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RESEARCH ARTICLE

Biological control of *Botrytis cinerea* and plant growth promotion potential by *Penicillium citrinum* in chickpea (*Cicer arietinum* L.)

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A total of 48 fungi were characterised for their antagonistic potential against *Botrytis cinerea* causing Botrytis Gray Mold (BGM) disease in chickpea by dual culture and metabolite production assays. The culture filtrate of the most promising isolate, VFI-51, was purified by various chromatographic techniques and identified as 'citrinin' by nuclear magnetic resonance and mass spectrometry studies. The efficacy of citrinin was demonstrated to control BGM in chickpea under greenhouse conditions. The sequences of 18S rDNA gene of the VFI-51 matched with *Penicillium citrinum* in BLAST analysis. The VFI-51 produced siderophore, hydrocyanic acid, indole-3-acetic acid, lipase, protease and β-1,3-glucanase; grew well in NaCl (up to 15%), at pH between 7 and 11 and temperatures between 20°C and 40°C; and was compatible with fungicides bavistin and thiram. Under greenhouse and field conditions, VFI-51 significantly enhanced the nodule number, nodule weight, root and shoot weight and stover and grain yield over the un-inoculated control. In the rhizosphere, VFI-51 also significantly enhanced total N, available P and OC over the un-inoculated control. Scanning electron microscopy analysis revealed that VFI-51 colonised on the roots of chickpea. This study concluded that VFI-51 has the potential for biocontrol of BGM and plant growth promotion in chickpea.

Keywords: *Penicillium citrinum*; citrinin; biocontrol; Botrytis Gray Mold; plant growth promotion; chickpea

Introduction

Chickpea (*Cicer arietinum* L.) is an important grain legume crop after common bean (*Phaseolus vulgaris* L.) and garden pea (*Pisum sativum* L.) on a world basis but of first importance in the south-east Asia and Mediterranean basin, with a total production of 11.6 M t from an area of 13.2 M ha and a productivity of 880 kg ha^{-1} (FAOSTAT, 2011). Among the south-east Asian countries, India is the largest chickpea growing country. The chickpea has been divided into '*desi*' (microsperma) and '*kabuli*' (macrosperma). Consumption of *desi* is restricted to South Asia and the

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middle east while *kabuli* is a popular around the world. Chickpea seed is mainly used as food because of its high protein (12–31%) and carbohydrate (52–71%) contents (Awasthi, Abidi, & Chowdhury, 1991). Productivity of chickpea may be improved if the adverse effects of abiotic (cold, heat, salinity and drought) and biotic [insect pests and diseases including Ascochyta blight, Fusarium wilt, collar rot and Botrytis Gray Mold (BGM)] stresses are reduced.

Botrytis cinerea Pers. ex. Fr. is a broad host range fungus that causes BGM. *B. cinerea* affects more than 200 agriculturally important plant species, including chickpea, which can devastate the crop up to 100% (Pande et al., 2005). Management of BGM is not easy as no single control measure is fully effective. Late sowing, wider spacing, intercropping with cereals or oil seed crops and use of pathogen-free seed are some of the cultural measures employed to control BGM (Pande et al., 2006), but with limited success. Seed treatment with fungicides such as thiram, captan, mancozeb and thiabendazole is effective in controlling BGM but such expansive fungicides are not widely used by the poor farmers. The most economical and efficient control measures against BGM are the use of resistant cultivars. However, the effectiveness of resistant cultivars is restricted by the adaptability and high genetic diversity of the pathogen, *B. cinerea*, which poses a risk of breakdown of host plant resistance (Pande et al., 2006). Biological control agents including essential oils such as oregano, thyme, dictamnus and marjoram and antagonistic microbes such as *Trichoderma viridae* and *T. harzianum* are reported to control BGM effectively (Agarwal & Tripathi, 1999; Daferena, Ziogas, & Polissiou, 2003).

Biological control agents are known to found commonly in forests, pasture soil, rhizosphere soil and compost/vermicompost (Tinatin & Nurzat, 2006; Torsvik, Ovreas, & Thingstad, 2002). Vermicompost not only benefiting crop plants by mobilising and acquiring nutrients from soil but also inhibiting phytopathogens (Gopalakrishnan, Humayun, et al., 2011; Gopalakrishnan, Pande, et al., 2011; Postma, Montanari, & Van den Boogert, 2003; Suthar, Choyal, Singh, & Sudesh, 2005). Such plant growth-promoting (PGP) microbes facilitate plant growth either by directly (by nitrogen fixation, phosphate solubilisation, iron chelation and phytohormone production) or by indirectly (by inhibition of phytopathogens) and thus promoting plant growth and development. PGP microbes have been widely reported to enhance productivity of various crops (Figueiredo, Martinez, Burity, & Chanway, 2008; Gopalakrishnan, Srinivas, et al., 2014; Vessey, 2003). The main objectives of this study were to isolate, characterise and evaluate fungi from vermicomposts and rhizosphere soils of chickpea for their ability to inhibit BGM disease and to promote plant growth in chickpea.

Materials and methods

Preparation of botanical vermicompost

Foliages of five different botanicals (*Jatropha curcas*, *Annona squamosa*, *Parthenium hysterophorus*, *Gliricidia sepium* and *Azadirachta indica*) were collected from ICRISAT Patancheru, air-dried at room temperature (30 ± 2°C) and vermicomposted as per the protocols of Gopalakrishnan, Pande, et al. (2011).

