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Bacillus subtilis Mediated Systemic Resistance in Chillies against *Colletotrichum capsici*

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A B S T R A C T

Die-back and fruit rot caused by *Colletotrichum capsici* (Syd.) Butl. and Bisby is the destructive disease in chillies. The effect of *Bacillus subtilis* talc based formulation in managing the fruit rot disease and inducing resistance in chillies was investigated. *B. subtilis* strain *Bs*1 significantly inhibited the growth of *C. capsici in vitro.* Seed treatment with *B. subtilis* @ 10 g/kg seed followed by foliar application of *B. subtilis* @ 10 g/l on 30 and 60 DAP was effective in managing the fruit rot disease and increasing the dry chillies yield under field conditions. The effect of biocontrol agent *B. subtilis* on the induction of defense enzymes *viz*., chitinase and β-1,3-glucanase in chilli plants infected with *C. capsici* was investigated. There was a marked increase in the induction of defense compounds in response to pathogen inoculation in *B. subtilis* treated chilli leaves.

Introduction

Chilli (*Capsicum annuum* L.) is an important commercial vegetable crop grown for domestic and export markets. Among the various biotic factors affecting chilli, fruit rot and die back caused by *Colletotrichum capsici* (Syd.) Butl. and Bisby is one of the serious diseases of chillies and occurs in severe form in all the southern states of India. The loss in yield was estimated as 30.7 per cent in Tamil Nadu. Use of chemicals against plant diseases leads to environmental pollution and toxic effects on human health and give possibility to pathogens for building-up resistance to chemicals. Hence biological control agents creating a longer lasting effect are a necessity besides their antiphytopathogenic potential of soils. The biocontrol agent, *Bacillus subtilis* produces several classes of broad spectrum lipopeptides antibiotics which are effective suppressors of many plant pathogens, including species of *Fusarium*, *Pythium*, *Phytophthora*, *Rhizoctonia*, *Sclerotinia*, *Septoria*, *Verticillium* etc. (Ongena and Jacques, 2008). Manikandan *et al.*, (2010) reported that the use of liquid bioformulation on tomato plants decreased the *Fusarium* wilt incidence due to the induction of defense enzymes. Collective function of several PR proteins may be effective in inhibiting pathogen growth, multiplication and spread of pathogen and be responsible for the state of induced resistance (Van Loon, 1997). Hence,

the present study was undertaken to evaluate the bioagent, *B. subtilis* on the management of die-back and fruit rot of chillies and induction of defense enzymes.

Materials and Methods

Isolation of pathogen

The pathogen *Colletotrichum capsici* was isolated from a fruit rot infected chillies and maintained on potato dextrose agar (PDA) slants (Rangaswami, 1972).

In vitro screening of *Bacillus subtilis* against *Colletotrichum capsici*

Using Nutrient Agar medium, *B. subtilis* isolates were isolated from the rhizosphere of various crop plants. The antagonistic potential of biocontrol agents against *C. capsici* was tested by dual culture method on PDA medium (Dennis and Webster, 1971).

A 9 mm actively growing culture disc of pathogen and antagonists were separately placed or streaked opposite to each other on sterilized PDA medium under aseptic conditions. The medium inoculated with pathogen alone served as the control. The radial growth of the pathogen was measured after seven days of incubation. Four replications were maintained.

Field studies

A talc-based powder formulation of *B. subtilis* was developed as described by Vidhyasekaran and Muthamilan (1995). Field eperiment was conducted in the farmers field at Thondamuthur, Coimbatore District, Tamil Nadu on the management of fruit disease of chillies. The seeds of chillies were treated with *B. subtilis* talc formulation at the rate of 10 g per kg of seed before sowing. Soil application of *B. subtilis* talc formulation at the rate of 2.5 kg/ha mixed with neem cake @ 250 kg/ha was done during the time of field preparation. Foliar application with *B. subtilis* talc formulation was done at 10 g per litre on 30 and 60 days after planting.

