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Induced defense response in small cardamom plants by *Bacillus subtilis* strain Bs against capsule rot pathogen, *Phytophthora meadii*

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Abstract

The objective of this study was to investigate the mechanism by which *Bacillus* strains confer plant protection. *Bacillus subtilis* strain Bs was identified as potential bacterial antagonist against cardamom capsule rot pathogen, *Phytophthora meadii*. This strain was tested for its ability to induce defense related enzymes *viz*., peroxidase (PO), polyphenoloxidase (PPO) and total phenols against *P. meadii* in cardamom plants. Cardamom plants treated with bacterial antagonist and challenge inoculated with *P. meadii* showed higher levels of defense related enzymes and phenols compared to antagonist alone, pathogen alone and untreated plants. *B. subtilis* strain Bs induced higher activities of total phenols (182 µg g^{-1} of tissue), PO (2.81 change in OD min^{-1} g^{-1} of tissue) and PPO activity (1.01 change in OD min^{-1} g^{-1}) in cardamom plants treated with *P. meadii*. The present study clearly indicated that the bacterial antagonist *B. subtilis* has the ability to induce defense related enzymes in cardamom plants against *P. meadii*.

Keywords: *Bacillus subtilis*, capsule rot, induced defense, *Phytophthora meadii*, small cardamom

Introduction

Capsule rot (Azhukal) caused by *Phytophthora meadii* Mc Rae of A2 mating type is a serious threat causing extensive damage during south west monsoon in many small cardamom growing areas of South India. The pathogen affects capsules at all ages and panicles. In severe cases, infection spreads over to the rhizomes and tillers. Decayed tillers break and fall off at the collar region. The loss in productivity due to this disease was up to 30% (Sivakumar *et al*. 2012). The disease is widely distributed and it is difficult to control with chemicals and cultural practices. Exploitation of antagonistic microorganisms against capsule rot pathogen is an alternative approach to produce cardamom on a sustainable basis and also to protect the cardamom ecosystem. Plant growth-promoting rhizobacteria (PGPR) improve plant health through mechanisms like antagonism against plant pathogens, improving host nutrition and stimulating plant or host defense mechanisms (Choudhary & Johri 2009). Plant possess a range of active

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defense compounds which act against invading pathogens and utilization of plant's own defense mechanism is the subject of current interest in management of plant diseases. Induced systemic resistance (ISR) is the enhancement of the plant's defense response by plant growth promoting rhizobacteria (PGPR) and systemic acquired resistance (SAR) is the defense response of plant against pathogen attack and other elicitors (Choudhary *et al*. 2007). Many *Bacillus* species like *B. amyloliquefaciens, B. subtilis, B.megaterium, B. pasteurii, B. cereus* etc., are potential antagonists and also induce defense response and reduce disease incidence in different host-pathogen combinations (Kloepper *et al*. 2004; Sivakumar *et al*. 2011 & 2013). These bacteria can activate plant's defense mechanisms by enhancing the levels of defense related enzymes like peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and phenolic compounds and make the plant resistant to pathogen. Phenols have been known to occur in all plants investigated so far. Some of these phenoloic enzymes occur constitutively, whereas others are formed in response to pathogen ingress and associated as part of an active defense response in the host (Nicholson & Hammerschmidt 1992). The constitutive phenolics are known to confer resistance either directly or indirectly through activation of post infection responses in the hosts (DeVecchi & Matta 1989). The antagonism and bioefficacy of promising bacterium *B. subtilis* strain Bs was proved against capsule rot under microplot conditions (Sivakumar *et al*. 2012). In the present study, this strain was tested for its ability to induce defense related enzymes and phenolic content in cardamom plants against capsule rot pathogen, *P. meadii*.

Materials and methods

Preparation of peat formulation of B. subtilis *strain Bs*

B. subtilis strain Bs was grown in nutrient broth for 48 h in shake culture at 150 rpm at room temperature (28 ± 2°C). After incubation, the broth culture was used for the preparation of peat formulation. The peat powder was autoclaved for 45 min at 137.3 kPa pressure. The pH was adjusted to 6.5-7.0 by using calcium carbonate ($CaCO_3$). About 500 mL of bacterial suspension containing 9×10^8 cfu mL^{-1} of broth was added to 1 kg of sterilized peat powder and mixed well under sterile conditions. The formulation was air-dried to 20% (w/v) moisture content, packed in polythene bags and incubated at 28 ± 2°C. The population of bacteria in peat formulation was estimated by serial dilution plate technique on nutrient agar medium.