Isolation of fungal strains from botanical vermicomposts and chickpea rhizosphere

Ten grams of either botanical vermicompost from each of the five samples or soils from chickpea rhizosphere were separately suspended in physiological saline (0.85% NaCl in water; 90 ml) in a flask and placed on an orbital shaker (at 100 rpm) at room temperature (28 ± 2°C) for 1 h. At the end of shaking, the samples were serially diluted up to 10^5 dilutions with physiological saline. Dilutions 10^3–10^5 were plated on potato dextrose agar (PDA) by spread plate technique and incubated at 28 ± 2°C for 4 days. The most prominent fungal colonies (the ones which were found produced pigments, inhibited the adjacent colonies and abundantly in the plate) were isolated and maintained on PDA slants at 4°C for further studies.

In vitro *antagonistic activity of fungal isolates against* B. cinerea

Fungal isolates were evaluated for their antagonistic activity against *B. cinerea* (acquired from legumes pathology, ICRISAT Patancheru) by dual-culture assay. In brief, a disc (6 mm diameter) of *B. cinerea* was placed on one edge (1 cm from the corner) of the PDA plate and a test fungal isolate was streaked on the other edge of the plate (1 cm from the corner). The plates were incubated at 28 ± 2°C for 6 days. The inhibition of *B. cinerea* was noted by measuring halo zone around the test fungal isolate.

***Effect of the culture filtrates of the test fungal isolate on* B. cinerea**

Culture filtrates (1 l) of the most promising fungus against *B. cinerea*, in the dual-culture assay, were prepared from 5-day-old cultures grown in starch casein broth (SCB) by collecting the supernatants after centrifuging the cultures for 20 min at 10,000 *g*. After adjusting the pH to 3.0, the supernatants were partitioned three times against ethyl acetate (EtOAc) resulting in organic and aqueous fractions. The organic fractions were combined, dried over anhydrous sodium sulphate and the EtOAc removed by film evaporation on a rotary evaporator (BUCHI V-850, Switzerland) at 35°C. The residues were dissolved in methanol (30 ml) and stored in a freezer at −20°C. Similarly, the aqueous fractions were combined, evaporated on a rotary evaporator at 35°C, dissolved in methanol (10 ml) and stored in a freezer at −20°C. Both the organic and aqueous samples were evaluated for their antagonistic potential against *B. cinerea*. For this, PDA plates were prepared by incorporating both organic and aqueous samples at a concentration of 0.5% (final volume) whereas control plates contained 0.5% methanol. A disc (6 mm) of *B. cinerea* bored from the actively growing plate was kept at the centre of the organic/aqueous samples incorporated PDA plate and incubated at 28 ± 2°C for 5 days. At the end of incubation, intensity of growth of fungus was measured and compared with control.

***Isolation of the active metabolites of the test fungal isolate antagonistic to* B. cinerea**

Culture filtrates (10 l) of the most promising fungus against *B. cinerea* were partitioned against EtOAc as described previously and the resultant organic (EtOAc) fractions were evaporated and collected in methanol (10 ml). A fraction of this (500 µl) was retained for assay and the remainder (9.5 ml) was injected onto a flash chromatography column packed with silica (30 g). The column was eluted with 500 ml of *n*-hexane followed by 500 ml each of incremental EtOAc (10–100%) concentrations in *n*-hexane. Each incremental fraction was evaporated on a rotary

evaporator and collected separately in a minimal volume of acetone (10 ml) and further assayed for their antagonistic potential against *B. cinerea* as described previously. The active fraction in the assay was evaporated and the sample sent to nuclear magnetic resonance (NMR) and mass spectrometry (MS) analysis for structural elucidation.

NMR and MS analysis

NMR data were recorded using a Bruker Avance 600 spectrometer operating at a proton frequency of 600.18 MHz with a 5 mm triple-resonance cryo probe equipped with a *z* gradient. The samples containing a solution of 10 mg of substance in $CDCl_3$ were measured at 298 K, using solvent signal as a reference. Following 1D and 2D pulse sequences from the Bruker user library was used for the NMR experiments: ^{1}H 1D (600 MHz), ^{13}C 1D (150 MHz), HSQC (600/150 MHz), HSQC–TOCSY (600/150 MHz), HMBC (600/150 MHz), DQF–COSY (600 MHz) and NOESY 2D (600 MHz).

Liquid chromatography–mass spectrometry (LC–MS) analysis was performed on a Waters Acquity UPLC system connected to a Waters Synapt G2-S High definition QTOF mass spectrometer. The QTOF was operated in positive Electrospray Ionization mode using leucine enkephalin as lock mass compound. Both crude and purified extracts were separated on an Acquity UPLC BEH C18 1.7 mm, 2.1 × 50 mm column using water with 0.01% formic acid as mobile phase A and acetonitrile with 0.01% formic acid as mobile phase B. The following gradient was used: 0–0.3 min: 100% A, 0.3–6 min: linear gradient from 100% to 5 5A, 6–6.1: back to 100% A, end after 8 min. One microliter of the sample was injected and a mobile phase flow rate of 0.4 ml min^{-1} was used.

