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In vitro Screening of Biocontrol Agents against *Alternaria alternata* (Fries) Keissler Causing Leaf Blight of Broad Bean in Manipur

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ABSTRACT

Leaf blight of broad bean caused by *Alternaria alternata* is one of the main foliar diseases of broad bean in Manipur. The disease predominantly affects above ground parts of the plant. Three techniques namely, dual culture, volatile and non volatile compounds assays were used to evaluate the efficacy of native biocontrol agents against the fungal pathogen. All the biocontrol agents used in this study potentially suppressed the growth of the fungal pathogen. NCIPMCAU-123 (*T. asperellum*) and NCIPMCAU-69 (*T. harzianum*) exceeded all the biocontrol agents in inhibiting the mycelial growth of *A. alternata* in all the techniques. In dual culture, NCIPMCAU-123 and NCIPMCAU-69 gave an inhibition of 94.07% and 91.85% respectively and displayed Class IV and Class VI of Bell's scale respectively. In volatile compounds assay, NCIPMCAU-123 and NCIPMCAU-69 showed an inhibition of 54.07% and 51.11% respectively. In non-volatile compounds assay, all the biocontrol agents performed better at highest concentration. At the concentration of 15%, NCIPMCAU-69 and NCIPMCAU-123 exhibited an inhibition of 87.04% and 84.81% respectively.

Keywords *Alternaria alternata*, Broad bean, Biocontrol agents, Dual culture, Volatile compounds, Non-volatile compounds.

INTRODUCTION

Broad bean (*Vicia faba* L.) is one of the significant legume crops cultivated in India. It is also referred to as faba bean, bakla bean and horse bean. It is an important source of food, animal feed and silage (Dhull *et al.* 2022). The worldwide cultivation of this bean accounts an estimated area of 255359 hectare, yield of 6255.8 kilogram per hectare and production of 1597477 tones in 2020 (Anonymous 2021).

Broad bean seed is a rich source of proteins and minerals including copper, phosphorus, potassium and zinc while the leaf is rich in magnesium, manganese, calcium and iron (Etemadi *et al.* 2018). The seed is also a good source of carbohydrates including fructose, glucose, sucrose, stachyose, verbascose and raffinose (Landry *et al.* 2016). Broad bean is also useful for treating parkinson disease as it is a natural

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Table 1. List of biocontrol agents with their accession number.

Treatments	Isolation code	Biocontrol agents	Accession no
1	NCIPMCAU-131	*T. harzianum*	KU933474
2	NCIPMCAU-123	*T. asperellum*	KU933476
3	NCIPMCAU-7	*T. asperellum*	KU933475
4	NCIPMCAU-96	*T. ovalisporum*	KU904456
5	NCIPMCAU-78	*T. harzianum*	KU904458
6	NCIPMCAU-18	*T. koningiopsis*	KU904460
7	NCIPMCAU-69	*T. harzianum*	KU933468
8	NCIPMCAU-118	*T. atroviride*	KU933475
9	NCIPMCAU-48	*Hypocrea lixii*	KX0113223
10	NCIPMCAU-109	*T. harzianum*	KU933471
11	NCIPMCAU-25	*T. asperellum*	KT601340
12	NCIPMCAU-100	*T. harzianum*	KU933469
13	WAN-K	*T. harzianum*	MH257330
14	TKS-H	*T. asperellum*	MH257327

source of carbidopa in addition to levo-dihydroxy phenylalanine which is a precursor of dopamine (Mehran and Golshani 2013).

Broad bean is affected by many pathogens. Leaf blight caused by *Alternaria alternata* is one of the significant foliar diseases of broad bean. This disease is also known as leaf spot. In Manipur, this disease remarkably affects the cultivation of broad bean. The disease attacks edible portions of the plant including leaves, pods and seeds. The yield, quality and flavor of the pod as well as seed are remarkably reduced. This fungal genus survives on infected plant debris, seed and infested soil. The pathogen also survives on other host plants and is airbone as well (Kumar *et al.* 2022). The disease initiates from the lower portion of the plants and progresses upwards.