Preparation of pathogen inoculum

Capsule rot pathogen of cardamom, *P. meadii* was isolated from infected cardamom capsules collected during monsoon season using PVPH medium (Tsao & Guy 1977). The culture was further transferred to carrot agar medium and maintained in slants for further use.

Analysis of defense related enzymes and total phenols

The promising antagonist *B. subtilis* strain Bs was tested for their efficacy to induce defense related enzymes *viz*., peroxidase (PO), polyphenol oxidase (PPO) and total phenols against capsule rot pathogen *P. meadii*. The following were the treatments. T_1: Rhizome bacterization with *B. subtilis* strain Bs @50 g $plant^{-1}$, T_2: Rhizome bacterization with *B. subtilis* strain Bs @50 g $plant^{-1}$ and challenge inoculated with *P. meadii* three days after the application of antagonist, T_3: Plants inoculated with the pathogen alone; T_4: Untreated control plants.

The experiment with the above treatments was conducted during 2008 & 2009 in a completely randomized design with five replications using variety 'Njallani'. Cardamom clones were treated with antagonistic peat formulation by rhizome bacterization and kept under shade for 1 h. The bacterized rhizomes were planted in the pits. The plants were challenge inoculated with the pathogen *P. meadii* after three days of antagonist treatment. The mycelial discs (5 mm diameter) of the freshly multiplied pathogen *P. meadii* was applied at the base of the plants into the soil.

Analysis of phenolic enzymes and total phenols

Shoot and leaf samples were collected at different time intervals (2, 4, 6, 8, 10 and 12 days after pathogen inoculation) for enzyme assays. One gram of sample from each treatment was homogenized with 2 mL of 0.1 M sodium phosphate buffer (pH 7.0) in a pre-chilled mortar and pestle under ice cold condition. The homogenate was centrifuged for 15 min at 10,000 rpm. The supernatant was used as a crude enzyme extract for assaying PO, PPO activity and 80% ethanol extracts were used for assaying total phenolic content (modified from Anand *et al*. 2010).

Assay of total phenols

An aliquot of 0.1 mL of ethanol extract was evaporated in hot water bath. After complete evaporation of ethanol, 6 mL water was added and shaken well before addition of 0.5 mL Folin-ciocalteu reagent (1 N). After 5 min, 2 mL of 20% sodium carbonate solution was added and incubated for 30 min in dark condition at room temperature. Absorbance was recorded at 660 nm in spectrophotometer and the phenol content in the sample was calculated using pyrocatechol as standard. The quantity of total phenols was expressed in μg g^{-1} of fresh plant weight (Malik & Singh 1980).

Assay of Peroxidase (PO)

The reaction mixture consisted of 100 μL enzyme extract, 1.5 mL of 0.05M pyrogallol in 0.1M sodium phosphate buffer (pH 6.5) and 0.5 mL of 1% hydrogen peroxide. Boiled enzyme preparation served as blank. The changes in absorbance at 420 nm was recorded at 30 sec interval for 3 min in spectrophotometer. The peroxidise (PO) activity was expressed as change in the absorbance of the reaction mixture min^{-1} g^{-1} of fresh plant weight at 420 nm (Hammerschmidt *et al*. 1982).

Assay of polyphenol oxidase

Polyphenol oxidase activity (PPO) was assayed by the change in colour intensity of catechol oxidation products. The reaction mixture consisted of 100 μL enzyme extract and 1.5 mL of 0.1M sodium phosphate buffer (pH 7.0), the reaction started when 200 μL of 0.01M catechol was added. The changes in the absorbance was recorded at 30 sec interval for 3 min at 495 nm and the enzyme activity was expressed as changes in absorbance at 495 nm min^{-1} g^{-1} of fresh plant tissue (Mayer *et al*. 1965).

Results and discussion

All the treatments showed increase in the activity of the peroxidase upto six days after inoculation except control where the activity was steady without much increase (Table 1). There was significant increase in peroxidase activity in the rhizome bacterized cardamom plants as compared to control and pathogen inoculated treatments. Highest activity of peroxidase (2.81 change in absorbance min^{-1} g^{-1} of tissue) was observed six days after inoculation in the bacterized plants and challenge inoculated with the pathogen as compared to bacterized plants alone (2.28 min^{-1} g^{-1}), pathogen inoculated alone (1.81 min^{-1} g^{-1}) and control (0.79 min^{-1} g^{-1}).

