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Evaluation of bacterial antagonists for the management of rhizome rot of cardamom (*Elettaria cardamomum* Maton)

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Abstract

Among the 90 rhizobacterial isolates screened against rhizome rot pathogens (*Pythium vexans, Fusarium oxysporum* and *Rhizoctonia solani*) of cardamom (*Elettaria cardamomum*) two bacterial strains viz., *Pseudomonas fluorescens* Pf 51 and *Bacillus subtilis* B45 were highly inhibitory. *P. fluorescens* Pf 51 exhibited highest inhibition (42.5%, 44.2% and 41.4% respectively) against *P. vexans, F. oxysporum* and *R. solani. B. subtilis* B45 also exhibited highest inhibition (43.2%, 41.2% and 42.4% respectively) against these three pathogens. *P. fluorescens* Pf51 was compatible with *B. subtilis* Bs 45. Peat formulation supported the survival of both the strains up to 270 days with a viable population of 4.3×10^7 cfu g^{-1} and 6.2×10^7 cfu g^{-1} respectively. Application of antagonists in combination with rhizome bacterization and soil application resulted in 54.0% reduction in rhizome rot over control as compared to single method such as rhizome bacterization (43.0%) or soil application (39.0%). Application of copper oxychloride and carbendazim resulted in 68.0% reduction of rhizome rot. Maximum height (167.21 cm) and number of tillers (30.14) were recorded due to the application of mixture of both the strains through rhizome bacterization and soil application.

Keywords: bacterial peat formulation, *Fusarium oxysporum, Pythium vexans, Rhizoctonia solani*

Introduction

Rhizome rot caused by *Pythium vexans* de Dary, *Fusarium oxysporum* and *Rhizoctonia solani* Kuhn is a serious disease throughout the cardamom (*Elettaria cardamomum* Maton) growing regions of south India. The disease is responsible for partial or total decay of plants of all stages and causes about 30%–60% crop loss in India (Thomas *et al*. 1988; Vijayan & Thomas 2002). Exploitation of antagonistic microorganisms against rhizome rot pathogens is an alternative approach to produce cardamom on a sustainable basis. Plant growth promoting rhizobacteria (PGPR) such as *Pseudomonas* spp. and *Bacillus* spp. have been widely used for biological control of several fungal, bacterial and viral pathogens. Some of the antagonistic *P. fluorescens* Migula and *B. subtilis* Cohn also act as inducers of systemic resistance in plants. The growth promotion and biocontrol activity of *P. fluorescens* and *B. subtilis* against cardamom pathogens *Phytophthora meadii* McRae and *Fusarium oxysporum* Schlecht. emend. Snyder and Hansen were proved (Thomas & Vijayan

2003; Bhai & Sarma 2003). In the present study, the bioefficacy of a peat formulation containing either a single strain or mixture of strains of bacterial antagonists was evaluated against rhizome rot of cardamom.

Materials and methods

Collection of pathogens and rhizobacteria

The studies were undertaken at Cardamom Research Station, Pampadumpara (Kerala) during 2007–08. Rhizome rot pathogens of cardamom (*P. vexans, F. oxysporum* and *R. solani*) were isolated from infected cardamom plants on potato dextrose agar (PDA) medium. The pathogenicity was established with pure cultures of the pathogens under sterile environment. Rhizobacteria were isolated on King's B and nutrient agar medium from fresh roots of cardamom, black pepper, coffee, vanilla and ginger from cardamom hill reserves of Idukki district of Kerala and Theni and Dindigul districts of Tamil Nadu. The rhizobacteria were characterized based on colony morphology, gram and endospore staining techniques. All the pure cultures of rhizobacteria were maintained on King's B and nutrient agar medium. The identity of highly promising strains *P. fluorescens* Pf51 and *Bacillus subtilis* Bs45 were confirmed with Project Directorate of Biological control (PDBC), Bengaluru.

In vitro screening of rhizobacteria

Ninety rhizobacterial isolates were tested *in vitro* for their antagonistic activity against *P. vexans, F. oxysporum* and *R. solani*. The antagonistic potential of the bacterial isolates against the pathogens was tested by dual culture method and growth of the fungal mycelia towards the bacterial colony and inhibition zone was recorded (Nagarajkumar *et al*. 2005). The bacterial antagonists were streaked at one end of the PDA placed in sterilized plates 24 h prior to pathogen inoculation. Just opposite to the bacterial streak, a 9 mm disc of the pathogen was placed. Three replications of each isolate including a control, (without inoculation of antagonists) were maintained. The plates were incubated at 28±°C. The linear growth of the fungal mycelia towards the bacterial colony was measured after 72 h and the per cent inhibition was calculated using the formula: I = C-T/C×100, where I is the per cent inhibition, C and T are radial growth of the pathogen in control and treatment respectively. The promising isolates of bacteria were maintained in glycerol stock.