Chemical pesticides managed several plant diseases efficiently. However, the continuous use of these chemicals confers several harmful impacts on human health, causes environmental pollution, confers non target effects on unrelated organisms and may even contributes to climate changes (Tudi *et al.* 2021). The biocontrol agents on the other hand, not only manages the plant diseases proficiently but is also is environmental friendly. Hence, the present study was undertaken to study the potential of biocontrol agents in managing broad bean leaf blight caused by *Alternaria alternata*.

MATERIALS AND METHODS

The present study was conducted at College of Agriculture, Central Agricultural University, Imphal, Manipur. The infected broad bean leaves were collected from the campus of College of Agriculture. The sample was cut into small pieces and surface sterilized by 1% sodium hypochlorite solution and subsequently rinsed with sterile distilled water for three times. The cut pieces were blot dried and inoculated into petri dishes containing sterilized solidified potato dextrose agar medium. Pure culture of the fungus was obtained by transferring pure mycelial strands into a fresh medium on the petri dishes. The pathogen was identified morphologically as well as molecularly at Unipath Specialty Laboratory Ltd, Ahmedabad, Gujarat. Fourteen isolates of biocontrol agent were obtained from the Department of Plant Pathology, College of Agriculture (Table 1). Both the pathogen and biocontrol agents were regularly subcultured and maintained on potato dextrose agar throughout the research period.

The efficacy of biocontrol agents were assayed by three techniques namely, dual culture, effects of volatile (Dennis and Webster 1971a) and non volatile compounds (Dennis and Webster 1971b) respectively. In dual culture assay, 5 mm mycelial disc of the fungal pathogen and the biocontrol agents were inoculated in opposite direction at 5 cm apart from each other on solidified sterilized potato dextrose agar medium. 5 mm mycelial disc of the test fungus were incubated on the center of the petri dishes and this served as a control. Three replications were maintained. The petri dishes were incubated at 28 ± 2°C until the radial mycelial growth of test fungus in control reached a periphery of the petri dishes.

For the evaluation of impact of volatile compounds released by biocontrol agents against the test pathogen, 5 mm mycelial discs of both the fungal pathogen and the biocontrol agents were inoculated on the center of petri dishes containing sterilized solidified potato dextrose agar medium. Top lid of each petri dishes inoculated with biocontrol agents were replaced by the bottom container of petri dishes centrally inoculated with the test pathogen and are bound tightly by parafilm. The lid of the petri dishes contain-

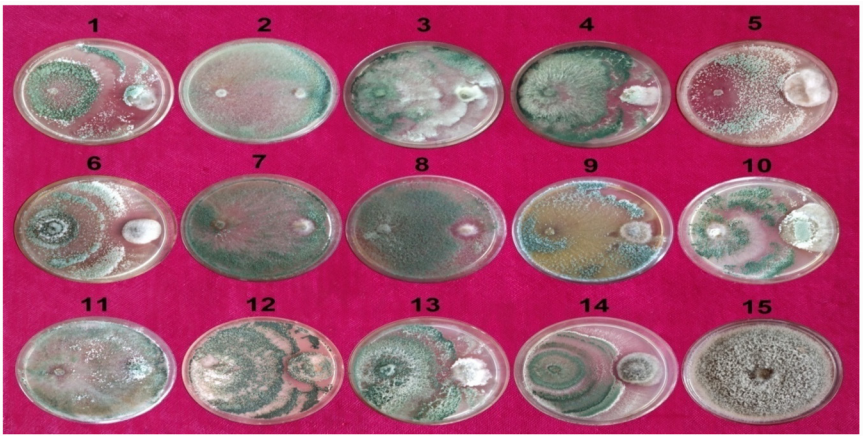

Fig. 1. Effect of dual culture in inhibiting the growth of *Alternaria alternata*.

ing only the culture medium were also replaced by the bottom container of petri dishes centrally inoculated with the test pathogen and this served as a control. Three replications were maintained for each treatment and the petri dishes were incubated at 28 ± 2°C until the radial mycelial growth of the test fungus in control reached a periphery of the petri dishes.