Effect of the purified compound for its antagonistic potential against **B. cinerea**

Seedlings of the BGM susceptible chickpea genotype ICC 4954 were grown in plastic pots filled with sterilised vermiculite and sand mixture (10:1) in a greenhouse, maintained at 25 ± 2°C for 10 days. The greenhouse trial was carried out in a completely randomised design with three replications (five plants/replication) and repeated once. A total of eight treatments were made which include: (1) control; (2) only *B. cinerea* spray at 0 h; (3) only purified compound spray at 0 h; (4) *B. cinerea* and purified compound spray at 0 h; (5) purified compound and *B. cinerea* spray at 0 h; (6) *B. cinerea* spray at 0 h while purified compound spray at 24 h; (7) *B. cinerea* spray at 0 h while purified compound spray at 48 h; and (8) *B. cinerea* spray at 0 h while purified compound spray at 72 h. Autoclaved flowers of *Tagetes erecta* (marigold) were used for mass multiplication of *B. cinerea* at 15 ± 1°C for 8 days with 12 h photoperiod. At the end of incubation, conidia of *B. cinerea* were collected into sterile distilled water and this conidial suspension (3×10^5 ml^{-1}) was used as inoculum. Ten-day-old seedlings of chickpea were transferred into a plant growth chamber maintained at 15 ± 1°C with a 12 h photoperiod of 2500–3000 lux intensity and 95–100% relative humidity for 24 h for acclimatisation. At the end of 24h incubation in the growth chamber, the seedlings were inoculated by spraying the inoculum of *B. cinerea*/purified compound (8 ppm), depending upon the treatment, on the foliage until run off using a hand-operated atomizer. The plants were kept in the growth chamber for 20 more days. At the end of incubation, the severity of the

disease was recorded using a 1–6 rating scale where, 1 is no infection on any part of the plant and 6 is extensive soft rotting, fungal growth on more than 70% of the leaves, branches and stems (Pande, Sharma, Nagavardhini, & Rameshwar, 2012).

Identification of the antagonistic fungus

Pure culture of the selected antagonistic fungus against *B. cinerea* was grown in SCB for 4 days and genomic DNA was isolated as per the protocol of Bazzicalupo and Fani (1995). The amplification of 18S rDNA gene was done with universal primers 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T-3′) and 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) according to the conditions described earlier (Pandey, Kang, & Maheswari, 2005). The polymerase chain reaction product was sequenced at Macrogen Inc. Seoul, Korea and the sequences were compared with those from the GenBank using the BLAST program (Altschul, Gish, Miller, Myers, & Lipman, 1990), aligned using the ClustalW software (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997), and phylogenetic trees inferred using the neighbour-joining method (Saitou & Nei, 1987) in the MEGA version 4 program.

Evaluation of the fungus for its biocontrol and PGP traits

The most promising fungus against *B. cinerea* was evaluated for production of biocontrol and PGP traits including siderophore, chitinase, lipase, protease, hydrocyanic acid (HCN), indole acetic acid (IAA) and β-1,3-glucanase. Siderophore and chitinase production was estimated on chrome azurol S agar (Schwyn & Neilands, 1987) and chitin agar (Hirano & Nagao, 1988), respectively. The lipase and protease production was done according to the previously published protocols (Bhattacharya, Chandra, & Barik, 2009). HCN was qualitatively assessed by sulfocyanate colorimetric method (Gopalakrishnan, Srinivas, et al., 2014; Lorck, 1948). All the treatments were replicated three times and the experiment was repeated three times. Observations of the selected fungus to siderophore, chitinase, lipase and protease were recorded on a 0–4 rating scale as follows: 0 = no halo zone; 1 = halo zone of <1 mm; 2 = halo zone of 1–3 mm; 3 = halo zone of 4–6 mm; 4 = halo zone of 7–9 mm and above. Observations of the selected fungus to HCN were recorded on a 0–3 rating scale (based on the intensity of the reddish brown colour) as follows: 0 = no colour; 1 = light reddish brown; 2 = medium reddish brown; and 3 = dark reddish brown.

IAA was quantitatively estimated by measuring the absorbance in a spectrophotometer at 530 nm (Patten & Glick, 1996). A standard curve was plotted to quantify the IAA (μg ml^{-1}) present in the culture filtrate. β-1,3-glucanase was done as per the protocols of Singh, Shin, Park, and Chung (1999) and Gopalakrishnan, Srinivas, et al. (2014) and is expressed as units, where one unit of β-1,3-glucanase activity was explained as the amount of enzyme that liberated 1 μmol of glucose h^{-1} at defined conditions. Both the IAA and β-1,3-glucanase tests were replicated three times and the experiment was repeated three times.

Evaluation of the fungus for its physiological traits

The most promising fungus against *B. cinerea* was evaluated for its physiological traits including salinity, pH, temperature and resistance to fungicides as per the protocols prescribed previously (Gopalakrishnan, Srinivas, et al., 2014). In brief, the

selected fungus was streaked on Bennett's agar having various concentrations of NaCl (0–25% at an interval of 5%), pH (3–13 at the interval of two units) and temperatures (20°C, 30°C and 40°C). The plates were incubated at 28°C (except for temperature traits) for 5 days and at the end of incubation, the intensity of growth was measured. For 50°C, the Bennett's broth was inoculated with the selected fungus and at the end of the 5-day incubation, the intensity of growth was measured at 600 nm in a spectrophotometer. The selected fungus was also evaluated for its tolerance to fungicides including bavistin, captan, ridomil, thiram and benlate at field application levels of 2500, 3000, 3000, 3000, 3000 and 4000 ppm concentrations, respectively. The plates were incubated at 28°C for 5 days and the intensity of growth was measured at the end of incubation. There were three replications for each test and the experiment was done thrice. Observations of the selected fungus to salinity, pH, temperature and fungicide tolerance were recorded as follows: 0 = no growth, 1 = little growth, 2 = medium growth and 3 = good growth.

