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In vitro compatibility of entomopathogenic fungus, *Cladosporium cladosporioides* with three plant extracts

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Abstract: The *in vitro* compatibility of the entomopathogenic fungus, *Cladosporium cladosporioides* BOU1, with the crude extract of three plants viz. *Calotropis gigantea*, *Vitex negundo* and *Carissa macrocarpa* at a concentration of 1% and 2% was investigated. The compatibility of the fungal isolate with the plant extracts was calculated using the biological index formula from the germination percentage, radial growth and spore production of BOU1. The compatibility of the plant extracts with the fungal isolate was also assessed based on the protease and lipase activities of the treated fungus. Both concentrations of plant extracts did not significantly inhibit the germination percentage, radial growth and spore production of BOU1. The average mean values of the biological index for the three plant extracts with two concentrations were more than 98%. The analyses of the data with the compatibility index formula suggest that irrespective of concentration, all three plant extracts were compatible with BOU1. When the plant extracts were added to the fungal isolate, the protease and lipase activities of the fungal isolate were not significantly inhibited compared to the untreated control. The reduction in all the variables of fungal growth and the enzymatic activity was less than 10% and 20%, respectively. From these findings, we can conclude that the fungal biocontrol agent *C. cladosporioides* BOU1 is compatible with the investigated plant extracts in terms of the fungal growth and enzymatic activities.

Keywords: biocontrol agent; biological index; virulence; enzymatic activity; IPM

The entomopathogenic fungus, *Cladosporium cladosporioides*, is one of the effective biological control agents against insects and mites. It is an important natural enemy of arthropods capable of infecting them directly through the integument (Shahid et al. 2012). Some *Cladosporium* species are efficient as biological insecticides, particularly against insects that have developed resistance to chemical insecticides (Baky & Salam 2003). It is well known that the entomopathogenic fungus is effective against a wide spectrum of insect pests and is commonly utilised in integrated pest management (IPM) as well as biological control programmes (Bedini et al. 2018). A fungal biocontrol agent has several advantages over chemical controls, such as the low likelihood of resistance development (Gao et al. 2017), safety

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to non-target organisms (Roy & Pell 2000), decreased negative impacts on human health (Baltazar et al. 2014), and lower risks of environmental contamination (Goettel & Johnson 1994).

Plant extracts and phytochemicals have already been recognised as antifeedants, repellents and insecticides (Rajendran & Sriranjini 2008). Plant extracts with hundreds of identified and isolated active substances are considered alternatives to synthetic chemicals for pest management against a wide range of pests (Isman 2006; Regnault-Roger & Philogène 2008). Because of the presence of the diverse chemical arsenal of secondary metabolites in plant extracts, the application of these natural materials limit damage in plants inflicted by herbivorous insects and plant pathogens (Jaber et al. 2018). *Calotropis gigantea*, *Vitex negundo* and *Carissa macrocarpa* are native medicinal plants in Bangladesh well-known for their insecticidal principles. The insecticidal activity of the *C. gigantea* L. flower extract (Habib & Karim 2016) and root bark extract (Alam et al. 2009) against *Tribolium castaneum* Herbst has been demonstrated. The extracts of *Calotropis* sp. are known to inhibit the stem and root borer of rice, soil-dwelling insects, the cotton caterpillar and sucking insects of sugarcane and the mustered saw fly (*Athalia proxima*) (Srivastava et al. 2006). Extracts of *C. gigantea* are inhibitory against many crop pests resistant to synthetic compounds (Mandepudi et al. 2012). Similarly, *V. negundo* extracts are effective in controlling *T. castaneum* (Haridasan et al. 2017) and *Aedes aegypti* (Chandrasekaran et al. 2019). *C. macrocarpa* extracts can control *Aphis fabae* Scopoli (Mohmed 2019).

By combining the entomopathogenic fungus with plant extracts, there is an increase in the efficiency and an acceleration in the insect mortality (Serebrov et al. 2005; Purwar & Sachan 2006; Asi et al. 2010). The appropriate use of an entomopathogenic fungus and plant extracts can play a significant role in sustainable crop production by providing a stable pest management programme. The technology of IPM operates throughout the world as a part of the management of pests in agriculture, forestry and greenhouse horticulture. With the increasing resistance of many insect pest species to chemical insecticides, pest control strategies are slowly shifting towards more sustainable, ecologically sound and economically viable options (Chidawanyika et al. 2012). Isman (2006) reported that the entomopathogenic fungus can be better applied in combination or rotation with plant extracts, rather than as a stand-alone. To reduce the chemical insecticides for pest control and minimise the environmental hazard, a biologically based pest management strategy is needed (Chidawanyika et al. 2012).