The combination treatment of seed treatment and and foliar application with bioagent, *B. subtilis* was also made. For comparison, the seeds were treated with fungicide carbendazim at the rate of 2 g per kg of seed.

Foliar application with carbendazim at 1 g per litre was also done on 30 and 60 days after planting. The combination of seed treatment with carbendazim at the rate of 2 g per kg of seed followed by foliar application with carbendazim at 1 g per litre was also done on 30 and 60 days after planting.The fruit root disease intensity was observed in scale 0-9 rating at the time of harvest by random selection of 25 plants per plot.

Per cent disease index (PDI) was calculated using the formula

$$PDI = \frac{\text{Sum of numerical ratings}}{\text{Number of plants observed} \times \text{Maximum grade}} \times 100$$

The yield of dry chillies was also recorded for each treatment and the data were statistically analyzed.

Induction of defense compounds in chillies due to foliar application of *B. subtilis*

Chilli plants grown in pots were sprayed with *B. subtilis* talc formulation at 10 g per litre at 45 days after planting and challenge inoculated with *C. capsici* at 47 days after planting. At various times after application, leaf samples were collected at 1, 3, 5 and 7 days after pathogen inoculation to assay the change in activities of defense related enzymes *viz*., chitinase and β-1,3-glucanase.

Assay of chitinase

The colorimetric assay of chitinase was carried out as per Boller and Mauch (1988). One gram of sample was extracted with 5 ml of 0.1 M sodium citrate buffer (pH 5.0). The homogenate was centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant was used as enzyme source. The reaction mixture consisted of 10 µl of 0.1 M sodium acetate buffer (pH 4.0), 0.4 ml enzyme source and 0.1 ml colloidal chitin (10 mg). After incubation for 2 h at 37ºC, the reaction was stopped by centrifugation at 3,000 rpm for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 µl of 1 M potassium phosphate buffer (pH 7.0) and incubated with 20 µl of 3% (w/v) snail gut enzyme for 1 h. Later, the reaction mixture was brought to pH 8.9 by the addition of 70 µl 0.1 M sodium borate buffer (pH 9.8). The mixture was incubated in a boiling water bath for 3 min and rapidly cooled. After addition of 2 ml of para-dimethyl aminobenzaldehyde (DMAB), the mixture was incubated for 20 min at 37°C and the absorbance was measured at 585 nm. N-acetylglucosamine (GlcNAc) was used as a standard and the enzyme activity was expressed as nmol GlcNAc equivalents min^{-1} g^{-1} of fresh tissue.

Assay of β-1,3-glucanase

One gram of sample was extracted in 5 ml of 0.05 M sodium acetate buffer (pH 5.0). The homogenate was centrifuged at 10,000 g for 10 min. at 4°C and the supernatant was used as enzyme source. β-1,3-glucanase activity was assayed by the laminarin-dinitrosalicylic acid method (Pan *et al.,* 1991). The reaction mixture consisted of 62.5 µl of 4% laminarin and 62.5 µl of enzyme source. The reaction was carried out at 40°C for 10 min and stopped by adding 375 µl of dinitrosalicylic acid and heating for 5 min in boiling water. The absorbance was measured at 500 nm and the enzyme activity was expressed as nmol GlcNAc equivalents min^{-1} g^{-1} of fresh tissue.

Results and Discussion

Among the various strains of *B. subtilis* tested against the pathogen *in vitro*, the strain *Bs*1 was the most effective one in inhibiting the fungal growth of *C. capsici* by recording least mycelial growth of 3.4 cm (Table 1). The strain *Bs2* was found to be the least effective in inhibiting the growth of *C. capsici* (6.1 cm). The maximum mycelial growth of 8.8 cm was observed in the control (Table 1). Meena and Marimuthu (2012) reported that *P. fluorescens* inhibited the growth of *Sclerotium rolfsii in vitro* effectively.