Evaluation of the fungus for PGP potential on chickpea under greenhouse and field conditions

The selected test fungus was evaluated for its PGP potential under greenhouse conditions on chickpea as monoculture. Pot mixture (1000 g) was prepared by mixing black soil, river sand and farmyard manure at 3:2:2 and placed in plastic pots (20 cm diameter). Chickpea seeds [ICCV-2 (Kabuli type), which matures in 90 days] were surface sterilised with sodium hypochlorite (2.5% for 3 min) and rinsed thoroughly (8 times) with sterilised water. The sterilised seeds were transferred into the test fungal culture broth (10^8 CFU ml^{-1}; grown in SCB separately) and incubated for 45 min. At the end of incubation, the inoculated seeds were sown (six seeds/pot) at a depth of 2.5 cm in the pot and after a week, a plant population of three/pot was maintained. Booster doses of test fungus (5 ml pot^{-1}, 10^8 CFU ml^{-1}) were applied at 15, 30, 45 and 60 days after sowing (DAS) by soil drench method. Control pots contained no test fungus. The pot experiment was carried out with six replications. Plants were irrigated once every 5 days with 30 ml sterilised water. At the end of 30 DAS, PGP parameters including plant height, leaf area, leaf weight, stem weight, root length and root volume were determined whereas at crop maturity, stem weight, pod weight and pod number were recorded.

The selected test fungus was also evaluated for its PGP potential on chickpea under on-station field conditions. The field trial was conducted in 2013−2014 post-rainy (Rabi) cropping season at ICRISAT Patancheru, India, with chickpea variety ICCV-2, which normally yields 1.1−1.2 t ha^{-1}. Soils at the experimental site are classified as Vertisol having 25% sand, 20% silt and 53% clay with alkaline pH (7.5−7.8) and organic carbon content of 0.4–0.5%. The mineral content of the top 15 cm soil includes 27.0 mg kg^{-1} soil of available N, 9.7 mg kg^{-1} soil of available P and 290 mg kg^{-1} soil of available K. The experiment was laid out in a plot size of 4 m × 3 ridges in a randomised complete block design with three replications. The test fungus was grown separately on SCB at 28°C for 5 days. Chickpea seed was incubated with the test fungus (containing 10^8 CFU ml^{-1}) for 50 min and sown on 2 November 2013 at a row-to-row spacing of 60 cm and a plant-to-plant spacing of 10 cm. The test fungus (1000 ml $replicate^{-1}$; 10^8 CFU ml^{-1}) was also applied once in 15 days to the soil close to the plant until flowering stage. The control plots

contained no test fungal strain. No serious phytopathogens or insect pest attack were observed during the cropping period. Irrigation was done on 23 and 51 DAS whereas weeding on 22 and 49 DAS. At 30 DAS, plant height, nodule number, nodule weight, shoot weight and root weight were noted and at 60 DAS, stem weight, pod number, pod weight, leaf weight and leaf area were noted and compared with un-inoculated control. The crop was harvested manually on 4 February 2014 and at harvest, stover yield, grain yield and total dry matter were noted.

Colonising ability of the test fungus

The colonising capability of the selected test fungus on the roots of chickpea was examined by scanning electron microscopy (SEM) analysis, as per the protocols of Bozzola and Russell, (1998). In brief, the seeds of chickpea (ICCV 2) were surface sterilised at first with sodium hypochlorite (2.5% in water for 3 min) followed by ethanol (70% in water for 3 min) and rinsed (7 times) thoroughly with sterilised water before being allowed to sproutovernight in a Petri dish under dark conditions at 30° C. At the end of overnight incubation, the sprouted seeds were incubated in test fungus (grown in SCB separately) for 45 min before being sown in plastic pots containing sterilised coarse sand (six seeds/8″ pot). Booster doze of the selected test fungus (10^8 CFU ml^{-1}; 5 ml per seedling) was applied upon germination of the seed by soil drench method. The pots were incubated at 24 ± 2°C for 2 weeks in a greenhouse. At the end of the 2 weeks incubation, seedlings of chickpea were removed carefully from the pots and the roots washed in phosphate buffer (0.1 M; pH 7.2). The tip of the roots were cut into 5 mm long pieces and fixed in glutaraldehyde (2.5%) in phosphate buffer for 24 h at 4°C. At the end of incubation, the cut-root samples were again washed with phosphate buffer, postfixed in osmium tetroxide (2%) for 4 h and dehydrated with a graded series of ethanol. The dehydrated cut-root samples were dried with critical-point liquid carbon dioxide as a transition fluid and adhered onto aluminium specimen mounts with double-stick adhesive tape. The mounted cut-root samples were coated with gold–palladium in an automated sputter coater (JEOL-JFC 1600) and examined under a SEM (JOEL-JSM 5600) as per the standardised protocols at RUSKA Laboratory, College of Veterinary Science, Rajendranagar, Hyderabad, India.

