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Antifungal ability of *Beauveria bassiana* (Balsamo) Vuillemin against *Curvularia lunata* causing leaf spot of rice

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Abstract
Beauveria bassiana (Balsamo) Vuillemin, a white muscardine fungus has widely drawn attention of crop protection practitioners as potential biocontrol agent against insect-pests since decades. In the present study, antagonistic potential of 53 native isolates of *B. bassiana* were evaluated against *Curvularia lunata* causing leaf spot as well as seed borne disease of rice. Results showed that all *B. bassiana* isolates were able to inhibit mycelial growth of *C. lunata* to the extent of 50-63.33% through varied mechanisms *viz.*, competition and diffusible non-volatile metabolites. However, potential isolates of *B. bassiana* also exhibited inhibition of mycelial growth of *C. lunata* to the maximum up to 67.78% through release of volatile inhibitory metabolites. These findings provide substantial evidences on multifarious potential of *B. bassiana* as plant disease antagonist in addition to a potential entomopathogen, thus paves the way of a newer domain in the arena of crop protection.

Keywords: Antifungal, *B. bassiana*, *C. lunata*, disease antagonist, entomopathogen

Introduction
Rice (*Oryza sativa* Linn.) is an important staple food crop for more than half of world's population, of which, Asian countries accounts for 90% of world's rice production. In global scenario, India stands second in rice production after China, accounting for 24% of world's rice production with an average production of 177 million tonnes from 43 million hectares area (FAO, 2019) [6]. Among several biotic and abiotic factors affecting production of rice, *Curvularia* disease paves devastating effect in rice cultivation, as one of the newly emerging disease of rice causing not only leaf spots symptoms but also leaf blights, kernel discoloration, grain lesions as well as grain discoloration in rice during seedling, vegetative as well as maturity stage (Kamaluddeen *et al.*, 2013) [15]. *C. lunata* (Ascomycota: Pleosporales: Pleosporaceae) have been reported as new seed-borne pathogen of rice associated with spots in grains as well as failure in germination of rice seeds (Sisterna and Dal Bello, 1988) [21]. In addition to that, it was also reported to cause new blight disease causing elliptical brown to black-colored spots in rice from Uttar Pradesh, India (Kamaluddeen *et al.*, 2013) [15]. However, records of *C. lunatus* causing leaf blight disease of rice were also reported from China showing typical leaf-streak symptoms at booting stage leading to 40-80% disease incidence (Liu *et al.*, 2014) [16]. Also, Majeed *et al.* (2015) [18] reported *C. lunata* to cause small, brown, round-ovoid leaf spots with chlorotic halo on rice leaf blade for first time from Punjab province of Pakistan. Similarly, Tann and Soytong (2017) [22] have reported *C. lunata* as causal agent for brown spot disease of rice from Cambodia.
However, management of *Curvularia* disease conventionally involves improvement of cultural practices and use of chemical protectants. But in recent scenario, use of microbial bio-inoculants are emerging as potential management strategy with an aim to minimize degrading impact on environment and human health. Studies on entomopathogenic fungus *B. bassiana* in controlling phytopathogens have introduced a new concept in plant disease management in addition to their promising biocontrol abilities against insect-pests of rice (Ownley *et al.*, 2008) [20]. *Beauveria bassiana* (Balsamo) Vuillemin, a white muscardine fungus is widely known as potential biocontrol agent against insect pests of tomato belonging to the order Lepidoptera, Hemiptera, Coleoptera, Hymenoptera, Homoptera and Orthoptera (Hazarika *et al.*, 2005) [10]. Recent studies showed that *B. bassiana*, often exclusively considered as insect pathogens, also plays additional beneficial roles in nature *viz.* plant endophytes, antagonists for