Integrated pest management (IPM) is a sustainable approach to crop protection using a decision support system to select pest control tactics, and integrate them into a management strategy, and environmental impacts (Kogan 1998). For the successful establishment of an IPM programme using a biocontrol agent with a plant extract, it is a prerequisite to evaluate the *in vitro* compatibility between the factors before being used in field conditions. Therefore, the present study was conducted to investigate the *in vitro* compatibility of *C. cladosporioides* BOU1 with three plant extracts viz. *C. gigantea*, *V. negundo* and *C. macrocarpa*.

MATERIAL AND METHODS

Study organism and plant extracts. *Cladosporium cladosporioides* (Davidiellaceae, Capnodiales) (Fresen.) de Vries, BOU1 is a novel fungal biocontrol agent that was screened by Islam et al. (2019), which was originally isolated from the brown planthopper, *Nilaparvata lugens* (Stål) (Hemiptera, Delphacidae), of rice from a farmer's rice field in Gazipur, Bangladesh; and characterised by a molecular analysis (GenBank accession number MG654669). The fungus was cultured on a PDA (potato dextrose agar) medium. The leaves of three plants, *Calotropis gigantea* (Family: Apocynaceae), *Vitex negundo* (Family: Lamiaceae) and *Carissa macrocarpa* (Family: Apocynaceae), were obtained from the Botanical Garden of Bangladesh Agricultural University, Mymensingh, Bangladesh. The leaves were dried in the shade, and 50 g of each dried plant leaves were pulverised using a mechanical blender. The powdered dried leaves were dissolved in 500 mL ethyl acetate and kept at room temperature for three days with occasional shaking. The ethyl acetate extracts were filtered and the filtrates were evaporated to dryness *in vacuo* using a rotary evaporator at 45 °C. The ethyl acetate extracts were dissolved in 50 mL MeOH and partitioned with *n*-hexane to remove the low polar oily substances. After partitioning, the *n*-hexane fraction was discarded and the MeOH

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fraction was evaporated *in vacuo* to dryness. After drying, 113, 130 and 124 mg extracts were obtained from *C. gigantean*, *V. negundo* and *C. macrocarpa*, respectively. These extracts were used for further studies.

Compatibility calculation. The compatibility of the biocontrol agent, *C. cladosporioides* BOU1 at two concentrations viz. 1% and 2% of three plant extracts, *C. gigantea*, *V. negundo* and *C. macrocarpa*, was estimated from the germination percentage of the conidia, the radial growth of the mycelia and the spore production containing 1 mL of the plant extract with the PDA into each Petri dish. The germination percentage of the conidia was assessed by inoculating 0.5 mL of the conidial suspension (1×10^6 conidia/mL) in each Petri dish containing PDA. The Petri dishes were incubated at 25 ± 1 °C, 70 ± 10% relative humidity and a light : dark of 12 : 12 photoperiod for 24 hours. Three separate microscopic fields on the Petri dishes were observed for germination at 40× magnification for each treatment and 100 conidia were observed randomly in each field. For the radial growth and spore production, the conidia of *C. cladosporioides* were suspended in the respective aqueous solution (1×10^5 conidia/mL) containing PDA into each Petri dish. The isolate was point inoculated into the centre of the agar with a 5 µL conidial suspension and the Petri dishes were incubated the same as the germination assay. The measurements of the radial growth of the mycelia were investigated after 14 days of inoculation. The conidia from six randomly selected plates from each treatment were dislodged by 50 mL of 0.02% Tween-80 and agitation for 15 minutes. Then, the conidia were counted using a compound microscope with a haemocytometer. Thereafter, the *in vitro* compatibility of the plant extracts with the fungal isolate was calculated using the biological index (*BI*) formula according to Rossi-Zalaf et al. (2008) as:

$$BI = \frac{47 \times VG + 43 \times SPO + 10 \times GERM}{100} \quad (1)$$

where: *BI* – biological index; *VG* – percentage of vegetative growth compared to the control; *SPO* – percentage of sporulation compared to the control; *GERM* – percentage of conidia germination compared to the control.