Statistical analysis

The data were subjected to analysis of variance (GenStat 10.1 version 2007, Lawes Agricultural Trust, Rothamsted Experimental Station) and isolate means were tested for significance and compared using Fisher's protected least significant difference.

Results

Selection of antagonistic fungal isolate against B. cinerea

A total of 48 fungi, the most prominent fungi which were found produced pigments, inhibited the adjacent colonies and abundantly in the PDA plate, were isolated from the five different herbal vermicomposts and chickpea rhizosphere soil samples. Of them, only 15 fungi were found to have antagonistic potential against *B. cinerea* (data not provided) in the *in vitro* dual-culture assay whereas the isolate VFI-51was

Table 1. Effect of VFI-51 on Botrytis Gray Mold disease of chickpea.

Pathogen	Duel culture assay (inhibition in mm)	Metabolite assay (growth in cm)
B. cinerea	9.3	3.3[a]
SE±	0.88	0.03

SE, standard error.
[a]Growth of fungus in control is 8 cm.

found to have most potential. The culture filtrates of VFI-51 were also found to have their antagonistic potential against *B. cinerea* (Table 1).

Isolation of the active metabolites of the fungus against B. cinerea

When the culture filtrates of VFI-51 were partitioned three times against EtOAc, organic (EtOAc) and aqueous fractions were obtained; however, the antagonistic activity, against *B. cinerea*, was found only in organic fraction. The active organic fraction was further fractionated on flash chromatography packed with silica gel and eluted with incremental EtOAc in *n*-hexane. Of all the fractions, only 20% EtOAc in *n*-hexane was found to be active against *B. cinerea* and this sample sent for NMR and MS analysis.

NMR and MS analysis

NMR data of the isolated compound from VFI-51 are given below. They support the structure of citrinin (Figure 1) and are in good agreement to those reported earlier (do Rosário Marinho and Rodrigues-Fo, 2011), except shifts of carbons C-9 and C-10. Our 2D experiments undoubtedly point out that C-9 has a lower shift than C-10 and we believe that the error in the previously published data is simply a typo.

^{1}H NMR ($CDCl_3$, 600.18 MHz, 298K), δ (ppm): 1.23 (d, 3H, J = 7.3 Hz, H-10), 1.35 (d, 3H, J = 6.7 Hz, H-9), 2.02 (s, 3H, H-11), 2.99 (q, 1H, J = 7.3 Hz, H-4), 4.78 (q, 1H, J = 6.7 Hz, H-3), 8.24 (s, 1H, H-1), 15.12 (bs, 1H, –COOH), 15.88 (s, 1H, OH). ^{13}C NMR ($CDCl_3$, 150.9 MHz, 298 K), δ (ppm): 9.7 (C-11), 18.4 (C-9), 18.7 (C-10), 34.8 (C-4), 81.9 (C-3), 100.5 (C-7), 107.6 (C-8a), 123.3 (C-5), 139.2 (C-4a), 163.0 (C-1), 174.7 (C-12), 177.4 (C-8), 184.0 (C-6).

The presence of citrinin as a dominant compound in the purified extract was also confirmed by LC–MS. A major peak was observed at 2.7 min in the LC–MS

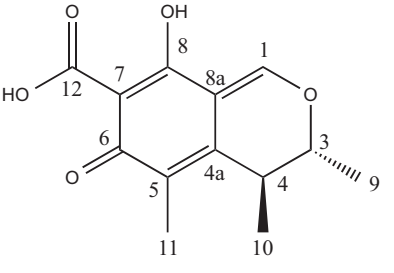

Figure 1. The structure of citrinin.

chromatogram with a *m*/*z* of 251.0919 which is 2 ppm of the theoretical $(M + H)^+$ ion for citrinin (251.0914).

Effect of the active compound for its antagonistic potential against B. cinerea

The active compound, citrinin, was evaluated for its antagonistic potential against *B. cinerea* under greenhouse conditions. When the pathogen, *B. cinerea*, was sprayed on the plants, the disease (BGM) occurred within a week indicating the potency of the pathogen while no toxic effect occurred in citrinin spray treatment indicating the compound is not phytotoxic. Upon spraying both pathogen and citrinin (8 ppm) together (at 0 h), irrespective of whichever is sprayed first, significant reduction of disease incidence was noted (<2.0 rating on 1–6 severity scale). As the application of citrinin was delayed by 24, 48 and 72 h after inoculation of *B. cinerea*, the efficacy of citrinin on BGM disease incidence also reduced significantly (Table 2).

Molecular identification of the fungus

The identity of the isolate VFI-51 was done by 18S rDNA analysis. A neighbour-joining dendrogram was prepared using VFI-51 sequence (1400 bp) and representative sequences from the databases. Phylogenetic analysis of sequences of the VFI-51 matched with *Penicillium citrinum* (100% similarity; Figure 2).

Evaluation of the fungus for its biocontrol, PGP and physiological traits

VFI-51 produced siderophore, HCN, IAA, lipase, protease and β-1,3-glucanase (Table 3). The isolate VFI-51 was also found to grow well up to 15% NaCl concentrations, pH 7–11 and temperatures between 20°C and 40°C. The fungus was found highly tolerant to bavistin, moderately tolerant to thiram and slightly tolerant to captan and ridomil but sensitive to benlate at field application levels (Table 3).