plant diseases, beneficial rhizosphere colonizers and plant growth promoters (Vega *et al.*, 2009; Jaber and Enkerli, 2016) [23, 12]. But there is substantial evidence that *B. bassiana* provides protection against both insect pests and plant pathogens, though mechanisms involved for later are not yet completely understood (Jaber and Ownley, 2018) [13]. In several studies, reports on *B. bassiana* as successful plant disease antagonists were published *viz.*, *Phytopthora infestans*, *Pythium myriotylum*, *P. debaryanum*, *P. irregular* and *P. ultimum*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Gaeumannomyces graminis* var. *tritici*, *Armillaria mellea*, *Rosellinia necatrix*, *Thielaviopsis bassicola*, *Botrytis cinerea*, *Septoria nodorum*, *Phoma betae*, *P. exigua* and *Colletotrichum falcatum*, *Xanthomonas campestris* pv. *malvacearum)* and *Meloidogyne incognita* (Griffin, 2007; Ownley *et al*., 2008; Vega *et al.*, 2009; Gothandapani *et al*., 2014; Jaber, 2015; Azadi *et al.*, 2016; Lozano-Tovar *et al.*, 2017; Yun *et al.*, 2017; Jaber and Ownley, 2018) [9, 20, 23, 8, 11, 1, 17, 26, 13]. Possible mechanisms of plant disease suppression incorporated by *Beauveria* sp. are employing direct mechanisms such as mycoparasitism, competition and antibiosis or complex indirect interaction by stimulating induced systemic resistance (ISR) as well as endophytic colonizing behaviour (Ownley *et al*. 2008; Vega *et al*. 2009; Jaber, 2015; Azadi *et al*. 2016; Lozano-Tovar *et al*. 2017) [20, 23, 11, 1, 17]. With this background, the present study was carried out to study the antagonistic potential of native *B. bassiana* isolates against *Curvularia lunata*, an emerging seed-borne and leaf spot disease causing pathogen of rice.

Materials and Methods

Fungal isolates

Fifty-three (53) native isolates of *B. bassiana* isolated from mulberry silkworm (*Bombyx mori*) from different locations of Meghalaya were used in the study. The ITS sequences for all the isolates (53) of *B. bassiana* are deposited in the NCBI GenBank nucleotide sequence database with accession numbers *viz.*, MW633007-MW633026, MW633206-MW633221, MW628496-MW628499, MW627305 and MW622068 respectively. All isolates of *B. bassiana* were sub cultured on Sabouraud Dextrose Agar (SDA- dextrose 40g, peptone 10g and agar agar 15g in 1000 ml distilled water) following 7-15 days incubation at 28±1 ºC under dark condition. Pure culture of *C. lunata* was obtained from microbial culture laboratory, SCP and sub cultured on Potato Dextrose Agar (PDA- potato infusion, 200g; dextrose, 20g and agar agar, 20g in 1000 ml distilled water) at 25±2 °C under an incubation period of three days.

Antagonistic potential of *B. bassiana* against *Curvularia lunata*

In vitro evaluation of *B. bassiana* for their antagonistic potential against *C. lunata* was studied by performing dual culture assay by following Dennis and Webster (1971a) [4]. Actively growing mycelia disc (7 mm) each of *B. bassiana* and test pathogen were placed simultaneously at the distance of 3 cm from the corner of petri plates (90 mm) poured with PDA. Triplicates were maintained and the control plate with only test pathogen placed at the centre of the plate (90 mm) were incubated at 28±1˚C until full growth was obtained. Periodic observations on radial growth of fungal mycelium were recorded and inhibition percentage of mycelial growth of test pathogens were calculated by the formula given by Vincent (1947) [24],

$$I = \left\{ \frac{R_1 - R_2}{R_1} \right\} X\ 100$$

Where

I = per cent inhibition in growth of test pathogen (%)
R_1 = Radial growth of test pathogen in control (cm)
R_2 = Radial growth of test pathogen in treatment (cm)

B. bassiana isolates were rated based on their ability to suppress mycelial growth of test pathogen by following modified Bell's scale (Bell *et al.*, 1982) [2]:

S_1: The antagonist completely overgrew the pathogen
S_2: The antagonist overgrew at least 2/3rd growth of the pathogen
S_3: The antagonist colonized half of the growth of pathogen and no one seems to dominate each other
S_4: The pathogen overgrew 2/3rd of the growth of antagonist and resist invasion
S_5: The pathogen completely overgrew the antagonist

Effect of volatile inhibitory substances released by *B. bassiana* against *C. lunata*

