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Characterization and Antagonistic Potential of Rhizospheric Microbes from Organic Cardamom Production System

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Abstract

Small cardamom (*ElettariacardamomumMaton*), the "Queen of Spices" is a zingiberaceous spice, native to the moist evergreen forests of Western Ghats, South India. Among the debilitating diseases, rhizome-root rot incited by *Rhizoctoniasolani*, *Pythium vexans* and *Fusarium oxysporum* is widespread across the cardamom growing tracts and possess enormous destructive potential. Non-judicious application of synthetic chemicals to manage this disease could create imbalance in the environmental equilibrium, contamination of soil, water bodies and might result in bio-magnification and organic-based production system could be the appropriate approach in order to nullify the deleterious effects of synthetic molecules. In the present study, attempts were made to isolate, characterize and assess antagonistic potential of microorganisms associated with cardamom rhizosphere against rhizome-root rot pathogens. Twelve fungi and six bacteria were isolated from the rhizosphere of cardamom grown under organic system. The fungal isolates were characterized morphologically based on colony characters, growth pattern and spore morphology. Whereas, the bacterial isolates were characterized based on the criteria such as colony appearance Gram's staining and response to various biochemical reactions. In the *in vitro* bioassay for antagonistic efficacy against the test pathogens, among the fungi isolate 3 was found to be superior in inhibiting all the test pathogens, while the bacterial isolate 2 proved to be effective against *R. solaniand P. vexans*. Further identification of the efficacious isolates *viz.*, fungal isolate 3 and bacterial isolate 2 based on ITS and rDNA sequencing, placed them in the genus *Trichoderma* and *Chromobacterium*, respectively.

Keywords: Cardamom; Rhizome rot; Biocontrol; *Rhizoctoniasolani; Pythiumvexans; Fusariumoxysporum; Trichoderma; Chromobacterium*

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Introduction

India is renowned as 'The Land of Spices' and the glory of Indian spices are celebrated throughout the world. Small cardamom (*Elettariacardamomum Maton*), the "Queen of Spices" is a *zingiberaceous* spice, native to the moist evergreen forests of Western Ghats, South India [1]. In general, cardamom-based ecosystem is considered to be the most viable and eco-friendly system, in which copious organic matter in the form of leaf litter of shade trees are constantly added to the soil continuum which helps to maintain the organic status. This versatile ecosystem provides a unique platform to a broad spectrum of soil microflora and fauna. Nevertheless, non-judicious application of plant protection chemicals in order to manage obnoxious pests as well as diseases could have adverse and far-reaching effects on cardamom cultivating tracts. Plant protection chemicals have been used extensively in cardamom as they suffer from the attack of various plant pathogens like *Phytophthora, Rhizoctonia, Fusarium and Pythium* [1]. The rhizosphere is considered as the most vibrant and hot spot of biodiversity. Competition for nutrient sources in the rhizosphere is found high. Therefore, different microorganisms have developed distinct strategies, ranging from antagonistic to synergistic interactions, both among themselves and with the plant to carve a niche in this microecosystem. The highest portion of microorganisms which inhabit the rhizosphere are fungi and bacteria [2].

Large-scale application of chemicals may have adverse effect on the proliferation and survival of beneficial rhizospheric microorganisms thus, reducing its ability to antagonize the pathogens and promoting plant growth. Apart from direct ill effects, it also results in residual toxicity, biomagnification and evolution of resistant strains of pathogens. In order to nullify the damage caused by plant protection chemicals to the nature, several alternatives have been developed which are being used widely [1]. Among the various alternatives, priority has been given to organic farming where the use of hazardous synthetic molecules is circumvented. Organic production system could be the appropriate approach to nullify the deleterious effects of chemical pesticides on the ecosystem aided by deployment of beneficial microbes possessing growth promotion activity and antagonistic potential against the target pathogens. Incorporation of organic matter into the soil helps in improving the soil health by altering the physical and chemical properties of soil. It also supports beneficial microbes which secretes secondary metabolites, which adds to the antagonistic ability to suppress the soil-borne plant pathogens. Biological control is an environment-friendly strategy to reduce crop damage caused by plant pathogens. Biological control of soil-borne pathogens with antagonistic bacteria and fungi has been intensively investigated. Rhizosphere-resident antagonistic microorganisms are considered to be the ideal biocontrol agents, as the rhizosphere provides frontline defence for roots against infection by the pathogens [3].

Biological control of plant pathogens by exploiting native as well as introduced microorganisms has been extensively documented in several economically important crops and efficacious microbes has been commercialized through several platforms including consortia mode. However, pertinent information on efficacious *rhizospheric* microbes associated with organic cardamom-based ecosystem, their characterization and antagonistic potentiality against major pathogens are scanty. Hence, the present study was formulated with the objectives to isolate the *rhizosphericmicroflora* (fungi and bacteria) associated with cardamom grown under organic production system, their characterization and evaluating the antagonistic potential against rhizome-root rot pathogens *viz.*, *Rhizoctonia solani*, Pythium *vexans* and *Fusarium oxysporum* of small cardamom.

Materials and Methods

Collection of samples

The soil samples were collected from the rhizosphere of cardamom clumps of organically maintained cardamom field at ICAR-Indian Institute of Spices Research Regional Station, Appangala, Kodagu, Karnataka (N 12o26' E 75o45' 920 m above MSL). The soil samples were collected at a depth of 5-10 cm from the root zone and brought to the laboratory in clean, sterilized polythene bags with appropriate labels. The soil samples collected were mixed thoroughly to get a composite soil mixture and further used for isolation of rhizospheremicroflora within 24 hours of collection.

Isolation of rhizospheric microflora

For the isolation of rhizospherec microflora, different media were used *viz.*, potato dextrose agar (PDA) for fungi and nutrient agar (NA) for bacteria. The PDA medium was amended with 100 ppm streptomycin sulphate to prevent bacterial growth.

10 g of respective soil samples were weighed and dissolved in 90 ml sterile distilled water (10-1 dilution) under aseptic conditions and shaken well. Further, 1ml of the suspension was transferred to 9 ml blank to obtain 10-2 dilution. Subsequently, serial fold dilutions were made up to 10-10 and aliquots of each dilution were cultured on plates of nutrient agar and potato dextrose agar for the isolation of bacteria and fungi, respectively employing pour plate method [4]. Isolation was carried out in duplicates in which the culture plates were swirled, allowed to solidify and incubated at 35°C for 48 hours at ambient temperature (25 ± 2°C) for 5 days. The most appropriate dilution was standardized for estimating the population and expressed as number of CFU (Colony Forming Units) per gram of soil using the formula:

CFU=Number of colonies × Dilution factor/Dry weight.