Table 2. Efficacy of citrinin against *B. cinerea* under greenhouse conditions.

Treatments	BGM severity (in 1–6 scale)[a]
Control	1.0
Only BGM spray	6.0
Only citrinin spray	1.0
BGM spray followed by citrinin spray at 0 h	1.7
Citrinin spray followed by BGM spray at 0 h	2.0
BGM spray at 0 h while citrinin spray at 24 h	3.3
BGM spray at 0 h while citrinin spray at 48 h	4.7
BGM spray at 0 h while citrinin spray at 72 h	6.0
Mean	3.3
SE±	0.37***
LSD (5%)	1.11
CV (%)	20

SE, Standard error; LSD, least significant differences; CV, coefficients of variation.
[a]The BGM severity of the disease was recorded after 20 days of inoculation using a 1–6 rating scale where, 1 is no infection on any part of the plant and 6 is extensive soft rotting, fungal growth on more than 70% of the leaves, branches and stems.
***Statistically significant at 0.001.

Table 3. Biocontrol, PGP and physiological traits of VFI-51.

Trait	Rating
Biocontrol and PGP traits	
Siderophore	1
HCN	1
IAA (μg ml^{-1})	0.4
Chitinase	0
Lipase	1
Protease	3
β-1,3-glucanase	2
Salinity (in %)	
0	3.0
5	3.0
10	3.0
15	2.0
20	1.0
25	0.0
pH	
3	0.0
5	1.0
7	3.0
9	3.0
11	2.3
13	0.0
Temperature (°C)	
50	0.0
40	2.0
30	3.0
20	2.7
10	0.0
Fungicide tolerance#	
Thiram (3000 ppm)	2.0
Bavistin (2500 ppm)	3.0
Benlate (4000 ppm)	0.0
Captan (3000 ppm)	1.0
Ridomil (3000 ppm)	1.0

Note: Responses of the VFI-51 to biocontrol and PGP traits were recorded as follows: 1 = halo zone of <2 mm; 2 = halo zone of 2–4 mm; 3 = halo zone of >4 mm; 0 = no halo zone. The responses of salinity, pH, temperature and fungicide tolerance were recorded as follows: 0 = no growth; 1 = poor growth; 2 = medium growth; 3 = good growth; # = at field application levels.

Evaluation of the fungus for PGP potential on chickpea under greenhouse and field conditions

Under greenhouse conditions, VFI-51 significantly enhanced PGP traits including the plant height (up to 6%), leaf area (up to 45%), leaf weight (up to 21%), stem weight (up to 49%), root length (up to 35%) and root volume (up to 89%) at 30 DAS and the stem weight (29%), pod weight (46%) and pod number (5%) at crop maturity over the control (Table 4).

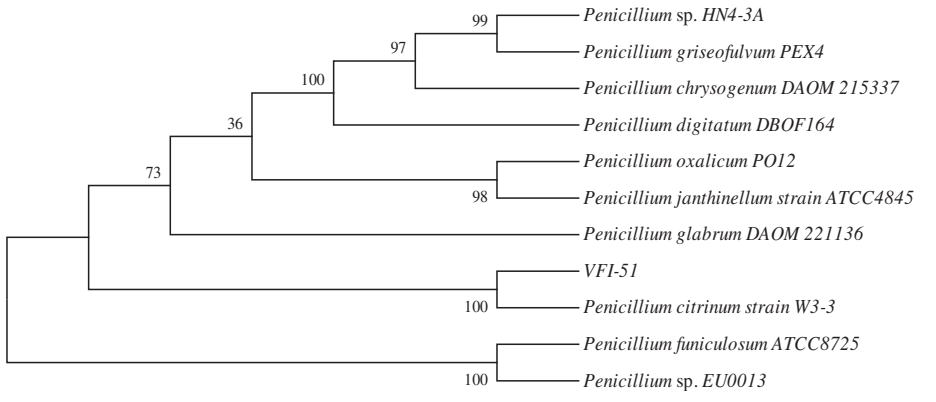

Figure 2. Phylogenetic relationship between VFI-51 and representative species based on full length 18s rDNA sequences constructed using the neighbour-joining method.

Under field conditions, plots treated with VFI-51 showed significantly enhanced agronomic performance of all the traits measured at 30 and 60 DAS and at crop maturity. At 30 DAS, the plots treated with VFI-51 significantly enhanced the nodule number (up to 16%), nodule weight (up to 12%), root weight (up to 16%) and shoot weight (up to 22%) and at 60 DAS, the stem weight (up to 11%), pod number (up to 22%), leaf weight (up to 19%) and leaf area (up to 12%) over the control plots. At crop maturity, VFI-51 inoculated plots also significantly enhanced the stover yield (up to 21%), grain yield (up to 15%) and total dry matter (up to 18%) over the control plots (Table 5).

Colonising ability of the fungus

SEM analysis of chickpea roots showed a remarkable degree of colonisation by VFI-51. Roots from VFI-51 inoculated plants exhibited significant surface colonisation by VFI-51 while those from un-inoculated plants did not. Further, the sporulation of the fungus on the surface cell layer of chickpea roots was also clearly evident. The hyphae of VFI-51 were also found to penetrate the surface cell layer of chickpea roots (Figure 3).