For the study of effect of non volatile compounds, the biocontrol agents were grown in sterilized 50 ml potato dextrose broth for 10 days in 100 ml Erlenmeyer flask at 28 ± 2°C accompanied with periodical shaking. Culture of biocontrol agents were filtered through Whatman filter paper no. 42 into sterilized Erlenmeyer flasks. The culture filtrates were centrifuged at 6000 rpm for 10 mins and the supernatant were filtered again through Celluose Millipore membrane filter (0.4 µm pore size). The

Table 2. Effect of dual culture in inhibiting the growth of *Alternaria alternata*. *Mean of three replications, Figures in the parenthesis are square root transformed.

Treatments	Biocontrol agents	Inhibition (%) over control*		Bell's scale	Duration for contact (days) between pathogen and biocontrol agents
1	NCIPMCAU-131	82.59	(9.12)	Class III	3
2	NCIPMCAU-123	94.07	(9.72)	Class IV	3
3	NCIPMCAU-7	88.52	(9.43)	Class I	4
4	NCIPMCAU-96	80.74	(9.01)	Class III	3
5	NCIPMCAU-78	70.74	(8.44)	Class IV	3
6	NCIPMCAU-18	80.00	(8.97)	Class IV	3
7	NCIPMCAU-69	91.85	(9.61)	Class VI	Inhibition zone of 0.2 cm
8	NCIPMCAU-118	91.11	(9.57)	Class VI	Inhibition zone of 0.3 cm
9	NCIPMCAU-48	85.93	(9.30)	Class IV	4
10	NCIPMCAU-109	64.07	(8.04)	Class III	3
11	NCIPMCAU-25	86.67	(9.34)	Class I	4
12	NCIPMCAU-100	72.96	(8.57)	Class II	3
13	WAN-K	74.44	(8.66)	Class V	3
14	TKS-H	72.22	(8.53)	Class VI	Inhibition zone of 0.3 cm
15	Control	-	-	-	
	SE(d) ±	0.07			
	CD (0.05)	0.15			

Table 3. Effects of volatile and non-volatile compounds in inhibiting the growth of *Alternaria alternata*. *Mean of three replications, Figures in the parenthesis are square root transformed.

Treatments	Biocontrol agents	Inhibition (%) over control* Volatile compounds	Non-volatile compounds 7%	11%	15%
1	NCIPMCAU-131	21.85 (4.73)	21.11 (4.65)	31.11 (5.62)	60.37 (7.80)
2	NCIPMCAU-123	54.07 (7.39)	53.33 (7.34)	73.70 (8.61)	84.81 (9.24)
3	NCIPMCAU-7	14.07 (3.81)	34.81 (5.94)	58.52 (7.68)	72.96 (8.57)
4	NCIPMCAU-96	47.41 (6.92)	23.33 (4.88)	39.26 (6.31)	60.00 (7.78)
5	NCIPMCAU-78	42.22 (6.53)	22.22 (4.76)	34.44 (5.91)	57.04 (7.59)
6	NCIPMCAU-18	38.89 (6.28)	26.30 (5.17)	43.33 (6.62)	62.96 (7.97)
7	NCIPMCAU-69	51.11 (7.18)	53.70 (7.36)	76.30 (8.76)	87.04 (9.36)
8	NCIPMCAU-118	12.96 (3.66)	44.44 (6.70)	58.52 (7.68)	75.56 (8.72)
9	NCIPMCAU-48	20.67 (4.60)	32.96 (5.78)	51.85 (7.24)	64.07 (8.04)
10	NCIPMCAU-109	48.89 (7.03)	17.04 (4.19)	25.19 (5.06)	51.48 (7.21)
11	NCIPMCAU-25	49.26 (7.06)	51.48 (7.21)	71.48 (8.48)	82.59 (9.12)
12	NCIPMCAU-100	22.22 (4.77)	27.04 (5.24)	36.30 (6.07)	55.19 (7.46)
13	WAN-K	39.63 (6.34)	27.78 (5.32)	50.74 (7.16)	56.30 (7.53)
14	TKS-H	30.37 (5.55)	24.81 (5.03)	33.70 (5.85)	54.07 (7.39)
15	Control	-	-	-	-
	SE(d) ±	0.15	0.12	0.11	0.11
	CD (0.05)	0.30	0.24	0.22	0.22