Pure culturing and morphological characterization

Representative bacterial and fungal colonies were sub-cultured on freshly prepared nutrient agar and potato dextrose agar, respectively. The cultures were incubated at 35°C for 24 hours and room temperature (25 ± 2°C) for 3 days for bacterial and fungal cultures, respectively. The fungal colonies were transferred to PDA slants by selecting single isolated colonies. The colonies were streaked on agar slants, incubated at room temperature and allowed to attain optimum growth. After attaining optimal growth, the fungi were sub-cultured on PDA, supplemented with 100 ppm streptomycin sulphate. Similarly, the single colonies of bacteria were transferred to NA slants and maintained at 4°C for further studies. The pure cultured fungal isolates were identified based on morphological and microscopic features. The fungal isolates were characterized based on colony characters *viz.*, diameter, color, texture and spore morphology. Whereas, for morphological characterization of bacteria, colony characters such as colony diameter, form of colony, structure, surface elevation, margin, topography, color and luster were employed as criteria.

Biochemical characterization-bacterial isolates

Gram's staining: The 24-hour old cultures was suspended in sterile water and a thin smear of the suspension was spread on a clean glass slide. The smear was heat fixed by passing over a flame and flooded with crystal violet (primary stain) for 1 minute and washed with water. The heat fixed smear was then flooded with Gram's iodine solution for 1 minute, washed with water and blot dried. The smear was decolorized with alcohol until only faint violet color remains. The slide was again washed with water and blot dried. It was counter stained with safranin for 30 seconds, washed with water, drained, air dried and examined at 100X (oil immersion objective) [4].

Potassium hydroxide (KOH) test: Two drops of 3% KOH were placed on a clean glass slide. With a sterile transfer loop, a loopful of test bacterial cultures was transferred to the KOH drops. The culture and the KOH solution were rapidly mixed by stirring with

a transfer loop for 5 to 10 seconds. The loop was raised and lowered off the slide surface to observe the formation of viscous strings. The reactions were graded as follows:

KOH (+)=viscous sting formation=Gram^{-ve}

KOH (-)=no viscous sting formation=Gram^{+ve}

Catalase test: 24 hours old bacterial cultures were smeared on glass slide and covered with few drops of 3% hydrogen peroxide. Release of free oxygen gas bubbles indicated a positive catalase reaction [5].

Citrate utilization: The bacterial cultures were streaked on the surface of simmon's citrate agar slants. Development of a blue color indicated utilization of citrate and green color indicated non-utilization of citrate [5].

H2S production: Sulfide-indole-motility (SIM) medium was inoculated with 24 hours old bacterial cultures by stab inoculation method and incubated at 35°C for 48 hours to check the production of hydrogen sulfide. Development of black color in the medium indicated reduction of sulfur and a positive result.

Starch hydrolysis: The test cultures were spotted on nutrient agar amended with soluble starch (0.2%). Hydrolysis of starch was analyzed 48 hours after incubation by flooding the agar surface with Lugol's iodine solution. A colorless zone around the bacterial growth in contrast to the blue background of the medium indicated a positive reaction.

Gelatin hydrolysis: The test medium containing beef extract (3 g), peptone (5 g), gelatin (120 g) and distilled water (1 litre) was dispensed in a test tube, autoclaved at 121°C for 15 minutes and cooled. After inoculation with the test isolates, the tubes were incubated at 20-22°C for 3 days and further, kept at 4°C for 30 minutes.

Methyl red test: The methyl red broth containing peptone (7 g), dextrose (5 g), potassium phosphate (5 g) and distilled water (1 litre) was prepared, dispensed in test tubes and autoclaved at 121°C for 15 minutes and cooled. The test isolates were inoculated on the medium and incubated at 35°C for 48 hours. Further, five drops of methyl red solution were added and change in the color was observed 5 minutes after incubation period [5].

In vitro bioassay to assess antagonistic efficacy

Dual culture: The fungal as well as bacterial isolates were evaluated under *in vitro* conditions employing dual culture technique to identify the most efficacious isolate against *Rhizoctonia solani*, *Fusarium oxysporum* and *Pythium vexans*. In the case of fungal isolates and pathogens, Petri dishes (90 mm) containing PDA were inoculated with 5 mm diameter mycelial discs derived from the advancing margins of seven days old cultures. The mycelial discs of test isolates and the pathogens were placed 5 cm apart and at equal distance from periphery of the Petri dish. For bacterial isolates, the colonies cultured for 48 hours on nutrient broth were streaked (single streak) 5 cm away from the mycelial discs of the pathogens. The mycelial discs were placed and bacterial colonies were streaked equidistantly from periphery of the Petri dish. The plates were incubated at (25 ± 2)°C for 4 days for bacteria and 7 days for fungi and the radial mycelia growth (mm) of the pathogens was recorded commencing from second day after incubation. The experiment was laid out with six replications (Petri dishes) in each treatment and was repeated twice to confirm the results. Per cent inhibition of radial growth of the pathogens was calculated after respective period of incubation using the formula:

$$\text{Per cent inhibition (I)} = \frac{C-T}{C} \times 100$$

Where, I=per cent inhibition, C=linear growth of the test pathogen in control (cm) and T=linear growth of test pathogen in treatment (cm).

Assessment of aggressiveness: For assessing aggressive nature of the fungal isolates, modified Bell's scale was employed:

R_1-antagonist completely overgrew the pathogen, R_2-75% overgrowth of antagonist, R_3-50% overgrowth of antagonist, R_4-25% overgrowth of antagonist, R_5-colonies contacted, R_6-pathogen overgrew the antagonist [6].

Effect of volatile inhibitors: The efficacy of volatile compounds produced by the test isolates against rhizome-root rot pathogens was evaluated by adopting the procedures described [7,8]. In the case of fungal isolates, the test isolates (3 mm) obtained from 4 days old culture was inoculated at the center of Petri dish containing solidified PDA amended with 100 ppm streptomycin sulphate. The test pathogens (3 mm discs derived from 4 days old culture) were also inoculated at the center of another Petri dish which was positioned exactly over the other half previously inoculated with the test isolate face to face in an inverted position preventing any contact between the pathogen and the isolates.The two halves were sealed together with adhesive tape to prevent the loss of volatile compounds. Similarly, in the case of bacterial isolates, bacterial suspension (100 µL) was placed at the center of one half of a Petri dish containing NA and test pathogens were inoculated on another half of the Petri dish. The plates were incubated at (25 ± 2)°C for 4 days for bacteria and 7 days for fungi and the radial mycelia growth (mm) of the pathogens was recorded commencing from second day after incubation. The experiment was laid out with six replications (Petri dishes) in each treatment and was repeated twice to validate the results. Percent inhibition of radial growth of the pathogens was calculated after respective period of incubation as described earlier:

Based on the inhibition studies and assessment of aggressiveness, the most effective fungal and bacterial isolates were shortlisted and nucleic acid-based protocols were further employed to identify and characterize the efficacious fungal as well as bacterial isolates.