The potential *B. bassiana* isolates were evaluated for their ability to produce volatile inhibitory substances against *C. lunata* under *in vitro* condition by following inverted plate technique as described by Dennis and Webster (1971b) [5]. Petri plates (90 mm) containing SDA (20 ml) were inoculated separately with actively growing (7 days old) mycelial disc (7 mm diam.) of *B. bassiana* at centre, sealed with parafilm and incubated at 28±1ºC for 3 days under dark condition. Separately, *C. lunata* in active growing stage (5 days old for fungus) was inoculated at centre of PDA plates (90 mm), sealed with parafilm and incubated at 28±1 ºC for 5 hours. After incubation, the lid of petri plates containing *B. bassiana* and test pathogens were aseptically removed and both bottoms were inverted over each other, double sealed with parafilm and further incubated at 28±1 ºC for 5 days under dark condition. Three replications were maintained and petri plates without antagonist served as control. Growth of test pathogen was measured after 5 days of incubation and percent inhibition was calculated as per formula given by Vincent (1947) [24]:

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

Where

PI = Per cent inhibition of mycelial growth of test pathogen (%)
C = Growth of test pathogen in control (mm)
T = Growth of test pathogen in treatment (mm)

Statistical analysis

The statistical design used in the present study was completely randomized design (CRD) and was analysed by using software ICAR Web Agricultural Statistical Package 2.0. The significant difference, if any, among the treatment means were compared by using critical difference (CD) at *P*=0.05 (Gomez and Gomez 1984) [7].

Results

Antagonistic potential of *B. bassiana* against *C. lunata*

The antifungal ability of *B. bassiana* isolates against *C. lunata* was evaluated by dual culture technique based on their potential for mycelial growth inhibition on PDA plates and their mode of action was studied. Inhibition of mycelial growth of test pathogen through intercultural competition as well as development of inhibition zone between interacting organisms were observed on 7th days post incubation (Plate 1). The mycelial inhibition percentage (%) of *B. bassiana* against *C. lunata* was found significantly higher with four (4) isolates *viz.*, Bb48 (63.33%), Bb16 (62.26%), Bb50 (61.48%) and Bb25 (60.00%) against radial growth of 33.00 mm, 33.97 mm, 34.67 mm, 36.00 mm respectively. Out of fifty-three (53) isolates, only twenty (20) isolates *viz.*, Bb53 60.00%), Bb11 (60.00%), Bb7 (59.26%), Bb14 (58.89%), Bb4 (57.78%), Bb44 (57.78%) and Bb18 (57.81%), Bb38 (56.48%), Bb31 (55.56%), Bb12 (55.37%), Bb24 (54.44%), Bb51 (53.33%), Bb28 (53.33%), Bb3 (51.11%), Bb8 (50.93%) and Bb45 (50.00%) showed >50% of inhibition efficiency over control (Table 1). While, majority of the isolates (33) were found ineffective in inhibiting mycelial growth of test pathogen.

