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Induction of plant defense mechanism by the effective isolate of *Bacillus subtilis* in coriander

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Abstract
Coriander, is an important spice crop affected by wilt disease caused by *Fusarium oxysporum.* A glass house trial was conducted to study the Induces Systemic Resistance (ISR) in coriander plants by *Bacillus subtilis.* The study revealed that the plants applied with *B. subtilis* as seed treatment @10g/kg of seed along with soil application as basal and topdressing on 30 DAS recorded maximum activity of PO (2.175 in absorbance min / g of fresh tissue), PPO (2.830 in absorbance min/g of fresh tissue), PAL (2.476 changes in absorbance min/g of fresh tissue) and phenol content (6.396 mg/g of fresh tissue) compared to control. This treatment also recorded minimum wilt incidence of 16.33 % against 35.56 % wilt incidence in control.

Keywords: Coriander, glass house and phenol

Introduction
Coriander (*Coriandrum sativum* L.) is one of the important seed spices belonging to the family Apiaceae, mainly cultivated in Rajasthan, Gujarat, Haryana, Punjab, Madhya Pradesh, Uttar Pradesh, Andhra Pradesh, Karnataka and Tamil Nadu. Pests and diseases are the major constraints in the production of coriander. Coriander cultivation is affected by several diseases like wilt caused by *Fusarium oxysporum* (Srivastava, 1972) [15], stem gall caused by *Protomyces macrospores* (Das, 1971) [2], grain mould diseases caused by *Helminthosporium* spp., *Fusarium* spp., *Curvularia* spp. and *Alternaria* spp. (Rajan *et al.*, 1990) [12], powdery mildew (*Erisyphe polygoni*) and leaf spots.
It has been found that members of genus *Bacillus* has successfully controlled plant diseases in a wide variety of crops including rice (Peng *et al*., 2014) [11], wheat (Liu *et al*., 2009) [9], potato (Balabel *et al*., 2013) [1], brinjal (Saha *et al*., 2012) [14] and cucumber (Huang *et al*., 2012) [7]. The involvement of ISR in disease suppression has been studied for a wide range of biological control microorganisms. Seed treatment and seedling root dipping induced early and enhanced levels of PO in rice plants (Nayar, 1996) [10].
The present study was conducted to reveal the ISR mechanism induced in coriander plants by *B. subtilis* under glass house condition.

Materials and Methods
Selection of effective strain of *Bacillus subtilis* for wilt pathogen
The wilt pathogen *F. oxyspoum* was isolated from infected plants and the different strains of *Bacillus subtilis* (isolated from rhizosphere region of coriander plant and strains obtained from the Dept. of Plant Pathology, TNAU) were tested for their inhibitory activity on mycelial growth of the pathogen by dual plate technique (Dennis and Webster, 1971) [3]. The effective strain of *B. subtilis* (VB1) was selected based on their inhibitory activity on the mycelial growth of the pathogen (table not shown).

Preparation of talc based formulation of the *B. subtilis*
A loop ful of effective *B. subtilis* (VB1) was inoculated into the sterilized NA broth and incubated in a rotary shaker at 150 rpm for 72 h at room temperature (28 ± 2ºC). After 72 h, 400 ml of bacterial broth suspension containing 9×10^8 cfu/ml, 1 kg of the carrier material (talc powder), 15 g calcium carbonate (to adjust the pH to neutral) and 5 g CMC (adhesive) were mixed under sterile conditions. The mixture was shade dried and packed in polythene bags and kept at room temperature condition (Vidhyasekaran and Muthamilan, 1995) [17].

Induced systemic resistance (ISR) in coriander plants under glass house condition

A glass house trial was conducted at TNAU Orchard to test the ISR mechanism in coriander plants by *Bacillus subtilis* (VB1) strain. The virulent isolate of wilt pathogen was mass multiplied in the sand maize medium and mixed with the sterilized potting soil at the ratio of 5% (w/w). Surface sterilized coriander seeds were treated with bacterial antagonistic formulations *(B. subtilis* @ 10 g / kg of seeds, *Pseudomonas fluorescens* @ 10 g / kg of seeds) and sown in pathogen inoculated 30 cm diameter pots. Ten seeds (CO 4) were sown per pot and three replications per treatment were maintained. Observations were recorded on germination percentage, plant height, disease incidence and seed yield.