Assessment of compatibility of P. fluorescens and B. subtilis in vitro

The compatibility of the two antagonistic organisms were tested *in vitro* through two methods. The mutual compatibility of two antagonistic organisms was tested by dual culture method, and the plates were assessed for inhibition zone after 48 h. In another method *P. fluorescens* culture was streaked on King's B medium. After 2 days, the *B. subtilis* suspension was sprayed over the *P. fluorescens* colonies. Similarly, the *P. fluorescens* suspension was sprayed over *B. subtilis* colonies and the plates were assessed for the inhibition zone after 48 h (Bharathi *et al*. 2004).

Shelf life in different formulations

Pseudomonas fluorescens Pf51 and *B. subtilis* B45 were highly inhibitory against cardamom rhizome rot pathogens and these strains were further studied for formulation development. Strains Pf51 and B45 were grown in King's B broth and nutrient broth respectively, for 48 h in shake culture at 150 rpm at room temperature (28±2°C). Shelf life of bacteria was tested in four different carriers: peat, talc, vermiculite and lignite. Carboxy methylcellulose (10 g) was added to 1 kg of the carrier as a sticker and mixed well. The carriers were autoclaved for 45 min at 137.3 kPa. Bacterial suspension (500 ml) containing 9 × 10^8 cfu ml^{-1} of broth was added to 1 kg of carrier and mixed well under aseptic conditions. The formulations were air-dried to 20% (w/v) moisture content, packed in separate polythene bags (three replicates) and incubated at (28±2°C). Samples were taken from each bag containing different carrier material at monthly intervals for up to nine months. The population of *P. fluorescens* Pf51 and *B. subtilis* B45 were done on King's B and nutrient agar

medium, respectively, using serial dilution method. The experiment was set up as a completely randomized design using four replications. The population of rhizobacteria were estimated at monthly intervals.

Efficacy of bioformulations in greenhouse conditions

The bioefficacy of effective strains of *P. fluorescens* Pf51 and *B. subtilis* B45 were evaluated individually as well as in combination against rhizome rot in the greenhouse. The experiment was conducted in a completely randomized block design in microplots with nine treatments and four replications using var. Greengold. Cardamom clones were planted in the pits of 75 cm × 75 cm × 45 cm size with a spacing of 1.5 m × 1.5 m. The following were the treatments: T_1: Rhizome bacterization with *P. fluorescens* Pf51 @ 50 g plant^{-1}, T_2: Rhizome bacterization with *B. subtilis* B45 @ 50 g plant^{-1}, T_3: Soil application with Pf51 @ 50 g plant^{-1}, T_4: Soil application with B45 @ 50 g plant^{-1}, T_5: Rhizome bacterization with Pf51 and B45 @ 50 g plant^{-1}, T_6: Soil application with Pf 51 and B 45 @ 50 g plant^{-1}, T_7: Rhizome bacterization and soil application with Pf51 and B45 @ 50 g plant^{-1}, T_8: Copper oxy chloride (0.25%) and Carbendazim (0.1%), T_9: Control. Sterile mixture consisting of river sand, soil and farm yard manure in the ratio of 1:1:1 was filled in the pits and cardamom clones were planted after treatment @ three clones per pit. Fresh peat formulations were prepared separately for two antagonists. In the case of rhizome treatment, 50 g of formulation (25 g from each antagonist) was mixed with required quantity of water and uniform thick paste was made which was coated uniformly on all sides of rhizome and kept under shade for 1 h. The bacterized rhizomes were planted in the pits. In case of soil application, the formulation of both the antagonists were incorporated uniformly in the potting mixture individually and in consortium basis (25 g from each) @ 50 g plant^{-1} at the time of planting. The cardamom clones were then planted in the mixture which contained the antagonist or antagonists. Copper oxychloride was drenched in the pits containing sterile potting mixture at the time of planting and carbendazim was drenched 4 days after planting. Control plots without any treatment were also maintained. The pathogens *P. vexans* and *F. oxysporum* were multiplied in sand-maize medium and *R. solani* in paddy grains. The freshly multiplied pathogens were artificially inoculated into the soil when the plants attained the tillering phase (7 months after planting). Observations on growth parameters and per cent tiller infection were recorded.