filtrates were added accordingly to 50 ml of sterilized and melted potato dextrose agar at the concentration of 7%, 11% and 15% respectively. The amended media were poured into three sterilized petri dishes and were centrally inoculated with 5 mm mycelial disc of the test pathogen after solidification. The test pathogen was also centrally inoculated on three petri dishes containing only potato dextrose agar and this served as a control. The petri dishes containing the test pathogen were incubated at 28 ± 2°C until the radial mycelial growth of the test fungus in control reached a periphery of the petri dishes.

The antagonistic activities for all the three techniques were evaluated by calculation of percent inhibition of mycelial growth of the test fungus (Vincent 1947) as follows :

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

Where C = Linear radial growth of the fungus in control

T = Linear radial growth of the fungus in treatment

The antagonistic activities in dual culture were further assessed by Bell's scale (Bell *et al.* 1982) developed as class I-VI with slight modifications as below :

Class I The antagonist completely overgrew the pathogen (100% over growth)

Class II The antagonist overgrew at least ⅔rd of the pathogen surface (75% over growth)

Class III The antagonist colonizes on half of the growth of the pathogen (50% over growth)

Class IV The pathogen and antagonist are locked at the point of contact

Class V The pathogen overgrew the antagonist

Class VI Formation of inhibition zone between the pathogen and the antagonist

RESULTS AND DISCUSSION

The pathogen was identified as *Alternaria alternata* by comparison with relevant monograph (Simmons and Roberts 1993). The DNA of the pathogen was also sequenced using ITS1 and ITS4 primers and the resultant sequence was analyzed by Nucleotide Basic Alignment Local Search Tool (blastn) at National Center for Biotechnology Information (NCBI) which further identified the pathogen as *A. alternata* (accession number OP893952). All the biocontrol agents in dual culture technique were able to suppress the growth of the fungal pathogen. Highest inhibition

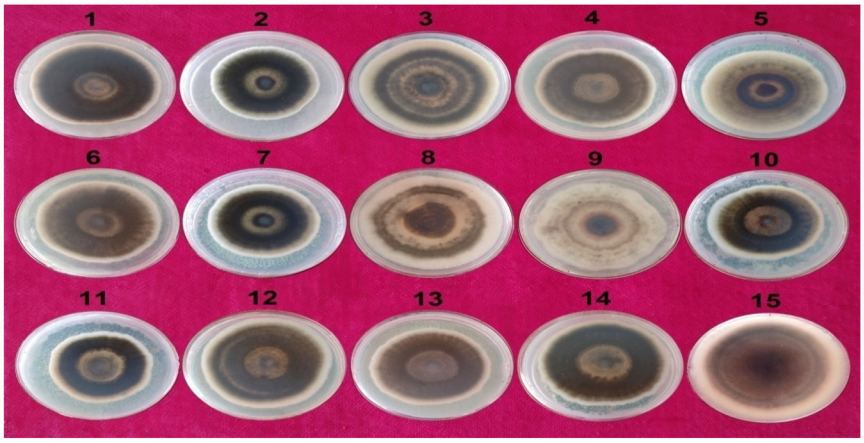

Fig. 2. Effects of volatile compounds in inhibiting the growth of *Alternaria alternata*.

of mycelial growth in dual culture was observed in NCIPMCAU-123 (94.07%) followed by NCIPMCAU-69 (91.85%) and NCIPMCAU-118 (91.11%) respectively (Table 2). The interaction of NCIPMCAU-123 with the fungal pathogen exhibited Class IV of Bell's scale. An inhibition zone was formed between the fungal pathogen and the biocontrol agents namely, NCIPMCAU-69, NCIPMCAU-118 and TKS-H, thus, exhibiting Class VI. NCIPMCAU-7 and NCIPMCAU-25 completely overgrew the fungal pathogen, thereby exhibiting Class I (Fig. 1).