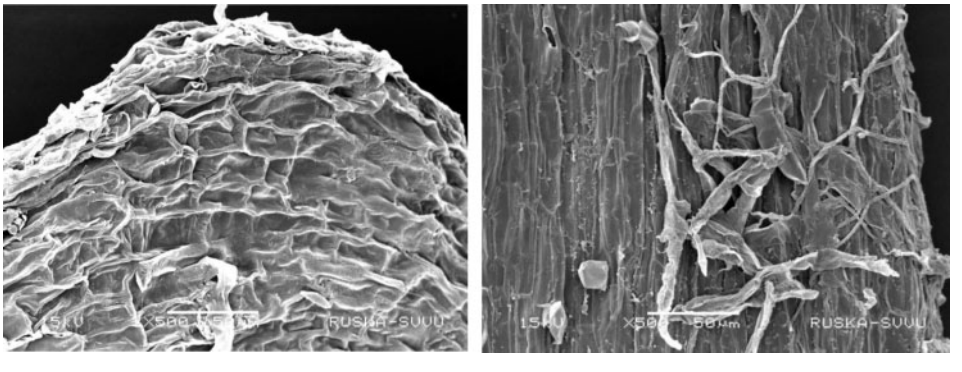

Figure 3. SEM photographs of the VFI-51 showing colonisation on the roots of chickpea.

Table 4. Evaluation of VFI-51 for its PGP potential in chickpea under greenhouse conditions.

	At 30 DAS						At crop maturity		
Isolate	Plant height (cm)	Leaf area (cm^{-2})	Leaf weight (g $plant^{-1}$)	Stem weight (g $plant^{-1}$)	Root length (cm)	Root volume (cm^3)	Stem weight (g $plant^{-1}$)	Pod weight (g $plant^{-1}$)	Pod number ($plant^{-1}$)
VFI-51	32.7	165	0.85	0.58	1792	4.43	1.77	3.90	16.9
Control	31.0	114	0.70	0.39	1323	2.34	1.37	2.68	16.1
Mean	31.8	140	0.78	0.49	1558	3.39	1.57	3.29	16.5
SE±	0.12**	6.4*	0.015*	0.020*	11.4***	0.107**	0.057*	0.103**	0.09*
LSD (5%)	0.72	39.1	0.089	0.123	69.6	0.650	0.345	0.625	0.52
CV (%)	1	8	3	7	1	6	6	5	1

SE, standard error; LSD, least significant differences; CV, coefficient of variation.
*Statistically significant at 0.05; **statistically significant at 0.01; ***statistically significant at 0.001.

Table 5. Effect of VFI-51 for its PGP and yield potential in chickpea under field conditions.

	At 30 DAS				At 60 DAS					At crop maturity		
Isolate	Nodule number ($plant^{-1}$)	Nodule weight (mg $plant^{-1}$)	Root weight (mg $plant^{-1}$)	Shoot weight (g $plant^{-1}$)	Stem weight (g $plant^{-1}$)	Pod number ($plant^{-1}$)	Pod weight (g $plant^{-1}$)	Leaf weight (g $plant^{-1}$)	Leaf area (cm^{-2} g $plant^{-1}$)	Stover yield (t ha^{-1})	Grain yield (t ha^{-1})	Total dry matter (t ha^{-1})
VFI-51	57	248	195	2.10	4.41	72	4.26	4.40	699	1.954	1.881	3.835
Control	49	222	168	1.72	3.96	59	4.21	3.71	626	1.612	1.631	3.244
Mean	53	235	182	1.91	4.19	65	4.24	4.06	663	1.783	1.756	3.540
SE±	0.4***	2.5*	2.3**	0.053*	0.051*	1.5*	0.008*	0.064*	8.4*	0.006***	0.014**	0.016***
LSD (5%)	2.5	15.1	13.8	0.323	0.313	9.0	0.050	0.388	50.9	0.037	0.087	0.096
CV (%)	1	2	2	5	2	4	1	3	2	1	1	1

SE, Standard error; LSD, least significant differences; CV, coefficients of variation.
*Statistically significant at 0.05; **statistically significant at 0.01; ***statistically significant at 0.001.

Discussion

Of the 48 fungal isolates obtained from five different herbal vermicomposts and chickpea rhizosphere soil samples, only 15 of them (31%) were found to have antagonistic potential against BGM caused by *B. cinerea* (data not shown). The most promising isolate, VFI-51 (based on the dual culture and metabolite production assays), was selected for purification of the active secondary metabolites responsible for inhibition of *B. cinerea*. The culture filtrates of VFI-51 were fractionated by solvent partitioning and the active EtOAc fraction was further purified on flash chromatography. The purified compound matched with the structure of 'citrinin' in the NMR and MS analysis (Figure 1) and this was in agreement with the earlier report (do Rosário Marinho & Rodrigues-Fo, 2011). Production of citrinin was reported by *Aspergillus* spp. and many species of *Penicillium*, including *P. citrinum* (Pitt, 2002). In the present investigation, under greenhouse conditions, when citrinin was sprayed against *B. cinerea at a concentration of 8 ppm,* significant reduction of BGM disease incidence was noted. Citrinin is already known for its antagonistic activity against many soil and seed-born plant pathogenic fungi including *Sclerotium rolfsii*, *Rhizoctonia solani* and *Sclerotinia minor* (Melouk & Akem, 1987). Though, citrinin is reported as mycotoxin, evaluation on sorghum and bean leaves did not show any changes in ATPase activity, respiration and photosynthetic rates (Damodaran, Kathirvel-Pandian, Seeni, Ganesan, & Shanmugasundaram, 1975). European Food Safety Authority (EFSA) reported the adverse effects and lethal doses associated with citrinin ingestion on various animal models. The LD_{50} of citrinin for rabbit was found to be 100 mg kg^{-1} body weight (b.w.) $oral^{-1}$ while in case of pigs, there were no adverse effects up to 20 μg kg^{-1} b.w. day^{-1}. Extrapolated results of animal models suggest that there would be no concern for adverse effects in humans at an exposure level of 0.2 μg kg^{-1} b.w. day^{-1} (EFSA, 2012). Hence, it can be concluded that the concentration of citrinin used in the present study to control BGM may not be having any adverse effects and safety issues.