Molecular characterization of efficacious isolates

Isolation of genomic DNA-Fungal isolate: For the isolation of genomic DNA, the isolate was cultured on potato dextrose broth. 0.1 g of mycelia was transferred to sterile microcentrifuge tube and 50 mg of sterile glass powder, 10 mg of polyvinyl polypyrrolidone and 750 µl extraction buffers was added and the mixture was ground to fine paste. The paste was centrifuged at 13000 rpm for 5 minutes and the supernatant was transferred to a sterile microcentrifuge tube. To this, 500 µl phenol: chloroform: isoamyl alcohol (25:24:1) was added and centrifuged at 13000 rpm for 5 minutes. The aqueous layer was transferred to a sterile microcentrifuge tube and equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 13000 rpm for 10 minutes. Aqueous layer was again transferred to a sterile microcentrifuge tube and the tube was filled with isopropanol (0.6 V), inverted gently and incubated at -20°C for 30 minutes and centrifuged at 13000 rpm for 10 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol. Inverted gently and allowed to stand for a minute and again centrifuged for 2 minutes. The pellets were air dried to remove ethanol and pellets were re-suspended in 50 µl of nuclease-free water. 3 µl of RNAse (5 mg/ml) was added and incubated at 37°C for 30 minutes and stored at -20°C for further studies.

Isolation of genomic DNA-Bacterial isolate: The culture was grown on 5 ml LB broth. 1 ml of the broth was transferred to sterile microcentrifuge tube and centrifuged at 14000 rpm for 2 minutes at room temperature. The supernatant was discarded and the pellet was washed three times with sterile distilled water. 550 µl of TE buffer+lysozyme was added, mixed well and incubated at 37°C for 30 minutes. 76 µl of 10% SDS+Proteinase K were added after incubation, mixed well and incubated at 65°C for 15 minutes. After incubation, 100 µl of 5MNaCl was added and thoroughly mixed. Further, 80 µl of CTAB/NaCl was added, mixed and incubated at 65°C for 10 minutes. After incubation, 660 µl of chloroform+isoamyl alcohol were added, thoroughly mixed and centrifuged for 15 minutes at 14000 rpm at room temperature. The aqueous layer was carefully transferred to 1.5 ml tube without disturbing the white middle layer. This step was repeated twice. Subsequently, equal volume of isopropanol was added, mixed by inverting and centrifuged for 15 minutes at 14000 rpm at room temperature. The supernatant was drained; 0.5 ml 70% ice cold ethanol was added and centrifuged for 15 minutes at 14000 rpm at 4°C. The supernatant was carefully removed and the remaining ethanol was evaporated in the laminar air flow chamber. 25 µl of 10:1 TE buffer was added to dissolve the DNA and stored at 4°C for overnight. To remove the RNA contamination, RNAse at a concentration of 200 µg/ml (stock 10 mg/ml) was added and incubated at 30°C for 30minutes and stored at-20oC [9].

Identification of the isolates: To identify the most promising isolate of bacteria (Isolate 2) 16S rDNA sequence analysis was carried out. The 16s rDNA was amplified using universal primer set 16s FP (5'-AGAGTTTGATCCGGCTCAG-3'); 16S RP (5'-AAGGAGGTGATCCAGCCGCA-3') primers. PCR reactions were carried out in 25 µl reaction mixture containing 10x Taq buffer2.5 µl; 2.5mM MgCl2, 3 µl; 2.5 mMdNTP mixture, 1 µl; 10 µM Forward primer, 2 µl; 10 µM Reverse primer, 2 µl; Taq DNA polymerase, 0.2 µl; H2O, 13.3 µl and 100 ng of template DNA samples were amplified on DNA thermal cycler (Eppendorf Master Thermal Cycler) using the PCR conditions consisted of an initial denaturation at 94°C for 2 min, 55°C for 1 min and 72°C for 1 min and final polymerization step at 72°C for 15 min. The total number of cycles was 35. The PCR products were resolved on 1 percent agarose at 85V stained with ethidium bromide, photographed and analyzed using geldocumentationsystem[10]. For the fungal isolate, (Isolate 3) was sequenced using ITS primes.The DNA was amplified using the primer set ITS4 (5'-TCCTCCGCTTATTGATATGC-3'); ITS 5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') primers. PCR reactions were carried out in 25 µl reaction mixture containing 10 × Taq buffer 2.5 µl; 2.5 mM MgCl2, 3 µl; 2.5 mMdNTP mixture, 1 µl; 10 pM/ µl Forward primer, 1 µl; 10 pM/ µl Reverse primer, 1 µl; Taq DNA polymerase, 0.2 µl; H2O, 14.3 µl and 100 ng of template DNA thermal cycler (Eppendorf Master Thermal Cycler) using the PCR conditions consisted of an initial denaturation at 95°C for 4 min, 60°C for 40 sec, 72°C for 60 sec and final polymerization step at 72°C for 10 min. The total number of cycles was 35. The PCR products were resolved on 1 per cent agarose at 85 V stained with ethidium bromide, photographed and analyzed using gel documentation system. The gel was excised and purified using Gene JET Gel Extraction kit (Thermo Scientific). The DNA sequencing was performed with SciGenom Labs Pvt Ltd, Kochi, Kerala.

Data analysis: The *in vitro* bioassay experiments were laid out in completely randomized design (CRD) and the data recorded in percent were transformed to arc sine transformation. The transformed data were statistically analyzed using the software package IASRI version 2010.

Results

Isolation and morphological characterization

The present study was carried out at ICAR-Indian Institute of Spices Research Regional Station, Appangala. Soil samples were collected from the rhizosphere of cardamom grown under organic production system during the month of June, 2015. *Rhizospheric* fungi and bacteria were isolated and each isolate were assigned with codes to facilitate characterization.

A total of 12 *rhizoshere* fungi were isolated and characterized based on colony morphological features like texture, color and shape. The growth rate was recorded at different intervals up to 7 days. Apart from the macroscopic studies, microscopic observations were also carried out as depicted in **Table 1**.

The fungal isolate 1 was characterized with a smooth textured colony, light green color with a white bottom. The colony attained a diameter of 31 mm after 7 days of growth. Isolate 2 was characterized with cottony texture, light pink color with light brown bottom and attained 32 mm after 7 days of growth. Isolate 3 was cottony, fast growing with greenish-white in color. The colony was found to be the most aggressive grower and attained

90 mm in 3 days of plating **(Figure 1)**. Isolate 4 was characterized with velvetty texture and green color. The colonies appeared orangish-brown from bottom and attained 26 mm after 7 days of growth. Isolate 5 was characterized with dark green velvetty textured colony and light-yellow color on the bottom surface which attained 27 mm diameter after 7 days of growth. Isolate 6 was characterized with cottony texture and attained 90 mm in 5 days of growth. The colony of isolate 7 was characterized with velvety texture and white color with arms radiating from center to periphery of the colony. The colony attained a diameter of 90 mm in 5 days of growth. Isolate 8 was characterized with white cottony colony with a diameter of 80 mm after 7 days of growth. Isolate 9 was found to be the slowest grower among all the isolates with a colony diameter of 24 mm after 7 days of plating. Isolate 10 was characterized by white cottony colony with sectoring and creamish white bottom which attained 65 mm on the 7th day after plating. Isolate 11 was characterized with velvety textured colony, white with yellowish brown and dark yellow bottom. The diameter of the colony was 37 mm after 7 days of plating. Isolate 12 was characterized with green velvety growth with whitish margin and a diameter of 29 mm after 7 days of growth **(Table 1)**.