The treatments are as follows

T1: ST with *Bacillus subtilis* VB1 @ 10g/kg of seeds
T2: SA with *Bacillus subtilis* VB1 (Basal) @ 2.5kg/ha
T3: ST (10g/kg of seeds) + SA with *Bacillus subtilis* VB1 (Basal) @ 2.5kg/ha
T4: ST (10g/kg of seeds) + SA with *Bacillus subtilis* VB1 (Basal & top dressing) @ 2.5kg/ha
T5: ST with *Pseudomonas fluorescens* Pf1 @ 10g/kg of seeds
T6: SA with *Pseudomonas fluorescens* Pf1 (Basal) @ 2.5kg/ha
T7: ST (10g/kg of seeds) +SA with *Pseudomonas fluorescens* Pf1 (Basal) @2.5kg/ha
T8: ST (10g/kg of seeds) + SA with *Pseudomonas fluorescens* Pf1 (Basal & top dressing) @ 2.5kg/ha
T9: ST with Carbendazim @ 2g/kg of seeds
T10: ST (2g/kg of seeds) + SD with Carbendazim @ 0.1%
T11: Untreated pathogen inoculated control

The experiment was conducted in completely randomized block design replicated thrice. The observation was taken wilt incidence. The per cent disease incidence was assessed using the following formula.

$$\text{Percent Disease Incidence} = \frac{\text{Number of infected plants}}{\text{Total number of plants}} \text{x100}$$

Enzyme extraction

Leaf samples from coriander plants were collected from 45 Days After Sowing (DAS) at 3 days interval. One gram of leaf sample was homogenized with 2 ml of 0.1M sodium citrate buffer (pH 5.0) at 4ºC. The homogenate was centrifuged for 20 min at 10,000 rpm. The supernatant was used for enzyme activity. Enzyme extracted in 0.1 M sodium phosphate buffer (pH 7.0) was used as the extraction buffer for the assay of Peroxidase (PO) and Polyphenol oxidase (PPO). Enzyme extract was stored in deep freezer (-70ºC) until used for biochemical analysis.

Assay of peroxidase (PO)

The reaction mixture consisted of 2.5 ml of a mixture containing 0.25% (v/v) guaiacol in 0.01 M sodium phosphate buffer, pH 6.0 and 0.1 M hydrogen peroxide. Enzyme extract (0.1 ml) was added to initiate the reaction, which was followed observation calorimetrically at 470 nm. Crude enzyme preparations were diluted to give changes in absorbance at 470 nm of 0.1 to 0.2 absorbance units/min. the boiled enzyme was used as blank. Activity was expressed as the increase in absorbance at 420 nm/min/g of fresh tissue.

Assay of polyphenoloxidase (PPO)

One gram of leaf sample was homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) at 4ºC. The homogenate was centrifuged at 20,000 rpm for 15 min at 4ºC. The supernatant served as enzyme source and polyphenoloxidase activity was determined as per the procedure and the enzyme activity was expressed as change in absorbance at 490 nm/min/g of fresh tissue.

Assay of phenylalanine ammonia lyase (PAL) activity

PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm. Sample containing 0.4 ml of enzyme extract was incubated with 0.5 ml of 0.1M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30 °C. Enzyme activity was expressed in fresh weight basis as nmol trans-cinnamic acid $min^{-1}mg^{-1}$ of sample (Dickerson *et al*., 1984) [5].