The effect of the plant extracts on the fungal isolate was classified based on: toxic = *BI* between 0 and 41; moderately toxic = *BI* between 42 and 66; and compatible = *BI* greater than 66.

Enzymatic activities of biocontrol agent to plant extracts. The activities of two enzymes, viz. protease and lipase of *C. cladosporioides*, to three plant extracts at two concentrations of 1% and 2% were evaluated for the virulence of this biocontrol agent (Islam et al. 2019). Without the plant extract served as the control. For both enzyme assays, 150 mL Erlenmeyer flasks containing 50 mL of PDA were sterilised by autoclave. After cooling, 5 mL of the fungal suspension (1×10^7 conidia/mL) was inoculated to each flask and incubated at 37 °C with continuous shaking at 180 rpm for five days; thereafter, the individual enzyme activity was determined as described by Islam et al. (2019).

Statistical analysis. The experiment was carried out in a complete randomised design (CRD) with six replications. Data on the germination percentage, radial growth and spore production were analysed with a two-way analysis of variance (ANOVA) between three plant extracts and two concentrations (plant extract × concentration). Data on the biological index were analysed with a one-way ANOVA. The mean value of the control treatment of three growth parameters and two enzymatic activities of *C. cladosporioides* were calculated from six replicates by MS Excel. Thereafter, the reduction percentages were calculated replicate wise individually as:

$$\%\text{Reduction} = \frac{\text{control} - \text{treatment}}{\text{control}} \times 100 \quad (2)$$

After calculating the reduction percentage of all the variables of *C. cladosporioides*, they were analysed individually with a one-way ANOVA. The statistical analyses were performed using SAS software (SAS Institute, Inc., version 3.6). The means were separated using the least significant difference test at a 5% level of significance.

RESULTS

Both concentrations of the plant extracts did not significantly inhibit the germination percentage, radial growth and spore production of BOU1 in a dose-depended manner. The interaction of the factors, i.e., plant extract × concentration was non-significant on the conidial germination (%), radial

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Table 1. Interaction (plant extract × concentration) effect of the three plant extracts, *Calotropis gigantea*, *Vitex negundo* and *Carissa macrocarpa*, with two concentrations, 1% and 2%, on the germination percentage, radial growth (cm) and spore production (1×10^5 conidia/mL) of the fungal isolate *Cladosporium cladosporioides* BOU1

Variable	*F*-value	df	*P*-value
Germination	0.773	7, 47	0.58
Radial growth	0.934	7, 47	0.35
Spore production	2.521	7, 47	0.062

Data were analysed with a two-way ANOVA between three plant extracts and two concentrations (least significant difference test, $P < 0.05$)

growth of mycelia and spore production of *C. cladosporioides* (Table 1). The germination (%) of *C. cladosporioides* exposed to all the plant extracts in both concentrations was higher than 95% (tabulated data were not shown). Incorporating the plant extracts into the PDA medium insignificantly reduced the mean radial growth and spore production of the fungal isolate incubated for 14 days (the tabulated data are not shown). The reduction (%) in the growth parameters of the fungal isolate were calculated from all the treatments over the control one. The reduction (%) in the germination (%) ($F = 5.13$; df = 5, 35; $P = 0.001\ 6$); radial growth ($F = 2.16$; df = 5, 35; $P = 0.017$); and spore production ($F = 7.18$; df = 5, 35; $P = 0.000\ 2$) of the fungal isolate were significantly different among the treatments (Table 2). Significant differences were also observed on the biological index among the treatments ($F = 2.75$; df = 5, 35; $P = 0.036\ 7$). The mean values of the biological index for all three plant extracts with two concentrations were more than 98%. According to the compatibility index formula, all three plant extracts with two concentrations are compatible with *C. cladosporioides* (Figure 1).

The physiological responses of the biocontrol agent *C. cladosporioides* to the two doses, 1% and 2% of the plant extracts, of *C. gigantean*, *V. negundo* and *C. macrocarpa* were assessed based on the protease and lipase activities of the treated fungus. When the plant extracts were added into the fungal isolate, the physiological responses as well as protease and lipase activities of fungal isolate were a little bit inhibited when compared with their respective controls. The one-way ANOVA results showed a reduction in the percentages of the protease ($F = 16.34$; df = 5, 35; $P < 0.000\ 1$) and lipase ($F = 16.26$; df = 5, 35; $P < 0.000\ 1$) activities were significantly different among the treatments (Table 2). The reduction in all the variables of the fungal growth and the enzymatic activity was less than 10% and 20%, respectively.