Volatile compounds secreted by all the tested biocontrol agents reduced the growth of the fungal pathogens. The highest inhibition (54.07%) of mycelial growth was recorded in NCIPMCAU-123. It was successively followed by NCIPMCAU-69 which recorded an inhibition of 51.11%. The lowest inhibition of mycelial growth were recorded in NCIPMCAU-118 (12.96%) and NCIPMCAU-7 (14.07%) respectively (Table 3 and Fig. 2).

Non-volatile compounds produced by all the

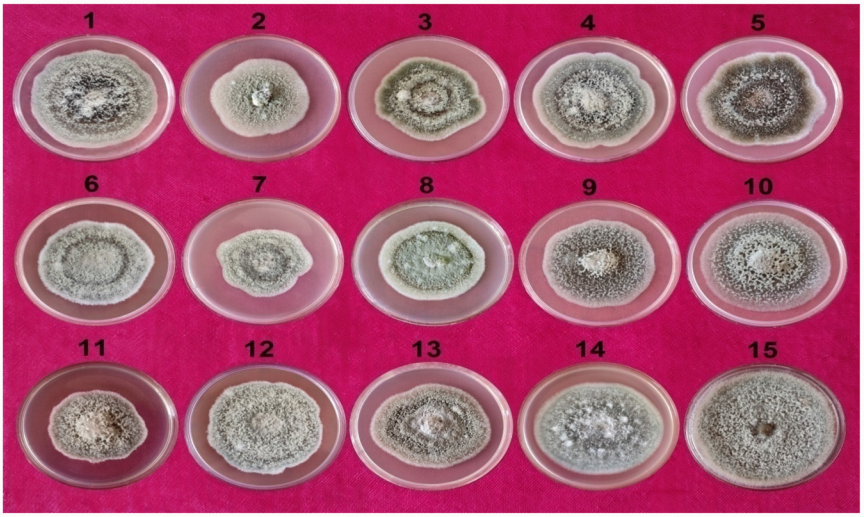

Fig. 3. Effects of non-volatile compounds at 7% in inhibiting the growth of *Alternaria alternata*.

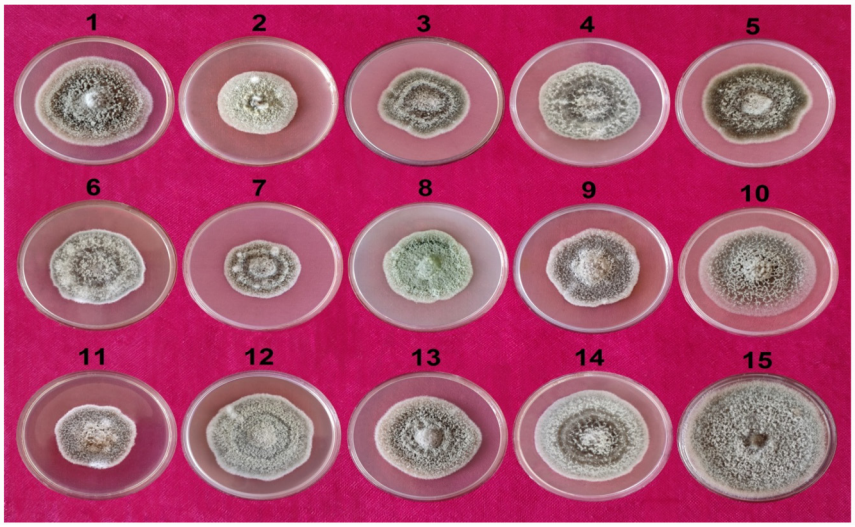

Fig. 4. Effect of non-volatile compounds at 11% in inhibiting the growth of *Alternaria alternata*.

tested biocontrol agents were effective in inhibiting the mycelial growth of the fungal pathogen. Among all the tested concentrations, the inhibition of the fungal pathogen was highest at 15% (Table 3). At all the concentrations, NCIPMCAU-69 gave highest inhibition of the mycelial growth of the pathogenic fungus and was subsequently followed by NCIPMCAU-123. At the concentration of 7%, inhibition of *A. alternata* by NCIPMCAU-69 was 53.70% and by NCIPMCAU-123 was 53.33% (Fig. 3). At 11% concentration, NCIPMCAU-69 gave an inhibition of 76.30% while NCIPMCAU-123 gave an inhibition of 73.70% (Fig. 4). At the concentration of 15%, NCIPMCAU-69 gave the highest inhibition of 87.04% and NCIPMCAU-123 gave an inhibition of 84.81% (Fig. 5).