Results and discussion

Among 90 rhizobacterial strains screened *in vitro* against *P. vexans, F. oxysporum* and *R. solani,* only 19 were inhibitory to the three pathogens. The short listed strains include *P. fluorescens* strains (7, 10, 15, 47, 51, 62, 75, 84 and 90) and *Bacillus* strains (9, 12, 13, 34, 45, 65, 71, 74, 85 and 87). Among 19 promising bacterial strains, *P. fluorescens* Pf51 and *B. subtilis* B45 were highly inhibitory. Strain Pf 51 exhibited highest inhibition (42.5%, 44.2% and 41.4%) against *P. vexans, F. oxysporum* and *R. solani* respectively. Similarly *B. subtilis* strain B45 also exhibited highest inhibition (43.2%, 41.2% and 42.4%) against those three pathogens respectively (Table 1). High degree of *in vitro* antagonism against different kinds of pathogens by fluorescent pseudomonads (Rangeshwaran & Prasad 2000; Sivakumar & Sharma 2007) and *Bacillus* spp. (Loganathan *et al*. 2011; Sivakumar *et al*. 2011) have also been reported earlier. The compatibility study revealed that there was no inhibition zone between *P. fluorescens* and *B. subtilis* indicating the compatible nature of both the antagonists. Earlier studies also reported that the both bacteria are compatible and the combination was highly successful in controlling crop diseases (Thilakavathi *et al*. 2007; Salaheddin *et al*. 2010).

The population of both the strains in different formulations was assessed at periodic intervals up to 270 days of storage. Among the carriers, peat supported the survival of both strains Pf51 and B45 for 270 days with a viable population of 4.3×10^7 cfu g^{-1} and 6.2×10^7 cfu g^{-1}, respectively (Table 2 & 3). Talc supported survival of strains

Table 1. *In vitro* screening of rhizobacteria against rhizome rot pathogens

Isolate	*In vitro* inhibition (%)		
	Pythium vexans	*Fusarium oxysporum*	*Rhizoctonia solani*
Pseudomonas fluorescens			
Pf 7	13.2	15.2	15.4
Pf 10	23.4	22.1	32.1
Pf 15	18.5	19.4	20.4
Pf 47	15.5	15.4	13.8
Pf 51	42.5	44.2	41.4
Pf 62	13.1	14.7	15.2
Pf 75	11.4	12.4	13.2
Pf 84	9.2	9.7	12.4
Pf 90	14.7	12.7	13.6
Bacillus subtilis			
B 9	18.6	17.4	13.6
B 12	19.2	14.6	17.7
B 13	24.7	23.5	20.4
B 34	15.3	14.6	17.2
B 45	43.2	41.2	42.4
B 65	13.9	15.2	14.2
B 71	14.2	16.1	14.7
B 74	9.8	9.1	12.5
B 85	21.4	22.2	19.7
B 87	13.2	13.2	14.1
Control	0.0	0.0	0.0
CD (P=0.05)	1.02	1.21	1.23

for 180 days with a population of 1.0 × 10^7 cfu g^{-1}, and 2.0 × 10^7 cfu g^{-1} respectively for both the strains (Tables 2 & 3). The population gradually declined in all the carrier media tested. However, they maintained minimum population of 0.1 x 10^7 cfu g^{-1} till nine months of storage. The results revealed that the bacterial strains survived with the required cfu (10^7 ml^{-1}) in the bio formulation up to 270 days of storage. The population of *B. subtilis* B45 was higher when compared to *P. fluorescens* Pf 51 after nine months of storage. It may be due to the production of endospores by *Bacillus* spp. that offer great advantage for long-term storage (Powell *et al*. 1999). Various carrier formulations of *Psueudomonas* and *Bacillus* have been developed for controlling soil and seed borne diseases. Satisfactory survival of *P. fluorescens* isolates in peat soil and its efficacy against chick pea wilt, banded leaf and sheath blight have been reported earlier by Vidyasekaran & Muthamilan (1995); Sivakumar *et al*. (2007); Nakkeeran *et al*. (2006). Similarly a good survival of *Bacillus* spp. in peat soil and its efficacy has also been reported earlier (Nakkeeran *et al*. 2006).

Peat based formulations of *P. fluorescens* Pf 51 and *B subtilis* B45 were evaluated individually and in combination under microplots in different methods for suppressing the rhizome rot disease of cardamom. Both the strains performed better and were on par in growth promotion and disease suppression activities as compared to control (Table 4). Mixture of both Pf 51 and B45 strains was best and resulted in 48% disease reduction over control as compared to single strain (Table 4). The most effective management was achieved when peat based

Table 2. Shelf life of *Pseudomonas fluorescens* strain Pf 51 in different formulations