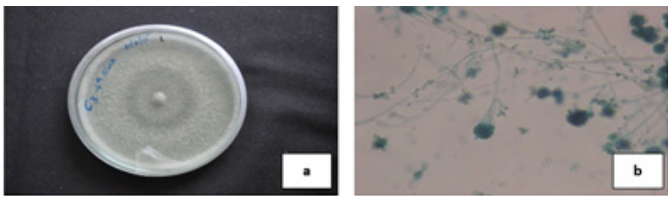

Figure 1: (a) Colony of fungal isolate 3 (b) Conidiophores (40X).

A total of six bacteria were isolated by serial dilution pour plate technique and were characterized based on the features presented in **Table 1**. Isolate 1 was characterized by yellowish smooth colony with raised surface and entire margin while, isolate 2 was characterized by its unique purple color with entire margin, smooth and slightly raised surface **(Figure 2)**. Isolate 3 was characterized with white, flat, irregular colony with smooth surface whereas, isolate 4 was characterized by dull white irregular colony with flat smooth surface. Isolate 5 was characterized by transparent creamish colony with flat contoured surface and filamentous structure and isolate 6 was characterized by transparent cream amoeboid colony with flat lobate margin with contoured topography **(Table 2)**.

Table 1: Morphological characteristics of rhizospheric fungal isolates.

Isolate code	Colony diameter in mm (days after inoculation)			Colony				Conidium(µm)		Conidiophore(µm)	
	3	5	7	Texture	Color	Sectoring	Coloration towards periphery	Range	Average	Range	Average
Isolate 1	20	27	31	Smooth	Light green, creamish white bottom	Absent	White	2.14-2.94	2.52	5.36-7.59	6.34
Isolate 2	13	23	32	Cottony	Light pink,light brownish bottom	Absent	White	1.71-2.91	2.43	5.06-5.88	5.48
Isolate 3	90	90	90	Cottony	Green and white, fast growing	Absent	-	2.91-4.44	3.56	6.00-8.09	6.3
Isolate 4	10	19	26	Velvety	Green, orangish brown bottom	Absent	White	1.69-2.81	2.19	5.22-5.74	5.41
Isolate 5	15	22	27	Velvety	Dark green, whitish margin, light yellowish bottom	Absent	White	1.69-2.91	2.12	5.06-5.99	5.43
Isolate 6	78	90	90	Cottony	Dull white, fast growing	Absent	-	3.02-4.59	3.94	10.71-16.25	10.86
Isolate 7	74	90	90	Cottony	White, radiating fast growing	Absent	-	2.49-3.92	3.19	-	-
Isolate 8	50	64	80	Cottony	White	Absent	-	4.79-8.34	6.47	-	-
Isolate 9	16	20	24	Velvety	Greyish green, brown bottom	Absent	White	1.68-2.67	2.18	5.72-7.86	6.43
Isolate 10	29	48	65	Cottony	White, creamish white bottom	Present	-	6.17-15.14	8.88	-	-
Isolate 11	18	27	37	Velvety	White with yellowish brown, dark yellow bottom	Present	White	1.96-2.76	2.39	5.38-7.89	6.34
Isolate 12	12	21	29	Velvety	Green with white margin, creamish bottom	Absent	White	4.34-7.8	5.74	11.94-17.53	13.84

Table 2: Morphological characteristics of rhizospheric bacterial isolates.

Isolate code	Isolate code	Isolate code	Isolate code	Isolate code	Isolate code	Isolate code	Isolate code	Isolate code	Isolate code	Isolate code
Isolate 1	2	3	Circular	Regular	Raised	Entire	Smooth	Yellow	Opaque	Glistening
Isolate 2	2	9	Circular	Regular	Slightly raised	Entire	Smooth	Purple	Opaque	Glistening
Isolate 3	2	30	Irregular	Irregular	Flat	Curled	Smooth	White	Opaque	Dull
Isolate 4	2	11	Circular	Regular	Flat	Entire	Smooth	Dull white	Opaque	Dull
Isolate 5	2	25	Irregular	Filamentous	Flat	Filiform	Contoured	Creamish	Transparent	Dull
Isolate 6	2	32	Amoeboid	Myceloid	Flat	Lobate	Contoured	Cream	Transparent	Glistening

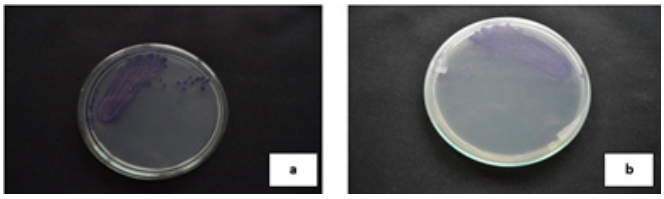

Figure 2: (a) Colony characteristics of bacterial isolate 2 (top) (b) Reverse.

Biochemical characterization-bacterial isolates: The bacterial isolate 3 was found to be Gram+ve which was purple in color after Gram's staining, while all the other isolates were found to be Gram-ve (pink color) **(Figure 3)**. Isolate 3 was found negative for H_2O_2 and KOH test, while the other isolates were positive for both H_2O_2 and KOH tests. In other biochemical tests, all the isolates were found negative for indole production and H_2S production. Isolate 3 was positive for methyl red test, isolates 2, 4, 5 and 6 were found positive for citrate utilization, isolates 1 and 3 was positive for starch hydrolysis, isolates 3 and 5 were found positive for gelatin hydrolysisand isolate 2 was found positive for nitrate reduction **(Table 3)**.

In vitro bioassay to assess antagonistic efficacy

Dual culture assay: Antagonistic efficacy of the fungal and bacterial isolates was evaluated under *in vitro* conditions by dual culture assay against rhizome-root rot pathogens. The fungal isolates 3, 6 and7 were found to be effective against *Rhizoctonia solani*, the isolate 3 being most effective with the maximum inhibition (85.32%), followed by isolate 6 (65.25%) and isolate 7 (64.27%) **(Table 4 and Figure 4)**. The fungal isolates 3, 7 and 8 were found effective against *Fusarium oxysporum*, with isolate 3 being most effective with the maximum per cent of inhibition (85.32%), followed by isolate 7 (66.09%) and isolate 8 (64.37%) **(Table 4 and Figure 5)**. Against the pathogen *Pythium vexans*, the tested fungal isolates 3,6 and 7 was found effective, isolate 3 being most effective with the maximum percentage of inhibition 76.44% followed by isolate 6 (78.57%) and isolate 7 (75.71%) **(Table 4 and Figure 6)**.

Table 3: Biochemical characterization of rhizospheric bacterial isolates.