Estimation of total phenols

One gram of leaf tissue were homogenized in 10 ml of 80% methanol and agitated for 15 min at 70ºC. To 5 ml of distilled-ciocalteauwater an reagent (1N), 1 ml of the methanolic extract was added and incubated at 25ºC for 3 min and after that 1 ml of saturated solution of 20% sodium carbonate was added and mixed well. Then the tubes were placed in a boiling water bath for 1 min and cooled. The absorption of the blue colour developed was measured at 725 nm and catechol was used as the standard.

Results and Discussion

The induced systemic resistance through biochemical analysis revealed that the increased level of activities of the defense enzymes *viz.,* peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and phenols were noticed in treated plants.

Generally the activity of peroxidase increased significantly upto seven days in all the treatments and thereafter declined. The maximum PO (2.175 changes in absorbance min/g of fresh tissue), PPO (2.830 changes in absorbance min/g of fresh tissue) and PAL (2.756 changes in absorbance min/g of fresh tissue) were observed in plants applied with *B.subtilis* as seed treatment @ 10g/kg and soil application as basal and top dressing (2.5 kg/ha)compared to control *viz.,* (1.775 changes in absorbance min/g of fresh tissue, 1.540 changes in absorbance min/g of fresh tissue and 0.895 changes in absorbance min/g of fresh tissue respectively (Table 1-3).

Table 1: Induction of Peroxidase (PO) activity in coriander plants applied with biocontrol agents under glass house conditions

S. No.	Treatments	Change in absorbance at 420 nm/min/g of sample				
		0 DAS	3 DAS	5 DAS	7 DAS	9 DAS
1	ST with *Bacillus subtilis* VB1 @ 10g/kg of seeds	0.687^{d}	0.891^{bc}	1.136^{f}	1.521^{e}	1.310^{d}
2	SA with *Bacillus subtilis* VB1 (Basal) @ 2.5kg/ha	0.783^{c}	0.972^{b}	1.239^{e}	1.257^{g}	1.10^{e}
3	ST (10g/kg of seeds) + SA with *Bacillus subtilis* VB1 (Basal) @ 2.5kg/ha	0.987^{a}	1.133^{a}	1.843^{b}	1.930^{b}	1.725^{b}
4	ST (10g/kg of seeds) + SA with *Bacillus subtilis* VB1(Basal & top dressing) @ 2.5kg/ha	0.892^{b}	1.153^{a}	1.991^{a}	2.175^{a}	1.967^{a}
5	ST with *Pseudomonas fluorescens* Pf1 @ 10g/kg of seeds	0.582^{e}	0.765^{d}	0.996^{g}	1.296^{g}	0.918^{f}
6	SA with *Pseudomonas fluorescens* Pf1 (Basal) @ 2.5kg/ha	0.334^{f}	0.658^{e}	1.099^{f}	1.375^{f}	1.017^{e}
7	ST (10g/kg of seeds) +SA with *Pseudomonas fluorescens* Pf1 (Basal) @2.5kg/ha	0.967^{a}	1.142^{a}	1.675^{c}	1.943^{b}	1.586^{c}

8	ST (10g/kg of seeds) + SA with *Pseudomonas fluorescens* Pf1 (Basal & top dressing) @ 2.5kg/ha	0.980[a]	1.101[a]	1.675[c]	2.031[a]	1.930[a]
9	ST with Carbendazim @ 2g/kg of seeds	0.279[f]	0.751[d]	1.491[d]	1.875[c]	1.567[c]
10	ST (2g/kg of seeds) + SD with Carbendazim @ 0.1%	0.582[e]	0.965[b]	1.296[e]	1.596[e]	1.118[e]
11	Untreated pathogen inoculated control	0.134[g]	0.828[c]	1.399[e]	1.775[d]	1.217[d]

ST – Seed treatment SA – Soil application SD – Soil drenching
Values are mean of three replications
Means followed by a common letter are not significantly different at 5 % level by DMRT.