DISCUSSION

A biocontrol agent is an essential component of an integrated pest management (IPM) programme

Table 2. Effect of the three plant extracts, *Calotropis gigantea*, *Vitex negundo* and *Carissa macrocarpa*, with two concentrations, 1% and 2%, on the reduction percentage (± standard error) of three growth parameters, germination percentage, radial growth (cm) and spore production (1×10^5 conidia/mL); and two enzymatic activities, protease and lipase, of the fungal isolate *Cladosporium cladosporioides* BOU1 over the control

Plant extract	%Reduction of fungal growth and enzymatic activity of *C. cladosporioides*				
	fungal growth			enzymatic activity	
	germination	radial growth	spore production	protease	lipase
C. gigantea (1%)	3.38 ± 0.03^{b}	4.59 ± 0.10^{b}	3.6 ± 0.09^{b}	9.61 ± 0.76^{b}	7.82 ± 0.59^{b}
C. gigantea (2%)	6.09 ± 0.06^{a}	5.82 ± 0.08^{ab}	9.44 ± 0.11^{a}	15.78 ± 1.13^{a}	14.59 ± 1.11^{a}
V. negundo (1%)	3.72 ± 0.05^{b}	5.21 ± 0.08^{b}	5.67 ± 0.22^{b}	9.51 ± 0.82^{b}	7.82 ± 0.62^{b}
V. negundo (2%)	6.09 ± 0.08^{a}	5.88 ± 0.10^{ab}	9.18 ± 0.06^{a}	16.47 ± 1.06^{a}	18.34 ± 1.06^{a}
C. macrocarpa (1%)	4.06 ± 0.07^{b}	5.21 ± 0.06^{b}	5.28 ± 0.05^{b}	9.84 ± 0.85^{b}	7.09 ± 0.65^{b}
C. macrocarpa (2%)	6.43 ± 0.06^{a}	7.40 ± 0.08^{a}	8.89 ± 0.08^{a}	18.14 ± 1.08^{a}	17.67 ± 1.08^{a}

^{a,b}Means with the same letter within the same column are not significantly different (least significant difference test, following a one-way ANOVA: $P < 0.05$)

The reduction percentages of the variables were calculated as: %reduction = [(control – treatment)/control] × 100, for details, please see the Material and methods section

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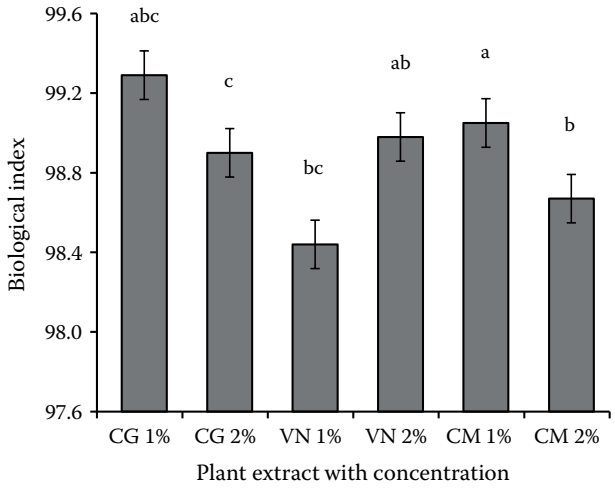

Figure 1. Biological index (*BI*) on the average (± standard error) of the *Cladosporium cladosporioides* isolate BOU1 with three plant extracts, *Calotropis gigantea* (CG), *Vitex negundo* (VN) and *Carissa macrocarpa* (CM), of two concentrations, 1% and 2%, according to Rossi-Zalaf et al. (2008) over the control

[a–c]Means with the same letter within the same bar are not significantly different (least significant difference test, following a one-way ANOVA: $P < 0.05$)

