasing yield. Foliar application of powder formulation of *Burkholderia* sp. strain TNAU-1 also significantly reduced the incidence of anthracnose and increased the yield in both the field trials. Foliar application of powder formulation of *Burkholderia* sp. strain TNAU-1 showed a comparable level of disease control to that of foliar application with Cabrio Top under field conditions. Control plots recorded the lowest yield of 2132 kg/ha and 2507 kg/ha in Trial I and Trial II, respectively (Table 3). Cabrio Top @1750 g/ha recorded the highest yield of 3091kg/ha and 3304 kg/ha in Trial I and Trial II, respectively.

Table 2. Efficacy of fungicides Cabrio Top and Mancozeb against the growth of *Colletotrichum capsici, C. gloeosporioides* and *Alternaria alternata in vitro*

Fungicide	Concentration (ppm)	*C. capsici*		*C. gloeosporioides*		*A. alternata*	
		Radial growth of mycelium (cm)	Percent inhibition over control	Radial growth of mycelium (cm)	Percent inhibition over control	Radial growth of mycelium (cm)	Percent inhibition over control
Cabrio Top	5	4.73	47.3	4.67	39.1	3.10	37.6
	10	3.40	62.1	4.07	46.9	2.70	45.7
	50	2.57	71.3)	3.0	60.9	2.27	54.3
	100	0.0	100	1.6	79.1	0.00	100
	250	0.0	100	0	100	0.00	100
Mancozeb	5	8	10.8	5.93	22.7	3.07	38.2
	10	5.33	40.6	5.13	33.1	2.83	43.1
	50	4.07	54.6	4.43	42.2	2.43	51.1
	100	0.00	100	4.20	45.2	1.83	63.8
	250	0.00	100	0.00	100	0.00	100
Control	-	8.97	-	7.67	-	4.97	-
	SE	0.27	2.27	0.20	1.41	0.16	1.90
	CD (P=0.05)	0.57	4.74	0.41	2.95	0.34	3.96

The data are mean of three replications

Table 3. Effect of *Burkholderia* sp. strain TNAU 1 and fungicides on the incidence of anthracnose of chilli caused by *Colletotrichum capsici* under field conditions

Treatments	Treatment details	Plant height (cm)		Percent Disease Index (PDI)		Yield(kg/ha)	
		Trial I	Trial II	Trial I	Trial II	Trial I	Trial II
T1	Cabrio Top @ 1250 g/ha	147.4^{bc}	161.2^{cd}	6.56^{bc}	6.38^{bc}	2912.5^{bc}	3004.8^{bc}
T2	Cabrio Top @ 1500 g/ha	148.6^{bc}	163.8^{bc}	6.40^{b}	6.29^{b}	2975.4^{ab}	3096.8^{b}
T3	Cabrio Top @ 1750 g/ha	146.2^{bc}	172.6^{b}	5.70^{a}	5.78^{a}	3090.8^{a}	3303.7^{a}
T4	Metiram @ 2000 g/ha	143.2^{bc}	164.6^{bc}	9.00^{e}	8.43^{g}	2761.2^{cd}	2871.1^{cd}
T5	Pyraclostrobin @ 500 g/ha	141.4^{bc}	158.3^{cd}	7.08^{d}	7.12^{de}	2818.2^{bc}	3038.1^{bc}
T6	Chlorothalonil @ 800 g/ha	140.4^{c}	156.2^{cd}	6.98^{cd}	6.30^{b}	2787.8^{cd}	2905.4^{cd}
T7	Mancozeb @ 2000 g/ha	143.4^{bc}	160.9^{cd}	6.21^{b}	6.13^{ab}	2852.3^{bc}	2965.9^{bc}
T8	*Burkholderia* sp. talc formulation @ 0.2%	165.2^{a}	193.4^{a}	7.11^{d}	7.48^{e}	2845.6^{bc}	2965.9^{bc}
T9	*P. fluorescens* (Pf1) talc formulation @ 0.2%	147.8^{bc}	190.0^{a}	7.33^{d}	7.94^{f}	2626.2^{d}	2749.2^{d}
T10	Chlorothalonil @ 800 g/ha	149.2^{b}	158.8^{ed}	6.90^{cd}	6.76^{cd}	2812.1^{bc}	2920.8^{cd}
T11	Untreated control	141.2^{bc}	152.6^{d}	21.77^{f}	19.58^{h}	2132.4^{e}	2507.5^{e}

In a column, means followed by a common letter are not significantly different at the 5% level by LSD. The data are mean of three replications.

Endophytic colonization of *Burkholderia* sp. strain TNAU-1

The colony PCR results showed that single amplification product of 417 bp was obtained from all the endophytic bacterial colonies, which confirmed the existence of *Burkholderia* sp. strain TNAU-1 in all parts of the foliar sprayed plant (Fig. 1).