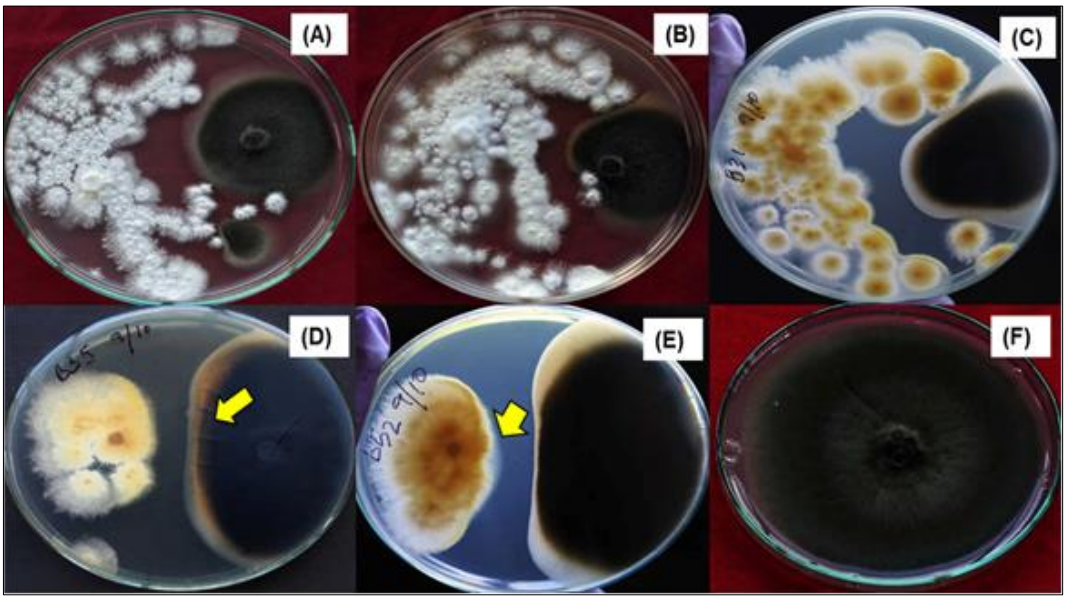

Plate 1: Mechanism of antagonism by *B. bassiana* against *C. lunata viz.*, (A, B) competition, (C, D) reverse Petri plate with inhibition zone (C, D), (E) reverse Petri plate showing diffusible secondary metabolite, and (F) as control Petri plate of *C. lunata*

Majority of *B. bassiana* isolates (27) *viz.*, Bb11, Bb14, Bb16, Bb25, Bb48, Bb53 exhibited competitive behaviour against *B. oryzae* by overgrowing the test pathogen through robust discharge of aerial conidia across the edges, thereby, delimiting the growth of pathogen. The mycoparasitic behaviour of potent *B. bassiana* isolates *viz.*, Bb31, Bb35, Bb52 were observed due to release of inhibitory diffusible substances, responsible for development of inhibition zone (1.1-1.4 cm) at their point of interaction (Plate 1).

Table 1: Per cent inhibition of *C. lunata* by *B. bassiana* isolates

Isolate code	Mycelial growth (mm)	*Inhibition (%)	**Bell's scale
Bb1	45.33	49.63 (44.70)	S3
Bb2	50.00	44.44 (41.76)	S4
Bb3	44.00	51.11 (45.61)	S3
Bb4	38.00	57.78 (49.45)	S3
Bb5	47.17	47.59 (43.60)	S4
Bb6	46.17	48.70 (44.23)	S3
Bb7	36.67	59.26 (50.31)	S3
Bb8	44.17	50.93 (45.51)	S3
Bb9	59.17	34.26 (35.80)	S4
Bb10	59.33	34.07 (35.69)	S4
Bb11	36.00	60.00 (50.74)	S2
Bb12	40.17	55.37 (48.06)	S3
Bb13	45.67	49.26 (44.55)	S3
Bb14	37.00	58.89 (50.10)	S2
Bb15	53.00	41.11 (39.86)	S4
Bb16	33.97	62.26 (52.08)	S2
Bb17	46.00	48.89 (44.34)	S3
Bb18	38.33	57.41 (49.24)	S3
Bb19	46.00	48.89 (44.34)	S3
Bb20	46.33	48.52 (44.13)	S3
Bb21	48.00	46.67 (43.04)	S4

Bb22	50.50	43.89 (41.47)	S4
Bb23	46.50	48.33 (44.02)	S3
Bb24	41.00	54.44 (47.53)	S3
Bb25	36.00	60.00 (50.74)	S2
Bb26	47.00	47.78 (43.70)	S4
Bb27	62.33	30.74 (33.65)	S4
Bb28	42.00	53.33 (46.89)	S3
Bb29	48.17	46.48 (42 (.96)	S4
Bb30	51.00	43.33 (41.15)	S4
Bb31	40.00	55.56 (48.17)	S3
Bb32	61.83	31.30 (34.00)	S4
Bb33	54.17	39.81 (39.10)	S4
Bb34	48.17	46.48 (42.96)	S4
Bb35	57.00	36.67 (37.25)	S4
Bb36	53.33	40.74 (39.64)	S4
Bb37	59.83	33.52 (35.35)	S4
Bb38	39.17	56.48 (48.70)	S3
Bb39	68.00	24.44 (29.61)	S5
Bb40	60.50	32.78 (34.90)	S4
Bb41	64.00	28.89 (32.49)	S5
Bb42	59.17	34.26 (35.80)	S4
Bb43	59.00	34.44 (35.92)	S4
Bb44	38.00	57.78 (49.45)	S3
Bb45	45.00	50.00 (44.98)	S3
Bb46	51.17	43.15 (41.04)	S4
Bb47	58.17	35.37 (36.47)	S4
Bb48	33.00	63.33 (52.71)	S2
Bb49	50.00	44.44 (41.79)	S4
Bb50	34.67	61.48 (51.62)	S2
Bb51	42.00	53.33 (46.89)	S3
Bb52	62.67	30.37 (33.42)	S4
Bb53	36.00	60.00 (50.74)	S2
Control	90.00	0.00 (0.28)	
SEm(±)	1.20	0.77	
CD0.05	3.38	2.18	