Isolate code	Indole production	Methyl Red test	Citrate utilization	Starch hydrolysis	Gelatin hydrolysis	Nitrate reduction	H2S production
Isolate 1	Negative	Negative	Negative	Positive	Negative	Negative	Negative
Isolate 2	Negative	Negative	Positive	Negative	Negative	Positive	Negative
Isolate 3	Negative	Positive	Negative	Positive	Positive	Negative	Negative
Isolate 4	Negative	Negative	Positive	Negative	Negative	Negative	Negative
Isolate 5	Negative	Negative	Positive	Negative	Positive	Negative	Negative
Isolate 6	Negative	Negative	Positive	Negative	Negative	Negative	Negative

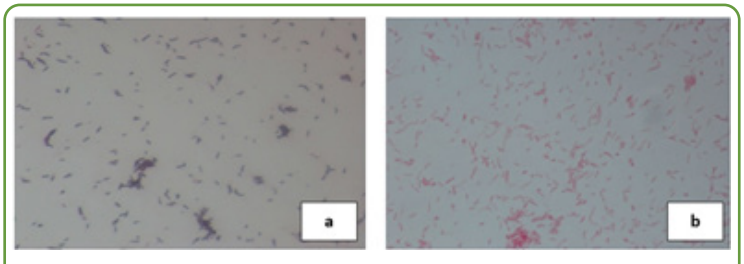

Figure 3: (a) Gram+ve (Isolate 3) (b) Gram–ve reaction (Isolate 1).

Table 4: Inhibition of mycelial growth of pathogens by rhizospheric fungal isolates (dual culture).

Isolate code	Percent inhibition*(R. solani)*	Percent inhibition*(F. oxysporum)*	Percent inhibition*(P. vexans)*
Isolate 1	50.07^{c***} (45.06)**	54.60CD*** (47.66)**	57.14D*** (49.14)**
Isolate 2	29.52^{FG} (32.92)	34.48^{E} (35.98)	41.43^{EF} (40.08)
Isolate 3	85.32^{A} (67.50)	76.44^{A} (61.00)	82.38^{A} (65.21)
Isolate 4	33.92^{EF} (35.63)	34.48^{E} (35.94)	41.43^{EF} (40.08)
Isolate 5	34.90^{E} (36.22)	31.03^{E} (33.86)	42.86^{E} (40.90)
Isolate 6	65.25^{B} (53.91)	58.05^{BCD} (49.83)	78.57^{AB} (62.48)
Isolate 7	64.27^{B} (53.34)	66.09^{AB} (54.43)	75.71^{B} (60.52)
Isolate 8	48.60^{C} (44.22)	64.37^{BC} (53.38)	65.24^{C} (53.90)
Isolate 9	31.96^{EF} (34.43)	32.76^{E} (34.93)	34.29^{G} (35.83)
Isolate 10	40.28^{D} (39.41)	48.85^{D} (44.36)	54.76^{D} (47.75)
Isolate 11	33.92^{EF} (35.64)	36.21^{E} (37.01)	37.14F^{G} (37.57)
Isolate 12	26.58^{G} (31.05)	36.21^{E} (36.99)	45.71^{E} (42.56)
Control (mm)	68.1	58	70
SE(d)=1.684 CV(%)=4.92 LSD at 1%=4.6801 *(R.solani)*; SE(d)=3.786 CV(%)=10.51 LSD at 1%=10.52 *(F.oxysporum)*, SE(d)=1.858CV(%)=4.51LSD at 1%=5.163 *(P.vexans)*			
**Figures in the parentheses are arcsine transformed values			
***Values with at least one letter in common are not statistically significant			

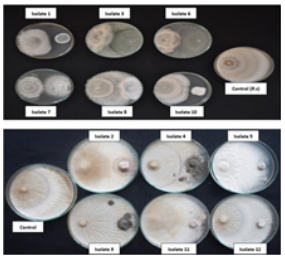

Figure 4: Antagonism of rhizospheric fungal isolates against *R. solani.*

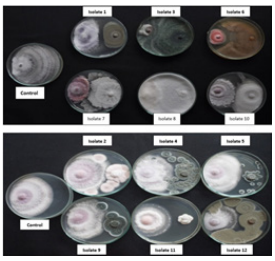

Figure 5: Antagonism of rhizospheric fungal isolates against *F. oxysporum.*

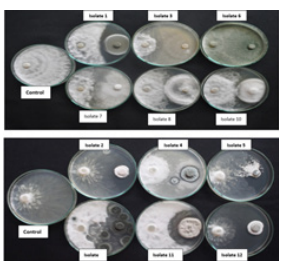

Figure 6: Antagonism of rhizospheric fungal isolates against *P. vexans.*

Among the bacterial isolates, isolate 2 was found effective against *R.solani* (37.62%) **(Table 5 and Figure 6a)** and *P.vexans* (36.19%) **(Table 5 and Figure 6b)** compared to other isolates.

Table 5: Inhibition of mycelial growth of pathogens by rhizospheric bacterial isolates (dual culture).

Isolate code	Percent inhibition*(R. solani)*	Percent inhibition *(F. oxysporum)*	Per cent inhibition *(P. vexans)*
Isolate 1	0.00^{B***} (0.00)**	8.84^{AB***} (17.20)**	25.24^{AB***} (30.17)**
Isolate 2	37.62^{A} (37.83)	4.76^{BC} (12.55)	36.19^{A} (36.99)
Isolate 3	4.29^{B} (7.01)	7.48^{AB} (15.11)	19.05^{BC} (21.55)
Isolate 4	10.95^{B} (19.27)	13.61^{A} (21.51)	24.29^{AB} (29.54)
Isolate 5	9.52^{B} (10.78)	3.40^{BC} (10.26)	30.95^{AB} (33.81)
Isolate 6	0.00^{B} (0.00)	5.44^{BC} (13.44)	0.00^{D} (0.00)
Control	70	49	70
**Figures in the parentheses are arcsine transformed values			
***Values with at least one letter in common are not statistically significant			

Assessment of aggressiveness: Aggressiveness of the fungal antagonists was assessed using modified Bell's scale and isolate 3 was found to be aggressive against all the pathogens tested, in which the antagonist completely over grew the pathogen **(Tables 6-8)**. Among the other isolates, isolate 6 found to be 75% aggressive on *R. solani* and *P. vexans*, isolate 8 was found to be aggressive on *F. oxysporum* **(Figure 7)**.

Table 6: Assessment of aggressiveness of rhizospheric fungal isolates against *R. solani*.

Isolate code	Duration required for contact (days)	*Bell's scale after (days)							
		2	3	4	5	6	7	8	9
Isolate 1	-	-	-	-	-	-	-	-	-
Isolate 2	4	-	-	R5	R6	R6	R6	R6	R6
Isolate 3	2	R5	R4	R4-R3	R4-R3	R3-R2	R2	R1	R1
Isolate 4	3	-	R5	R6	R6	R6	R6	R6	R6
Isolate 5	4	-	-	R5	R6	R6	R6	R6	R6
Isolate 6	2	R5	R4	R4	R4-R3	R3	R2	R2	R2
Isolate 7	2	R5	R4	R4	R4	R4	R4	R4	R4
Isolate 8	3	-	R5	R5	R5	R5	R5	R5	R5
Isolate 9	4	-	-	R5	R6	R6	R6	R6	R6
Isolate 10	-	-	-	-	-	-	-	-	-
Isolate 11	5	-	-	-	R5	R6	R6	R6	R6
Isolate 12	5	-	-	-	R5	R6	R6	R6	R6

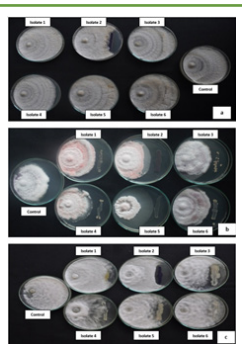

Figure 7: Antagonism of rhizospheric bacterial isolates against (a) *R. solani* (b) *F. oxysporum*(c) *P. vexans.*

Table 7: Assessment of aggressiveness of rhizospheric fungal isolates against *F. oxysporum*.