The toxicity classifications are: *BI* between 0 and 41 = toxic; *BI* between 42 and 66 = moderately toxic and *BI* higher than 66 = compatible. All the *BI* values were more than 66; therefore, all the concentrations of the plant extracts are compatible with the fungal isolate *C. cladosporioides* BOU1

in multiple ecosystems that can provide more comprehensive management than any single approach in the IPM (Chidawanyika et al. 2012; Roubos et al. 2014). The integrated use of plant extracts with a biocontrol agent can enhance the efficacy of any insect pest control. When the *C. cladosporioides* isolate was exposed to the both the 1% and 2% concentrations of the three plant extracts, *C. gigantea*, *V. negundo* and *C. macrocarpa*, the three major virulence parameters of the fungal isolate, viz. the conidia germination (%), radial growth of mycelia and spore production, were slightly affected. However, according to the value of the biological index, these two different types of pest control agents were compatible. An average mean value of the biological index ranging from 98.16 to 99.01 was observed in our study. A similar value in the biological index between a neem extract and *Beauveria bassiana* using the formula of Alves et al. (1998) ranged from 78.58 to 90.38 (Wisuda et al. 2019) and 68.7 to 86.6 (Nana et al. 2016). Based on the compatibility index using the formula of Rossi-Zalaf et al. (2008), the values of the biological index ranged from 80.52 to 106.10 for *B. bassiana*; from 93.43 to 155.56 for *Isaria fumosorosea* and from 64.83 to 96.86 for *Metarhizium anisopliae* (Ribeiro et al. 2014).

The radial growth of the mycelia, conidial yield, mycelia dry weight and conidial viability were not affected by the aqueous leaf extract of *Calpurnia aurea* at all by the used concentrations of *M. anisopliae*. Therefore, that biocontrol agent was considered compatible with the *C. aurea* extract (Nana et al. 2016). A similar phenomenon was employed by Mohan et al. (2007) and Ribeiro et al. (2012) concerning *B. bassiana* with the *Azadirachta indica* extract. Ribeiro et al. (2014) reported that the three entomopathogenic fungi, *B. bassiana*, *I. fumosorosea* and *M. anisopliae* were compatible with the *Annona mucosa* Jacq. seed extract. On other hand, the higher concentration viz. 5% or greater than the 5% active ingredient of the commercial formulation of *A. indica* has a significant inhibitory effect on the germination, vegetative growth and conidiogenesis of *B. bassiana* (Castiglioni et al. 2003). The spore density and growth of *B. bassiana* increased from 6% to 17% and 19% to 23%, respectively, due to the addition of a neem extract on the fungal colony, meaning that the *B. bassiana* was compatible with the neem extract (Wisuda et al. 2019). The different species of entomopathogenic fungus with different species of plant extract, viz. *B. bassiana* with a *V. negundo* L. extract (Sahayaraj et al. 2011); *M. anisopliae* with an *A. indica* L. extract (Schumacher & Poehling 2012); *Fusarium solani*, *M. robertsii*, *Nigrospora sphaerica* and *Penicillium citrinum* with an *A. indica* L. extract (Hernandez-Trejo et al. 2019); *B. bassiana* and *M. anisopliae* with *A. indica* L. and *Eucalyptus camaldulensis* D. extracts (Ali et al. 2018); *B. bassiana* and *M. brunneum* with *C. procera* and *Inula viscosa* extracts (Jaber et al. 2018) were also compatible. Considering the finding of these previous reports, our experimental findings suggest that BOU1 is compatible with extracts of the medicinal plants tested in this study.

The fungal growth, such as the radial growth, germination percentage and spore production of the biocontrol agent, is not enough to evaluate the virulence activity as, sometimes, the variation of these variables depend on environmental factors. Therefore, the combination of the fungal growth and the physiological mechanism is much better than

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as just a stand-alone. The internal activity, such as the enzymatic activity, indicates the aliveness of any living organism, like an entomopathogenic fungus, which was also found as a virulence indicator of a biocontrol agent (Castellanos-Moguel et al. 2007). The reduction in the protease and lipase activity of *C. cladosporioides* was as high as 18.14% and 18.34%, respectively. These results indicate that the fungal isolate was not completely inhibited by the three plant extracts with two tested concentrations. Furthermore, the fungal enzymes were active enough in the presence of the plant extracts. Based on the mycelial growth, the spore germination and enzymatic activities of *C. cladosporioides* BOU1 in the presence of the MeOH extracts of three medicinal plants, viz. *C. gigantea*, *V. negundo* and *C. macrocarpa*, we conclude that BOU1 is compatible with the tested plant extracts.

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