The results of the field experiments conducted on the management of fruit rot disease of chillies showed that seed treatment with *B. subtilis* @ 10 g/kg seed followed by foliar application of *B. subtilis* @ 10 g/l on 30 and 60 DAP was effective in managing the fruit rot disease which recorded the lowest disease intensity of 23.6 PDI. It was found to be on par with seed treatment with carbendazim at 2 g per kg seed followed by foliar application of carbendazim at 1 g per litre on 30 and 60 days after planting which recorded disease intensity of 24.7 PDI. Seed treatment alone with *B. subtilis* @ 10 g/kg seed recorded the disease intensity of 31.8 PDI. The disease intensity of 32.3 PDI was observed in streatment alone with carbendazim at 2 g per kg seed.

Soil application of *B. subtilis* @ 2.5 kg/ha mixed with neem cake @ 250 kg/ha with the disease intensity of 34.3 PDI was not effective in managing the fruit rot disease. The highest root rot disease intensity (46.6 PDI) was observed in the control (Table 2). Meena (2016) reported the effectiveness of *Pseudomonas fluorescens* in the management of root rot disease of *Coleus forskohlii*.

Table.1 *In vitro* inhibition of *Colletotrichum capsici* by *Bacillus subtilis*

B. subtilis strains	Mycelial growth of *C. capsici** (cm)	Inhibition zone (mm)
*Bs*1	3.4	14.6
*Bs*2	6.1	7.2
*Bs*3	4.2	10.4
*Bs*4	3.8	12.3
*Bs*5	5.6	8.1
Control	8.8	-
CD (P=0.05)	2.4	

*Mean of four replications

Table.2 Effect of *Bacillus subtilis* talc based formulation on the management of fruit rot disease of chillies

Treatments	Fruit rot disease intensity PDI	Dry chillies yield (t/ha)
T_1- Soil application of *B. subtilis* @ 2.5 kg/ha mixed with neem cake @ 250 kg/ha	34.3	1.97
T_2- Seed treatment with *B. subtilis* @ 10 g/kg seed	31.8	2.39
T_3- Foliar application of *B. subtilis* @ 10 g/l on 30 and 60 DAP	27.5	2.65
T_4- Seed treatment with *B. subtilis* @ 10 g/kg seed + Foliar application of *B. subtilis* @ 10 g/l on 30 and 60 DAP	23.6	2.83
T_5- Seed treatment with carbendazim @ 2 g/kg seed	32.3	2.14
T_6- Foliar application of carbendazim @ 1 g/l on 30 and 60 DAP	28.4	2.48
T_7- Seed treatment with carbendazim @ 2 g/kg seed + Foliar application of carbendazim @ 1 g/l on 30 and 60 DAP	24.7	2.71
T_8- Control	46.6	1.81
CD (P=0.05)	2.8	0.16

The values are mean of three replications

Table.3 Induction of chitinase activity upon treatment with *B. subtilis* formulation

S. No.	Treatments	Chitinase activity (nmol GlcNAc/min/g)			
		Days after inoculation with *C. capsici*			
		1	3	5	7
1.	*B. subtilis* + *C. capsici*	31.39^{a}	40.38^{a}	51.46^{a}	46.23^{a}
2.	*B. subtilis*	27.58^{b}	31.26^{c}	42.83^{c}	38.27^{c}
3.	*C. capsici*	28.41^{b}	34.87^{b}	46.71^{b}	41.26^{b}
4.	Untreated Control	24.63^{c}	27.25^{d}	31.42^{d}	29.84^{d}

The values are mean of five replications.
In a column, mean followed by a common letter are not significantly different at 5% level by DMRT.