Table 2: Induction of Poly peroxidase (PPO) activity in coriander plants applied with biocontrol agents under glass house conditions

S. No.	Treatments	Change in absorbance at 420 nm/min/g of sample				
		0 DAS	3 DAS	5 DAS	7 DAS	9 DAS
1	ST with *Bacillus subtilis* VB1 @ 10g/kg of seeds	0.884	1.461[d]	1.960[c]	2.256[e]	1.944[e]
2	SA with *Bacillus subtilis* VB1 (Basal) @ 2.5kg/ha	0.804[e]	1.361[e]	1.776[d]	2.017[f]	1.762[f]
3	ST (10g/kg of seeds) + SA with *Bacillus subtilis* VB1 (Basal) @ 2.5kg/ha	0.952[d]	1.791[b]	2.323[b]	2.503[c]	2.445[b]
4	ST (10g/kg of seeds) + SA with *Bacillus subtilis* VB1(Basal & top dressing) @ 2.5kg/ha	1.246[a]	1.893[a]	2.456[a]	2.830[a]	2.509[a]
5	ST with *Pseudomonas fluorescens* Pf1 @ 10g/kg of seeds	0.834[e]	1.321[e]	1.843[d]	2.031[f]	2.376[c]
6	SA with *Pseudomonas fluorescens* Pf1 (Basal) @ 2.5kg/ha	0.932[d]	1.454[d]	1.932[c]	2.385[d]	2.068[d]
7	ST (10g/kg of seeds) +SA with *Pseudomonas fluorescens* Pf1 (Basal) @2.5kg/ha	1.043[c]	1.543[c]	1.864[d]	2.458[d]	2.142[d]
8	ST (10g/kg of seeds) + SA with *Pseudomonas fluorescens* Pf1 (Basal & top dressing) @ 2.5kg/ha	1.154[b]	1.743[b]	2.244[b]	2.643[b]	2.245[d]
9	ST with Carbendazim @ 2g/kg of seeds	0.787[f]	1.100[f]	1.572[e]	2.044[f]	1.702[f]
10	ST (2g/kg of seeds) + SD with Carbendazim @ 0.1%	0.792[ef]	1.021[g]	1.402[f]	1.672[g]	1.503[g]
11	Untreated pathogen inoculated control	0.691[g]	0.931[h]	1.281[g]	1.540[h]	1.401[h]

ST – Seed treatment SA – Soil application SD – Soil drenching
Values are mean of three replications
Means followed by a common letter are not significantly different at 5 % level by DMRT.

Table 3: Induction of Phenyl alanine ammonia lyase (PAL) activity in coriander plants applied with biocontrol agents under glass house conditions

S. No.	Treatments	Change in absorbance at 420 nm/min/g of sample				
		0 DAS	3 DAS	5 DAS	7 DAS	9 DAS
1	ST with *Bacillus subtilis* VB1 @ 10g/kg of seeds	0.742[d]	0.847[f]	0.993[fg]	1.356[g]	1.023[e]
2	SA with *Bacillus subtilis* VB1 (Basal) @ 2.5kg/ha	0.695[e]	0.783[fg]	0.902[g]	0.957[h]	0.873[f]
3	ST (10g/kg of seeds) + SA with *Bacillus subtilis* VB1 (Basal) @ 2.5kg/ha	0.796[d]	1.320[c]	2.033[a]	2.205[c]	2.092[b]
4	ST (10g/kg of seeds) + SA with *Bacillus subtilis* VB1(Basal & top dressing) @ 2.5kg/ha	1.036[a]	1.654[a]	1.929[ab]	2.75a	2.534[a]
5	ST with *Pseudomonas fluorescens* Pf1 @ 10g/kg of seeds	0.723[de]	0.884[f]	1.032[f]	1.442[f]	1.032[e]
6	SA with *Pseudomonas fluorescens* Pf1 (Basal) @ 2.5kg/ha	0.748[d]	1.088[e]	1.487[e]	1.754[e]	1.533[d]
7	ST (10g/kg of seeds) +SA with *Pseudomonas fluorescens* Pf1 (Basal) @2.5kg/ha	0.801[c]	1.139[d]	1.638[d]	2.023[d]	1.836[c]
8	ST (10g/kg of seeds) + SA with *Pseudomonas fluorescens* Pf1 (Basal & top dressing) @ 2.5kg/ha	0.988[b]	1.454[b]	1.856[c]	2.476[b]	2.043[b]
9	ST with Carbendazim @ 2g/kg of seeds	0.689[e]	0.774[g]	0.867[h]	0.946[h]	0.845[f]
10	ST (2g/kg of seeds) + SD with Carbendazim @ 0.1%	0.698[e]	0.786[fg]	0.799[i]	0.841[i]	0.765[g]
11	Untreated pathogen inoculated control	0.667[f]	0.789[fg]	0.791[i]	0.895[h]	0.691[h]