Isolate code	Duration required for contact (days)	*Bell's scale after (days)					
		4	5	6	7	8	9
Isolate 1	-	-	-	-	-	-	-
Isolate 2	5	-	R5	R5	R5	R5	R5
Isolate 3	4	R5	R5-R4	R4	R3-R2	R2	R1
Isolate 4	5	-	R5	R5	R5	R5	R5
Isolate 5	-	-	-	-	-	-	-
Isolate 6	6	-	-	R5	R5	R5	R5
Isolate 7	4	R5	R5	R5	R5	R5	R5
Isolate 8	5	-	R5	R5	R4	R3-R2	R2
Isolate 9	6	-	-	R5	R5	R5	R5
Isolate 10	-	-	-	-	-	-	-
Isolate 11	-	-	-	-	-	-	-
Isolate 12	6	-	-	R5	R5	R5	R5

Table 8: Assessment of aggressiveness of rhizospheric fungal isolates against *P. vexans*.

Isolate code	Duration required for contact (days)	*Bell's scale after (days)							
		2	3	4	5	6	7	8	9
Isolate 1	-	-		-		-	-	-	-
Isolate 2	-	-		-		-	-	-	-
Isolate 3	2	R5	R4	R3	R2-R1	R2-R1	R1	R1	R1
Isolate 4	4	-	-	R5	R5	R5	R5	R5	R5
Isolate 5	-	-		-	-	-	-	-	-
Isolate 6	2	R5	R5-R4	R4	R3	R3-R2	R3-R2	R3-R2	R3-R2
Isolate 7	3	-	R5	R5-R4	R5-R4	R4-R3	R4-R3	R4-R3	R4-R3
Isolate 8	4	-	-	R5	R5	R5-R4	R4-R3	R4-R3	R4-R3
Isolate 9	4	-	-	R5	R5	R5	R5	R5	R5
Isolate 10	4	-	-	R5	R5-R4	R5-R4	R5-R4	R5-R4	R5-R4
Isolate 11	4	-	-	R5	R5	R5	R5	R5	R5
Isolate 12	-	-		-		-	-	-	-

Effect of volatile inhibitors: Antagonistic efficacy was also studied *in vitro* by production of volatile inhibitors by the isolates against rhizome-root rot pathogens. The fungal isolates 1, 3, 7 and 10 was found to be effective against the pathogen *Rhizoctonia solani* with the per cent inhibition of 29.44%, 28.89%, 34.82% and 33.34%, respectively. Isolate 3 was found effective against *F. oxysporum* with the per cent inhibition 38.84%, whereas, none of the isolates showed any effect against *P. vexans* **(Table 9)**.

Table 9: Inhibition of mycelial growth of pathogens by rhizospheric fungal isolates (volatile inhibitors).

Isolate code	Percent inhibition *(R. solani)*	Percent inhibition*(F. oxysporum)*	Percent inhibition*(P. vexans)*
Isolate 1	29.44^{A***} (32.01)**	19.72^{B***} (24.62)**	0.00L
Isolate 2	-0.00^{C}	17.43^{BC} (24.56)	0.00K
Isolate 3	28.89^{A} (32.46)	38.84^{A} (38.19)	0.00J
Isolate 4	-0.00^{C}	5.96^{BC} (13.28)	0.00I
Isolate 5	-0.00^{C}	5.96^{BC} (13.28)	0.00H
Isolate 6	$6.67B^{C}$ (12.13)	15.14^{BC} (22.75)	0.00G
Isolate 7	34.82^{A} (36.16)	10.55^{BC} (18.67)	0.00M
Isolate 8	12.59^{B} (20.73)	15.90^{BC} (22.73)	0.00^{F}
Isolate 9	0.00	12.84^{BC} (21.01)	0.00^{E}
Isolate 10	33.34^{A} (35.27)	16.67^{BC} (23.38)	0.00^{D}
Isolate 11	0.00^{C}	12.84^{BC} (20.87)	0.00^{C}
Isolate 12	0.00^{C}	19.72^{B} (26.35)	0.00^{B}
Control (mm)	45	43.6	45
SE(d)=2.642 CV(%) =28.85 LSD at 1%=7.3426 (*R. solani*); SE(d)=6.848 CV(%)=56.93 LSD at 1%=19.029 (*F. oxysporum*) SE(d)=-CV(%)=0.00 LSD at 1% =-(*P. vexans*)			
**Figures in the parentheses are arcsine transformed values ***Values with at least one letter in common are not statistically significant			

Among the bacterial isolates, isolate 3 (35.66%) and isolate 6 (51.94%) were found effective against *R. solani*, isolate 2 was found effective against *F. oxysporum* with the percentage inhibition of 45.93% over the other isolates. Against the pathogen *Pythium vexans*, the isolates 2 and 5 were found effective, with the percentage of inhibition 36.60% and 31.85% respectively **(Table 10)**.

Table 10: Inhibition of mycelial growth of pathogens by rhizospheric bacterial isolates (volatile inhibitors).

Isolate code	Per cent inhibition (R. solani)	Per cent inhibition(F. oxysporum)	Per cent inhibition(P. vexans)
Isolate 1	$19.38B^{C***}$ (25.75)**	11.85^{B} (12.21)	16.30^{C} (23.82)
Isolate 2	17.05^{BCD} (22.67)	45.93^{A} (42.18)	36.60^{A} (37.05)
Isolate 3	35.66^{AB} (35.97)	-0.00^{B} (0)	16.30^{C} (23.62)
Isolate 4	13.18^{BCD} (19.71)	-0.00^{B} (0)	0.00^{D} (0.00)
Isolate 5	4.65^{CD} (11.77)	-0.00^{B} (0)	31.85^{B} (34.36)
Isolate 6	51.94^{A} (45.72)	7.41^{B} (9.38)	0.00^{D} (0.00)
Control	43	45	45
SE(d)=8.338 CV(%)=98.87LSD at 1%=22.88-(*R. solani*),			
SE(d)=6.744 CV(%)=174.02LSD at 1%=18.505-(*F. oxysporum*),			
SE(d)=1.315 CV(%)=21.95LSD at 1%=3.6071-(*P. vexans*)			
**Figures in the parentheses are arcsine transformed values			
***Values with at least one letter in common are not statistically significant			

Molecular Identification

Based on the *in vitro*studies, the most efficient isolates *viz*., fungal isolate 3 and bacterial isolate 2 were further used for molecular identification. The DNA was extracted from the isolates using standard isolation procedure. For bacteria, the 16S rDNA and for fungiITS4 and ITS 5 regions were amplified using universal primers and the PCR product was run through Gel electrophoresis and bacterial DNA was amplified in the region of 1500 bp and fungal DNA was amplified in the region of between 500-750 bp. The gel with the bands was excised and purified using Gene JET Gel Extraction kit (Thermo Scientific). The DNA sequencing was performed with SciGenom Labs Pvt Ltd, Cochin, Kerala. Sequence was subjected to BLAST analysis and compared

with the registered sequences in Gene Bank database using NCBI Blast server. The ITS and rDNA sequencing placed them in the genus *Trichoderma* and *Chromobacterium* **(Figure 8)**.