Formulation	Population (× 10^7 cfu g^{-1})								
	Days after storage								
	0	30	60	90	120	150	180	240	270
Peat	66.0	56.0	49.2	38.0	26.0	18.1	14.2	9.2	4.3
Talc	59.5	43.1	40.3	31.6	19.3	4.1	1.0	0.1	0.1
Vermiculite	52.0	41.0	37.4	20.2	10.2	1.0	0.1	0.1	0.1
Lignite	52.2	40.0	35.2	17.2	5.3	1.0	0.1	0.1	0.1
CD (P=0.05)	1.01	0.99	1.15	0.88	1.10	0.74	1.31	0.04	0.01

Table 3. Shelf life of *Bacillus subtilis* strain B45 in different formulations

Formulation	Population (× 10^7 cfu g^{-1})								
	Days after storage								
	0	30	60	90	120	150	180	240	270
Peat	76.0	63.0	52.2	42.0	28.0	20.0	15.4	11.2	6.2
Talc	68.5	58.0	43.0	33.6	20.3	6.3	2.0	1.0	0.1
Vermiculite	62.0	44.0	38.5	22.2	16.3	1.1	0.1	0.1	0.1
Lignite	64.0	42.0	36.1	19.2	10.2	1.0	0.1	0.1	0.1
CD (P=0.05)	1.1	1.02	1.12	1.25	2.21	0.31	1.21	0.03	0.02

Table 4. Bioefficacy of bacterial antagonists on growth and rhizome rot incidence of cardamom under microplots

Treatment	No. of tillers	Plant height (cm)	Rhizome rot incidence (%)	Percent reduction over control
T_1: Rhizome bacterization with *P. fluorescens* Pf 51 @ 50 g $plant^{-1}$	24.27	142.27	40.15	42.74
T_2: Rhizome bacterization with *B. subtilis* B 45 @ 50 g $plant^{-1}$	23.14	142.17	40.65	42.04
T_3: Soil application with *P. fluorescens* Pf51 @ 50 g $plant^{-1}$	21.56	140.24	42.35	39.60
T_4: Soil application with *B. subtilis* B45 @ 50 g $plant^{-1}$	20.21	141.15	42.34	39.61
T_5: Rhizome bacterization with *P. fluorescens* Pf 51 and *B. subtilis* B45 @ 50 g $plant^{-1}$	27.12	151.76	38.14	45.60
T_6: Soil application with *P. fluorescens* Pf 51 and *B. subtilis* B45 @ 50 g $plant^{-1}$	26.27	150.27	36.34	48.17
T_7: Rhizome bacterization and soil application with *P. fluorescens* strain Pf51 and *B. subtilis* strain B45	30.14	167.21	32.12	54.19
T_8: Copper oxychloride (0.25%) and Carbendazim (0.1%)	32.30	140.14	22.31	68.18
T_9: Control	13.21	90.34	70.12	0.00
CD (P=0.05)	3.01	5.13	4.12	

formulations of both strains Pf51 and B45 were applied as rhizome bacterization and soil application which resulted in 54% reduction of rhizome rot over control as compared to individual treatments as rhizome bacterization (43%) and soil application (39%). Application of copper oxychloride and carbendazim resulted in 68% reduction of rhizome rot. Better growth of cardamom plants was noticed following application of the bacterial antagonists as compared to chemicals. Maximum height (167.21 cm) and number of tillers (30.14) were recorded due to the application of mixture of both the strains through rhizome and soil (Table 4) as compared to the single strain and individual

application method. The increase in plant growth might be due to the growth-promoting compounds such as gibberellins, cytokinins, auxin from tryptophan produced by biocontrol agents (Pal *et al*. 2000). Several approaches have been used to control crop diseases which include the combined application of two or more biocontrol strains to enhance the level and consistency in disease control. Biological control with multi-mechanisms may be achieved by using one biocontrol agent exhibiting several mechanisms or by applying more than one biocontrol agent in a mixture. *P. fluorescens* was found compatible with *B. subtilis* (Salaheddin *et al*. 2010) and the compatible combination was effective in controlling tomato damping off (Nakkeeran *et al*. 2006), dry root rot of green gram (Thilakavathi *et al*. 2007) ground nut root rot (Ramesh & Korikanthimath 2010) and bacterial blight of cotton (Salaheddin *et al*. 2010). Studies on the mode of action of *B. subtilis* have shown that increase in crop growth parameters is due to the release of bacterial metabolites having precursors of auxin (indole-3-pyruvic acid) or inducers (G3 fraction) for auxin synthesis (Bochow & Dolej 1999). Biosynthesis of antibiotics, production of lytic enzymes, production of siderophores, production of hydrogen cyanide, competition for substrates and induced systemic resistance are the major mechanisms responsible for the bicontrol activity of various fluorescent pseudomonads (Bloemberg & Lugtenberg 2001). The present study confirmed the compatibility between these two bacteria and application of this mixture through rhizome bacterization and soil application could effectively control rhizome rot of cardamom.

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