Trichoderma isolates are reported to be effective against *A. alternata* in dual culture test, inhibitory assay test of both volatile and non-volatile compounds

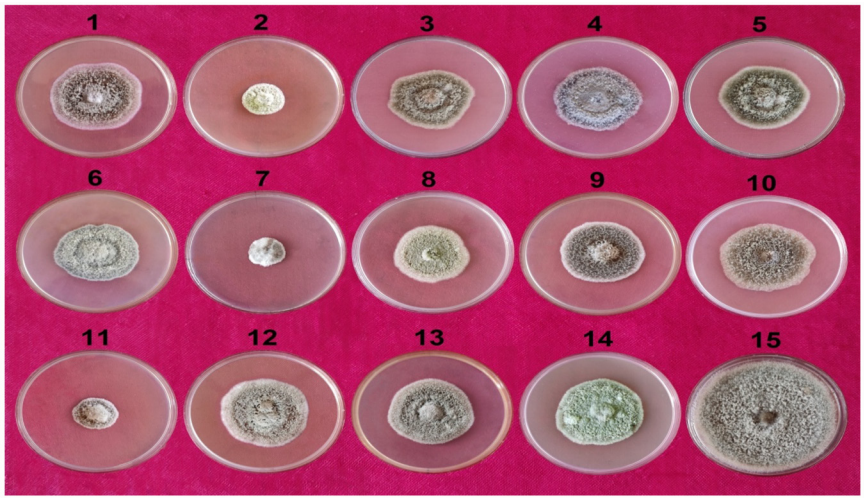

Fig. 5. Effect of non-volatile compounds at 15% in inhibiting the growth of *Alternaria alternata*.

(Arzanlou *et al.* 2014, Meena *et al*. 2017, Ferreira *et al.* 2020). The formation of clear inhibition zone in between the pathogen and the biocontrol agents in dual culture may specify antibiosis through secretion of antifungal metabolites by the biocontrol agents (Gajera *et al.* 2016, Aoki *et al.* 2020). Shafique *et al.* (2019) reported the fungicidal efficiency of *T. koningii*, *T. harzianum* and *T. viride* in reducing fungal biomass of *A. alternata*. Mukhopadhyay and Kumar (2020) outlined the system of mechanisms employed by *Trichoderma* during interaction with plant pathogens which comprises of parasitism, competition for nutrients and space, production of antibiotics, and several hydrolytic enzymes including glucanase, chitinase and protease. They also described the activation of signalling pathways namely, MAP kinase, G protein and cAMP which are associated with formation of infection structures and production of several antifungal metabolites. Similarly, Ghosh *et al.* (2022) described the efficacy of *T. harzianum* and *T. asperellum* in reducing the radial growth of *A. alternata* and also delineated different mechanisms employed by *Trichoderma* against the pathogen which includes hyperparasitsm, production of siderophores and secretion of volatile compounds namely, HCN and CH_3.

CONCLUSION

All the biocontrol agents repressed the growth of the fungal pathogen in varying extents. The tested biocontrol agents showed different antagonistic activities on the fungal pathogen. The isolate of *Trichoderma asperellum* (NCIPMCAU-123) and *T. harzianum* (NCIPMCAU-69) outperformed all the tested biocontrol agents in dual culture, effects of volatile and non-volatile compounds. Hence, the biocontrol agents can be significantly used for managing leaf blight of broad bean and serves as a potential alternative for chemical fungicides and antifungal antibiotics. The potential biocontrol agents can be used individually or in combination with other compatible biocontrol agents.

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