Table.4 Induction of β-1,3-glucanase activity upon treatment with *B. subtilis* formulation

S. No.	Treatments	β-1,3-glucanase activity (µmol glucose released/min/g)			
		Days after inoculation with *C. capsici*			
		1	3	5	7
1.	*B. subtilis* + *C. capsici*	14.83[a]	27.35[a]	38.91[a]	29.72[a]
2.	*B. subtilis*	11.38[b]	17.82[b]	26.74[c]	21.28[c]
3.	*C. capsici*	12.64[b]	18.53[b]	31.46[b]	25.81[b]
4.	Untreated Control	9.12[c]	12.53[c]	15.93[d]	13.81[d]

The values are mean of five replications.
In a column, mean followed by a common letter are not significantly different at 5% level by DMRT.

In addition to disease reduction, the dry chillies yield was also found to be increased in the effective treatment. The maximum dry chillies yield of 2.83 t/ha was recorded in seed treatment with *B. subtilis* @ 10 g/kg seed followed by foliar application of *B. subtilis* @ 10 g/l on 30 and 60 DAP. Seed treatment with carbendazim @ 2 g/kg seed followed by foliar application of carbendazim at 1 g per litre on 30 and 60 days after planting recorded the dry chillies yield of 2.71 t/ha. The dry chillies yield of 2.39 t/ha was observed in seed treatment alone with *B. subtilis* @ 10 g/kg seed. In control, the lowest yield of 1.81 t/ha was observed (Table 2). Meena and Rajamani (2015) observed the plant growth promotion and enhancement of yield parameters in glory lily by dipping the tubers followed by foliar application of *P. fluorescens* talc formulation.

Synthesis and accumulation of PR proteins have been reported to play an important role in plant defense mechanisms. Chitinases (PR-3 protein) and β-1,3-glucanases (PR-2 protein) have been reported to associate with resistance in plants against pests and diseases (Van Loon, 1997). In general, fungal cells contain chitin and glucan as their cell wall constituents. The main mode of antagonistic activity of microbes is production of lytic enzymes which act on cell walls or organisms which have chitin and glucan as their cell wall component (Singh *et al.,* 1999) and also through induced systemic resistance (ISR) in plant system. In the present study, the elevated levels of chitinase and β-1,3-glucanase in plants treated with bioformulation containing endophytic bacteria against pathogen was observed. Chitinases are PR-proteins which hydrolyze chitin, a major cell wall component (3-10%) of higher fungi. Chitinases cleave a bond between C1 and C4 of two consecutive N-acetyl glucosamine (GlcNAc) either by endolytic or exolytic mechanisms. The treatment with foliar application of *B. subtilis* formulation and challenge inoculated with pathogen led to the enhanced activity of chitinase compared to other treatments (Table 3). The maximum chitinase activity of 51.46 nmol GlcNAc min^{-1} g^{-1} was observed in foliar application of *B. subtilis* talc formulation and challenge inoculated with pathogen. The chitinase activity was found to be increased up to five days after inoculation of the pathogen and thereafter declined. All the treatments were significantly different from control (Table 3). Meena *et al.,* (2000) reported that *P. fluorescens* induced various defense compounds in groundnut.

Significant increase in β-1,3-glucanase activity (38.91 µmol glucose released min^{-1} g^{-1} was observed in the treatment of foliar application of *B. subtilis* talc formulation and challenge inoculated with pathogen. *B. subtilis* treatment challenge inoculated with pathogen recorded higher levels of β-1,3-glucanase activity up to five days after inoculation of the pathogen and declined thereafter (Table 4). Elicitation of ISR in cotton by *B. subtilis* EPCO 102 with chitin led to the lowest bacterial blight incidence due

to the induction of chitinase, β-1,3-glucanase, peroxidase and polyphenol oxidase (Rajendran *et al.,* 2006). Ramyabharathi *et al.,* 2012 highlighted the induction of defense enzymes *viz*., chitinase and β-1,3-glucanase in tomato plants infected with *Fusarium oxysporum* f. sp. *lycopersici* by the biocontrol agent *B. subtilis* EPCO16.

Thus, in conclusion, the current study reveals the potential of talc formulation of *B. subtilis* in inducing the plant's own defense mechanism to suppress the fruit rot disease of chillies.

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