Figures in data are mean of three (3) replications; *Data within parentheses are angular transformed values; **Bell's Scale: S1- Antagonist completely overgrew pathogen, S2- Antagonist overgrew 2/3rd growth of pathogen, S3- Antagonist colonized half of pathogen, S4- Pathogen overgrew 2/3rd growth of antagonist and S5- Pathogen completely overgrew antagonist

Out of fifty-three (53) isolates, only fifeteen (15) isolates of *B. bassiana* were screened based on their antagonistic potential against *C. lunata* in dual culture assay for further study on production of volatile metabolites against *C. lunata* under *in vitro* condition.

Effect of volatile inhibitory substances against *C. lunata*

The ability of potential *B. bassiana* isolates to produce volatile inhibitory substances under *in vitro* condition was evaluated against *C. lunata* of rice by following inverted plate technique (Plate 2). Volatile diffusible substances derived from *B. bassiana* isolates exhibited growth inhibition of test pathogen was determined at 7th days post incubation, when control plate attained full growth (90 mm). Majority of the *B. bassiana* isolates (13) showed significant effect in reducing radial growth of *C. lunata* through the production of toxic volatile metabolites. The mycelial inhibition percentage (%) against *C. lunata* was found significantly higher with *B. bassiana* isolates *viz.*, Bb53 (67.78%) followed by Bb16 (61.11%), Bb25 (60.00%), Bb4 (57.78%), Bb44 (56.67%) and Bb13 (51.16%) against radial growth of 29.00 mm, 35.00 mm, 36.00 mm, 38.00 mm, 39.00 mm and 43.42 mm respectively (Table 2). Whereas, the mycelial inhibition percentage (%) of 13.70% and 17.78% was recorded with Bb48 and Bb28 against radial growth of 77.67 mm and 74.00 mm respectively, indicated no volatile metabolite production.

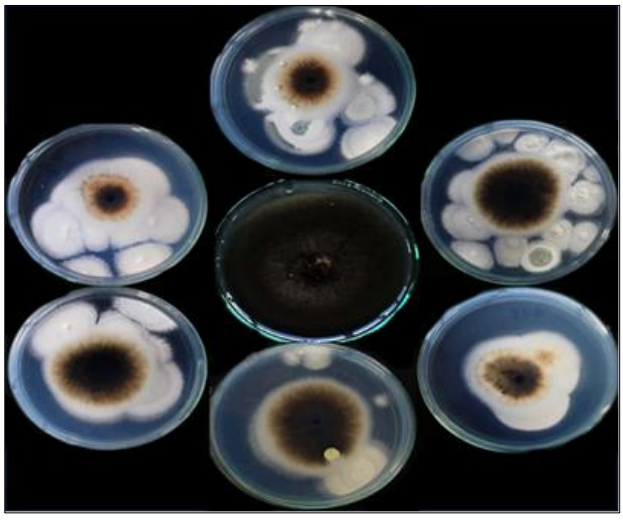

Plate 2: Inhibitory effect of volatile inhibitory metabolites released by *B. bassiana* in inverted plate assay against growth of *C. lunata*

Table 2: Per cent inhibition of *C. lunata* under the influence of volatile inhibitory substances released by *B. bassiana* isolates