The maximum increase in PO activity six days after inoculation of the pathogen was observed in the rhizome bacterized cardamom plants with challenge inoculation of pathogen (1.01

Table 1. Peroxidase (PO) activity in cardamom plants treated with *B. subtilis* under microplots

Treatments	Peroxidase (PO) activity (absorbance min^{-1} g^{-1} of tissue)					
	Days after inoculation					
	2	4	6	8	10	12
Rhizome bacterization with *B. subtilis*	1.61	1.71	2.28	1.81	1.59	1.53
Rhizome bacterization with *B. subtilis* + *P. meadii*	1.82	1.89	2.81	2.19	2.01	1.93
P. meadii	1.17	1.67	1.81	1.63	1.41	1.33
Control	0.54	0.72	0.79	0.81	0.79	0.67

change in absorbance min^{-1} g^{-1} of tissue) followed by bacterized plants alone (0.89), pathogen alone (0.59) and control (0.15). In control plants there was not much variation in the activity. The activity of the enzyme declined significantly eight days after inoculation of the pathogen (Table 2).

Table 2. Polyphenol oxidase (PPO) activity in cardamom plants treated with *B. subtilis* under microplots

Treatments	Polyphenol oxidase activity (PPO) (absorbance min^{-1} g^{-1} of tissue)					
	Days after inoculation					
	2	4	6	8	10	12
Rhizome bacterization with *B. subtilis*	0.43	0.55	0.89	0.87	0.72	0.52
Rhizome bacterization with *B. subtilis* + *P. meadii*	0.77	0.89	1.01	0.89	0.76	0.54
P. meadii	0.29	0.43	0.59	0.41	0.32	0.19
Control	0.12	0.15	0.15	0.17	0.15	0.13

There was significant increase in phenol content in the antagonist treated cardamom plants as compared to control. The phenol content increased after inoculation and attained maximum at six days after inoculation of pathogen.The maximum increase in phenol content was observed from 161-182 μg g^{-1} of plant tissue in the bacterized cardamom plants with challenge inoculation of pathogen followed by bacterized plants alone from 152-169 μg g^{-1}, pathogen inoculated plants alone (147-161 μg g^{-1}). In control plants the phenol content increased gradually upto six days after inoculation and declined thereafter (Table 3).

Induction of systemic resistance in plants by application of any bioagent is thought to be the best alternative for plant protection from pathogens. Our present study clearly proved that the bacterial antagonist *B. subtilis* induced defense enzymes against the capsule rot pathogen *P. meadii*. Many studies have shown that members of bacterial genera can induce systemic resistance in different plants for control of soil-borne diseases (Nagorska *et al*. 2007). Some members of *Bacillus* genera are able to produce various lytic enzymes (e.g. chitinase and μ-1,3 glucanase) and antibiotics, along with induction of systemic resistance of plants, such as increasing the activities of plant defense related enzymes of peroxidase, PPO and PAL (Jayaraj *et al*. 2004). Oxidative enzymes such as PO and PPO, can catalyze the formation of lignin and other oxidative phenols, and contribute in formation of defense barriers by changing the cell structure defense system against pathogens (Thilagavathi *et al*. 2007). These enzymes have been reported to correlate with the defense activities against pathogens in several plant species (Thilagavathi *et al*. 2007). The present study proved that there was increase in the activity of peroxidase and PPO

Table 3. Phenol content in cardamom plants treated with *B. subtilis* under microplots

Treatments	Phenol content (μg g^{-1} of plant tissue)					
	Days after inoculation					
	2	4	6	8	10	12
Rhizome bacterization with *B. subtilis*	152	161	169	154	148	141
Rhizome bacterization with *B. subtilis* + *P. meadii*	161	172	182	179	165	151
P. meadii	147	152	161	144	143	139
Control	120	121	124	123	119	117

and phenol content in the bacterized cardamom plants upon inoculation of pathogen. This results are in accordance with the earlier reports (Ramanujam *et al*. 2012; Nakkeeran *et al*. 2006) which revealed that the application of bacterial antagonists *P. fluorescens* and *B. subtilis* increased the level of peroxidase, PPO and PAL, 3-4 days after inoculation of pathogen. Elicitation of induced systemic response by use of *Bacillus* strains has been documented on tomato against fungal and bacterial diseases. *B. subtilis* strain FZB-G was shown to produce defence related biochemical enzymes in tomato against Fusarium wilt disease (Gupta *et al*. 2000). The present study revealed that application of peat formulation of *B. subtilis* strain Bs increased the activity of phenolic enzymes and phenols in cardamom plants against capsule rot pathogen and this bacterial strain could be effectively utilized for the management of capsule rot disease in cardamom.

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