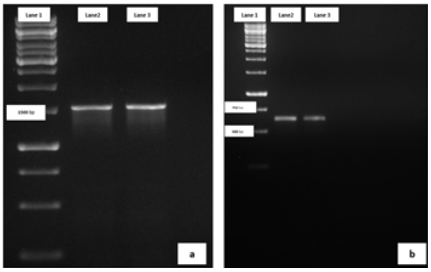

Figure 8: (a) Amplification profiles with primers 16s FP and 16s RP of bacterial isolate2; (b) Amplification profiles of ITS regions with primers ITS 4 and ITS 5 of fungal isolate 3[lane 1: Marker DNA (1 kb ladder) and lane 2 and 3: template DNA].

Discussion

Sustainable food production to meet the requirement of growing population demands the indispensable use of organic matter and a reduction in the use of agro-chemicals in the farming systems. Extensive and non-judicious application of plant protection chemicals to manage plant diseases has disturbed the delicate ecological balance of the nature leading to ground water contamination, development of resistant races of pathogens and health risks to human beings. Soil microbial diversity is considered to be critical for the maintenance of soil health and quality and agricultural practices influence soil microbial diversity and the ability to suppress plant diseases [11]. However, indiscriminate use of synthetic molecules would adversely affect various beneficial microflora residing in the rhizosphere of crops. These beneficial microbes interact both with the plants and other microbes resulting in the suppression of deleterious organisms through various mechanisms thus protecting the crop from pathogenic microbes besides enhancing plant growth. Trophic interactions between plants and soil microflora include direct intake of root exudates, cell contents and plant debris, grazing of plant roots, predation, symbiosis, antibiosis, parasitism and competition. They control the patterns and rates of organic matter decomposition, nutrient cycling, nutrient immobilization and nutrient uptake by plants. Together with abiotic factors of the soil environment these microbes regulate soil fertility and thus favors plant growth. Organic matter can affect the vigor of the host by improving the structure, moisture and nutrient holding capacity of the soil, but its principle effect is to provide a more complex antagonistic microflora and fauna [12].

In the present study, bacteria and fungi isolated from the rhizosphere of cardamom grown under organic production system were characterized and isolate codes were assigned to each isolate. Antagonistic potential of 6 predominant bacterial isolates and 12 fungal isolates were assessed against the three major pathogens of cardamom i.e., *Rhizoctoniasolani, Fusariumoxysporum and Pythiumvexans*. The rhizosphere bacteria were characterized based on twelve criteria described by [13]. Besides these criteria, investigations on their reaction to catalase test, Gram's staining, KOH test, citrate utilization, H2S production, starch hydrolysis, gelatin hydrolysis and Methyl red tests further strengthened identity of each of the isolates. The efficient bacterial isolate 2 and fungal isolate 3 were identified based on morphology, biochemical reaction (for bacterial isolates), ITS and rDNA sequencing and found to be *Chromobacterium* and *Trichoderma*, respectively.

Rhizobacteria have been found to suppress the diseases caused by various pathogenic bacteria and fungi and have the potential for use as biocontrol agents. The rhizosphere bacteria are ideal for use as biocontrol agent as they can provide the front-linedefense for plant roots against the attack by various plant pathogens. Disease suppression by biocontrol agents occurs due to interactions among the biocontrol agents with members of the rhizosphere. The mechanisms by which rhizobacteria inhibit the growth of phytopathogenic microorganisms includes: (i) antibiotic production (ii) production of bacteriocins; (iii) production of siderophores; (iv) production of hydrolytic enzymes such as β-1,3-glucanase and chitinases (v) production of other metabolites; (vi) Phytoalexins production; (vii) interference in quorum sensing; (viii) reduction in ethylene production; and (ix) induction of systemic resistance [14].

The twelve rhizosphere fungi isolated were characterized based on colony characteristics and other microscopic features. These conventional methods that have been in use for several decades were considered as efficient tools for identifying unknown fungi belonging to different groups. However, with the advent of modern molecular techniques revolutionized the field of fungal identification. The molecular tools like restriction-fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), cloning and sequencing [15] have been extensively used to characterize wide range of fungi belonging to all groups and ecosystems.

In the present study, twelve fungal isolates *viz.*, isolate 1, isolate 2, isolate 3, isolate 4, isolate 5, isolate 6, isolate 7, isolate 8, isolate 9, isolate 10, isolate 11 and isolate 12 were evaluated for their antagonistic potential against *R. solani*, *F. oxysporum and P. vexans*. Among the isolates, isolate 3 (*Trichoderma spp*.) resulted in maximum mycelial growth inhibition of *R. solani* (85.32%), *P. vexans* (82.38%) and *F. oxysporum*(76.44%), respectively. These findings are in conformity with Dar [16] who noticed *T. harzianum causing* maximum mycelial growth inhibition (92.5%) in dual culture followed by, *T. viride* (86.2%). Similar findings were also reported [17] in which the mycelial inhibition ranged from 33% to 73.3% and 29.5% to 70.8% for *F. oxysporum* and *R. solani*, respectively with *T. harzianum* proving more effective against both the pathogens, followed by *T. viride* (70.5% and 64.8%) and *Gliocladium roseum* (50% and 54.7%). *Trichoderma* species proved to be highly antagonistic and exhibited strong mycoparasitic activity. *Trichoderma spp*. has been the cynosure of many researchers who have been contributing to biological control pursuit through use of fungi. Fungi of the genus *Trichoderma* are important biocontrol agents of several soil-borne phytopathogens. *Trichoderma* use different mechanisms for the control of phytopathogens which include mycoparasitism,

competition for space and nutrients, secretion of antibiotics and fungal cell wall degrading enzymes [18]. The biocontrol agent is able to parasitize the pathogen and derive nutrition from the host pathogen. Several fungal antagonists such as Trichodermavirens may function as an aggressive mycoparasitefo fungal pathogen. It may parasitize not only Hyphae of many fungal species, but can also penetrate and destroy the resting bodies (sclerotia) that can help the pathogen overwinter and resist adverse environmental conditions. *T. virens* penetrates the hyphae and forms haustoria for absorption of nutrients from *Rhizoctonia solani* causing root rot diseases [19].