Isolate code	Mycelial growth (mm)	*Inhibition (%)
Bb4	38.00^{hi}	57.78 (49.47)^{cd}
Bb5	48.33^{ef}	46.30 (42.87)^{fg}
Bb11	53.00^{d}	41.11 (39.87)^{i}
Bb13	43.42^{g}	51.76 (46.00)^{e}
Bb16	35.00^{j}	61.11 (51.42)^{b}
Bb17	49.00^{e}	45.56 (42.44)^{g}
Bb18	53.00^{d}	41.11 (39.87)^{i}
Bb25	36.00^{ij}	60.00 (50.76)^{bc}
Bb28	74.00^{c}	17.78 (24.83)^{j}
Bb31	49.33^{e}	45.19 (42.23)^{gh}
Bb44	39.00^{h}	56.67 (48.83)^{d}
Bb45	46.00^{fg}	48.89 (44.36)^{ef}
Bb48	77.67^{b}	13.70 (21.70)^{k}
Bb50	52.00^{d}	42.22 (40.52)^{hi}
Bb53	29.00^{k}	67.78 (55.42)^{a}
Control	90.00^{a}	0.00 (0.28)^{l}
SEm(±)	0.92	0.61
$CD_{0.05}$	2.66	1.78

Figures in data are mean of three (3) replications; *Data within parentheses are angular transformed values; Data followed by same alphabets are statistically at par

Discussion

B. bassiana operates more than one mechanism for antagonistic interaction as well as suppression of plant diseases through direct mechanisms *viz.,* mycoparasitism, competition and antibiosis or complex indirect interaction by stimulating induced systemic resistance as well as promotion of plant growth (Vega *et al.*, 2009; Ownley *et al.*, 2010) [23, 19]. It also includes production of toxins as well as secondary metabolites having insecticidal, antimicrobial and antioxidant properties, responsible for antibacterial and antifungal activities against plant pathogens. *B. bassiana* produces wide array of biologically active metabolites mainly volatile organic compounds (VOCs) *viz.*, diisopropyl napthalenes, ethanol, sesquiterpenes etc., alkaloids (tennelin, bassianin, pyridovericin, pyridomacrolidin), non-peptide pigment (oosporein), non-ribosomally synthesized cyclodepsipeptides (beauvericin, allobeauvericins) and cyclopeptides (beauveriolides) (Crespo *et al.*, 2008; Xu *et al.*, 2009) [3, 25]. Inhibition of mycelial growth through phenomenon of antibiosis by *B. bassiana* was reported against several phytopathogens *viz., Gaeumannomyces graminis* var. *tritici, Armallaria mellea, Rossellinia necatrix, Fusarium oxysporum, Botrytis cinerea, Phoma* sp., *Pythium ultimum* and *R. solani* (Ownley *et al.*, 2010) [19].

However, Azadi *et al.* (2016) [1] reported mycelial inhibition of *R. solani* was executed due to volatile compounds and culture filtrates containing secreted metabolites of *B. bassiana.* In the present study, diffusible inhibitory metabolites released by *B. bassiana* isolates was found to interact with hyphae of pathogens leading to collapse of mycelia before direct contact. However, similar findings were observed in case of *Trichoderma* spp. by Zeilinger and Oman (2007) [27] and John *et al.* (2010) [14]. Production of cell wall degrading enzymes (CWDEs) *viz.*, chitinases, glucanases, proteases, caesinases, lipases and cellulases by *B. bassiana* governs various physiological processes such as morphogenesis, parasitism and growth regulation, therefore, facilitating pathogenesis against wide range of phytopathogens (Griffin, 2007; Vega *et al.*, 2009; Jaber and Enkerli, 2016) [9, 23, 12]. In the present study, production of CWDEs by potential *B. bassiana* isolates led to the development of inhibition zone surrounding the colony through lysis of cell wall component of fungus *C. lunata*.

Conclusion

The native isolates of *B. bassiana viz.*, Bb16, Bb50, Bb25 and Bb53 were found to exhibit antagonistic activities against *C. lunata* causing seed-borne and leaf spot disease in rice in addition to their entomopathogenic behaviour. Biocontrol potential of native *B. bassiana* isolates as plant disease antagonist was found to be attributed towards varied mechanisms *viz.*, competition, production of volatile as well as non-volatile metabolites. However, the efficacy of the potential isolate must be further evaluated in field condition for their role in disease management, plant growth promotion and enhancement of crop yield. *B. bassiana* can also be established as dual purpose biocontrol agent due to their antagonistic potential against both insect pests and phytopathogens, though further studies need to be done in this respect.

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