In the present investigation, phylogenetic analysis of 18S rDNA sequences of the fungal isolate VFI-51 matched 100% with *P. citrinum* (Figure 2). *P. citrinum* had been isolated from agricultural fields and forest soils as endophytic fungus of legumes such as soybean and wheat. After the discovery of citrinin from *P. citrinum*, the fungus was explored for the secondary metabolites it produce and their associated benefits (Dutta et al., 2007; Wakiyama, Tanaka, Yoshihara, Hayashi, & Ohta, 2008).

In the present study, the *P. citrinum* VFI-51 produced siderophore, IAA, HCN, lipase, protease and β-1,3-glucanase. Siderophores function as solubilising agents for iron from minerals under conditions of iron limitation (Indiragandhi, Anandham, Madhaiyan, & Sa, 2008). Microbes producing IAA are known to stimulate seed germination, root formation and increase root surface area and length, thereby providing the host plant greater access to water and soil nutrients (Ahemad & Kibret, 2014). Bacteria producing HCN are reported to help in disease suppression, as in the case of tobacco where *Pseudomonas fluorescens* was reported to suppress black root rot disease (Haas et al., 1991). Protease-producing microorganisms are reported to play a role in nutrient mineralisation and thus help the plants in growth promotion (Lima, Marco, & Felix, 1998). β-1,3-glucan is found in the cell walls of plant pathogens, fungi in particular, and lysis of this by β-1,3-glucanase-producing

microorganisms leads to leakage of cell contents and collapse of the pathogenic fungi (Singh et al., 1999). Similarly, Khan et al. (2008) reported *PGP ability of P. citrinum through the production of various* gibberellins GA_1, GA_3, GA_4 and GA_7. Hence, it is concluded that the *P. citrinum* VFI-51 contains multi-trait of PGP and can be exploited for PGP in chickpea.

The fungus *P. citrinum* VFI-51 was found to grow well up to 15% NaCl concentrations, pH 7–11, temperatures 20–40°C, tolerant to bavistin and thiram whereas found sensitive to benlate at field application levels. Thus, it can be concluded that *P. citrinum* VFI-51 is capable of surviving in harsh environments, particularly on saline soils.

In the present study, under both greenhouse and field conditions, *P. citrinum* VFI-51 enhanced morphological and yield parameters of chickpea such as plant height, leaf area and weight, stem weight, root length, volume and weight, nodule number, nodule weight, stover yield and grain yield over the un-inoculated control. PGP by *P. citrinum* was reported on rice and *Artiplex gemelinii* seedlings (Khan et al., 2008). Citrinin was reported to induce the swarming motility of PGP *Paenibacillus polymyxa* (Park et al., 2008), thereby indirectly helping the plant growth. The mechanism by which the *P. citrinum* VFI-51 enhanced the plant growth and yield parameters of chickpea could be attributed to their siderophore, IAA, lipase, protease, HCN and β-1,3-glucanase producing capabilities and/or to their ability to survive under harsh environments. Colonisation by PGP microbes is a pre-requisite for PGP activity. Host-microbe interaction is essential for colonisation which involves sufficient population of microbe, rhizosphere competence of the microbe, colonising ability of the microbe on roots and finally PGP ability of the microbe (Lugtenberg & Dekkers, 1999). In the present investigation, SEM analysis demonstrated colonisation of *P. citrinum*VFI-51 on the roots of chickpea (Figure 3). Therefore, the SEM analysis in addition to the data for grain and stover yield, root and other agronomical traits strongly suggest that the *P. citrinum* VFI-51 had colonised on chickpea roots.

It is concluded that the active secondary metabolite of the fungal strain, *P. citrinum* VFI-51 that inhibited *B. cinerea*, was identified as citrinin and demonstrated for their efficacy against BGM disease of chickpea. Under both greenhouse and field conditions, VFI-51 was apparently found to promote plant growth and yield in chickpea. Further, the strain produces a broad range of PGP traits such as siderophore, IAA, protease, HCN, lipase and β-1,3-glucanase indicating its broad spectrum activity. The PGP ability of *P. citrinum* VFI-51 may help in conservation of the rapidly eroding agricultural lands, particularly on saline soils. Therefore the fungal strain used in this study is a potential candidate for integrated BGM disease and nutrient management programmes. There is a need to do additional comprehensive research for identifying mode of action of citrinin in controlling BGM and the role of P. citrinum as PGP agent under field conditions (multi-location trials).

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Disclosure statement

No potential conflict of interest was reported by the authors.

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