In the present study, 6 bacterial isolates *viz.*, isolate 1, isolate 2, isolate 3, isolate 4, isolate 5 and isolate 6 were evaluated for antagonistic potential against the rhizome-root rot pathogens of cardamom. Among the isolates, isolate 2 was found to have superior antagonistic activity against *R.solani* and *P. vexans* compared to other isolates. Native isolates of rhizobacteria have been used against several plant pathogens including *Phytophthora capsici* [20], *R.solani* [21], *A.flavus*, *F.verticillioides* [22] and *M.phaseolina* [23]. These bacteria with excellent antagonistic potential employ several mechanisms to subdue the plant pathogens. These mechanisms include production of antifungal metabolites such as pyoluteorin, pyrrolnitrin, phenazines, 2, 4 diacetylphloroglucinol and siderophores which scavenge iron in the rhizosphere environment and thus suppress the target pathogens [24]. Currently, the role of biological control agent is a well-established fact and has become increasingly crucial, and in several cases, complementary where antagonistic fungi play an important part. The mechanisms of fungal biocontrol may involve (i) antibiosis, (ii) competition, (iii) mycoparasitism, (iv) cell wall degrading enzymes, and (v) induced resistance. However, these mechanisms of biological control may include one and more processes [25].

Chromobacterium usually constitute only a small proportion of the total micro-flora in soil, the ability of certain strains to produce antibiotics, hydrogen cyanide, proteases and a number of chitinolytic enzymes indicate they have the potential as biological control agents. A chitinolytic bacterium, the genus *Chromobacterium*, strain C-61 was strongly antagonistic to *Rhizoctonia solani*, a causal agent of damping off of eggplant [26].

Conclusion

Hence, these microbes could be further employed as integral components in organic cardamom production system to manage rhizome-root rot efficiently, economically and eco-friendly in a sustainable manner.

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References

1. Ravindran PN (2002) Cardamom. The genus Elettaria, Indian Institute of Spices Research, Kozhikode, Kerala.
2. Toppo SR, Naik UC (2015) Department of Microbiology and Bioinformatics, Bilaspur University, Bilaspur. Isolation and characterization of bacterial antagonist to plant pathogenic fungi (Fusarium spp.) from agro based area of Bilaspur. IJRSB.
3. Deshwal VK, Dubey RC, Maheshwari DK (2003) Isolation of plant growth promoting strains of Bradyrhizobium (Arachis) sp. with biocontrol potential against Macrophominaphaseolina causing charcoal rot of peanut. Currsci India. 84: 443-448.
4. Aneja KR (2003) Experiments in Plant Pathology, Microbiology and Biotechnology, 269-274.
5. Schaad NW (1992) Laboratory Guide for identification of Plant Pathogenic Bacteria Eds. N. W. Schad, The American Psychopathological Society. Minneapolis, USA.
6. Bell DK, Wells HD, Markham CR (1982) *In vitro* antagonism of *Trichoderma spp*. against six fungal plant pathogens. Phytopathology 72: 379-382.
7. Dubey SC, Patel B (2001) Evaluation of fungal antagonists against Thanatephoruscucumeris causing web blight of urd and mung bean. Ind Phytopatho 54: 206-209.
8. Asha BB, Nayaka CS, Udaya Shankar AC, Srinivas C, Niranjana R (2011) Biological control of F. oxysporumf.sp. lycopersici causing wilt of tomato by Pseudomonas fluorescens. Inter J Microbiol Resear. 3: 79-84.
9. Kumar A, Sharma YR, Anandaraj M (2004) Evaluation of genetic diversity of Ralstoniasolanacearum causing bacterial wilt of ginger using REP-PCR and PCR-RFLP. Currsciindia 87: 1555-1561
10. Dinesh R, Anandaraj M, Kumar A, Bini YK, Subila KP, et al. (2015) Isolation, characterization and evaluation of multi-trait plant growth promoting rhizobacteria for their growth promoting and disease suppressing effect son ginger. Microbiol Res. 173: 34-43.
11. Garbeva P, Postma J, Van Veen JA, van Elsas JD (2006) Effect of above ground plant species on soil microbial community structure and its impact on suppression of Rhizoctoniasolani AG3. Environ Microbiol 8: 233-246.
12. Broadbent P, Baker KF (1975) Soils suppressive to phytophthora root rot in eastern Australia. 152-157.
13. Buchanan RE (1967) Bacteriology (5). pp: 175-186.
14. Sindhu SS, Rakshiya YS, Sahu G (2009) Department of microbiology, ccs haryana agricultural university hisar, india, biological control of soilborne plant pathogens with rhizosphere bacteria. Pest Technology 3: 10-21.
15. Bowen JK, Franicevic SC, Crowhurst RN, Templeton MD, Stewart A (1996) Differentiation of specific Trichoderma biological control agent by restriction fragment length polymorphism (RFLP) analysis. New Zealand J Crop Horticul Sci 24: 207-217.
16. Dar WA, Beig MA, Ganie SA, Bhat JA, Shabir-u-Rehman, et al. (2013) *In vitro* study of fungicides and biocontrol agents against Fusariumoxysporumf.sp. pini causing root rot of Western Himalayan fir (Abiespindrow). Sci Res Essays 8: 1407-1412.
17. Gh Hassan Dar MA, Beig Ahanger FA, Nadeem A Ganai, Ashraf Ahangar M (2011) Management of root rot caused by Rhizoctoniasolani and

Fusariumoxysporum in Blue Pine (Pinuswallichiana) through use of fungal antagonists. Asian J Plant Patho 5: 62-74.

18. Ouda SM (2014) Biological control by microorganisms and ionizing radiation. Inter J Advanc Resear 2: 314-356.

19. Narayanasamy P (2013) Mechanisms of Action of Fungal Biological Control Agents. pp:99-100.

20. Diby P, Saju KA, Jisha PJ, Sharma YR, Kumar A, et al. (2005) Mycolyticenzymes produced by Pseudomonas fluorescence and Trichoderma spp. Against Phytophthoracapsici, the foot rot pathogen of black pepper (Piper nigrum L.). Ann Microbiol55: 129-133.

21. Bautista G, Mendoza H, Uribe D (2007) Biocontrol of Rhizoctoniasolani in native potato (Solanumphureja) plant susing native Pseudomonas fluorescens. Acta Biol Colombo 12: 19-31.

22. Palumbo JD, O'Keeffe TL, Abbas KK (2007) Isolation of maize soil and rhizosphere bacteria with antagonistic activity against Aspergillus flavus and Fusarium verticillioides. J Food Protect 70: 1615-1621.

23. Arora NK, Kang SC, Maheshwari DK (2001) Isolation of siderophores producing strains of Rhizobium meliloti and their biocontrol potential against Macrophomina phaseolina that causes charcoal rot of groundnut. Currsciindia. 81: 673-677.

24. Dwivedi D, Johri BN (2003) Antifungal from fluorescent pseudomonads: Biosynthesis and regulation. Current Science 85: 1693-1703.

25. Chaur-Tsuen Lo (1998) Taiwan Agricultural Research Institute, 189 Chung-cheng Rd. Wufeng, Taichung, Taiwan, General mechanisms of action of microbial biocontrol agents. Plant Pathology Bulletin 7: 155-166.

26. Seur Kee Park, Myung-Chul Lee, Herman GE (2005) The biocontrol activity of Chromobacterium sp. strain C-61 against Rhizoctoniasolani depends on the productive ability of chitinase. Plant Pathology J 21: 275-282.