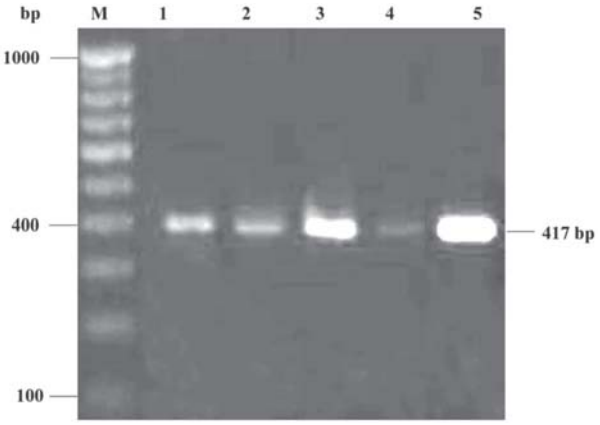

Fig. 1. Detection of endophytic colonization of *Burkholderia* sp. strain TNAU1 in chilli by PCR using SCAR marker. Lane M - DNA ladder; Lane 1 - bacterial colony from leaf; Lane 2 - bacterial colony from fruit; Lane 3 - bacterial colony from stem; Lane 5 - bacterial colony from root; Lane 5 - Purified DNA from Burkholderia sp. strain TNAU1

DISCUSSION

It has been well documented that variety of fungicides, plant extracts and biocontrol agents can inhibit the growth of phytopathogenic fungi and bacteria. Anand and Bhaskaran (2009) reported that spraying with aqueous extract from leaves of *Abrus precatorius* (10%) twice, the first spray at the time of fruit set and the second spray 20 days after fruit set caused significant reduction in the incidence of chilli anthracnose under field conditions. Lewis-Ivey *et al.* (2004) reported that application of Pyroclostrobin alternated with Maneb resulted in significantly higher yield by reducing the fruit rot disease incidence of bell pepper. Gopinath *et al*. (2006) demonstrated that application of propiconazole at 0.1% caused a dramatic reduction of chilli anthracnose disease incidence by 70% when compared to difenoconazole at 0.05% (58%) and carbendazim at 0.1% (44%) and the fruit yield increased in the range of 86%, 63% and 60% for propiconazole, difenoconazole and carbendazim, respectively, when compared to unsprayed controls. Cabrio Top inhibited the mycelial growth of all the three pathogens with minimal inhibitory at 250 ppm. Foliar application of Cabrio Top chemical @ 1750 g/ha immediately after appearance of disease symptom followed by two sprays at 15 days interval was found to be most effective in controlling anthracnose and increasing yield compared to other treated and control plots.

Biological control of chilli anthracnose also has been addressed using bacterial and yeast antagonists in recent years (Chanchaichaovivat *et al.,* 2007; Anand *et al*., 2010). Bharathi *et al*. (2004) demonstrated that chilli seed treatment and foliar application with a talc-based powder formulation of *Pseudomonas fluorescens* (Pf1) significantly reduced the severity of anthracnose with a simultaneous increase in chilli yield both in the glass house and in the field. Chanchaichaovivat *et al.* (2007) demonstrated that application of *Pichia guilliermondii* showed significant reduction of *C. capsici* disease incidence on chilli in storage. Paramasivam and Kalaimani (2008) demonstrated that application of *P. fluorescens* (0.2%) significantly reduced the chilli anthracnose and increased the yield. Anand *et al*. (2010) recently reported that combined use of talc based formulation of *P. fluorescens* Pf1 (2.5 kg/ha) and azoxystrobin (250 ml/ ha) was more effective against chilli anthracnose disease than treatments with azoxystrobin (500 ml/ha) and Pf1 (2.5 kg/ha) applied individually. Similarly foliar application of azoxystrobin in combination with *Pseudomonas* and *Bacillus* sp. effectively controls the foliar diseases of wheat and increased the grain yield (Singh *et al.,* 2016).

Species belonging to the genus *Burkholderia* have been identified as biocontrol agents of many phytopathogenic fungi, such as *Pythium aphanidermatum, P. ultimum, Fusarium* sp., *Rhizoctonia solani* and *Ganoderma* (Sapak *et al.,* 2008). Vijayasamundeeswari *et al*. (2010) demonstrated the potential of *Burkholderia* sp. strain TNAU-1 in controlling *Aspergillus flavus* infection and aflatoxin contamination in groundnut. The same antagonistic bacteria inhibited the growth of all the isolates of pathogen causing anthracnose disease on chilli. Further, foliar application of powder formulation of *Burkholderia* sp. strain TNAU-1 showed a comparable level of disease control to that of foliar application with Cabrio Top under field conditions and also it enhances the height of plants. The reduction in the anthracnose disease incidence and enhanced plant height in *Burkholderia* sp. strain TNAU-1 treated plants might be due to direct antagonistic effect of the bio-control agent on *C. capsici*, induction of defense compounds *viz.*, peroxidases, polyphenol oxidases, phenol and thaumatin-like protein in response to treatment with *Burkholderia* sp. strain TNAU-1 and plant growth promoting activity of the antagonist through endophytic colonization in the treated plants, respectively. This result was supported by foliar application of *Burkholderia* sp. strain TNAU-1 which leads to activation of defense responses in chilli (Madhavan *et al*., 2011). The endophytic colonization of foliar sprayed bioagents is most important for antagonistic activity fungal pathogens in plants (Singh, 2016).

Use of bio control agents and combination (systemic and contact) of fungicides has been suggested as an approach for the management of fungicide resistance. Hence, results from the present study indicated that use of bio control agent *Burkholderia* sp. strain TNAU-1 and the fungicide Cabrio Top, combination of two chemical molecules [Metiram (ethylene bis dithiocarmate) + Pyraclostrobin (Strobilurin)] can be effectively employed to control the anthracnose disease in chilli with probable reduction of fungicide resistance and environment hazard caused by heavy application of fungicide.

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