ST – Seed treatment SA – Soil application SD – Soil drenching
Values are mean of three replications
Means followed by a common letter are not significantly different at 5 % level by DMRT.

The total phenol content was 6.396 mg/g of fresh tissue in plants applied with *B.subtilis* as seed treatment @ 10g/kg and soil application as basal and top dressing (2.5 kg/ha) compared to control (5.26 mg/g of fresh tissue) (Table 4).

Table 4: Induction of Phenol content in coriander plants applied with biocontrol agents under glass house conditions

S. No.	Treatments	Phenol content (mg/g of sample)				
		0 DAS	3 DAS	5 DAS	7 DAS	9 DAS
1	ST with *Bacillus subtilis* VB1 @ 10g/kg of seeds	4.274[f]	4.876[d]	5.531[c]	5.839[d]	5.231[f]
2	SA with *Bacillus subtilis* VB1 (Basal) @ 2.5kg/ha	4.514[c]	4.893[d]	5.439[d]	5.717[e]	5.482[d]
3	ST (10g/kg of seeds) + SA with *Bacillus subtilis* VB1 (Basal) @ 2.5kg/ha	4.393[e]	4.984[c]	5.432[d]	5.849[d]	5.029[g]
4	ST (10g/kg of seeds) + SA with *Bacillus subtilis* VB1(Basal & top dressing) @ 2.5kg/ha	4.712[a]	5.289[b]	5.832[a]	6.396[a]	5.99[a]
5	ST with *Pseudomonas fluorescens* Pf1 @ 10g/kg of seeds	4.309[e]	4.790[e]	5.056[f]	5.467[f]	5.065[g]
6	SA with *Pseudomonas fluorescens* Pf1 (Basal) @ 2.5kg/ha	4.401[d]	4.788[e]	5.478[d]	5.976[c]	5.687[b]
7	ST (10g/kg of seeds) +SA with *Pseudomonas fluorescens* Pf1 (Basal) @2.5kg/ha	4.603[b]	4.988[c]	5.487[d]	5.978[c]	5.530[c]
8	ST (10g/kg of seeds) + SA with *Pseudomonas fluorescens* Pf1 (Basal & top dressing) @ 2.5kg/ha	4.754[a]	5.580[a]	5.854[a]	6.238[b]	5.965[a]
9	ST with Carbendazim @ 2g/kg of seeds	4.384[e]	4.734[e]	5.642[b]	5.893[d]	5.391[e]
10	ST (2g/kg of seeds) + SD with Carbendazim @ 0.1%	4.390[e]	4.832[d]	5.358[e]	5.958[c]	4.732[i]
11	Untreated pathogen inoculated control	4.274[f]	4.637[f]	5.028[f]	5.268[g]	4.890[h]

ST – Seed treatment SA – Soil application SD – Soil drenching
Values are mean of three replications
Means followed by a common letter are not significantly different at 5 % level by DMRT.

The wilt incidence was 16.33 % in plants applied with *B.subtilis* (VB1) as seed treatmemt @ 10g/kg + soil application as basal and top dressing @ 2.5 kg/ha and followed by *P.fluorescens* (19.32%) as seed treatmemt @ 10g/kg + soil application as basal and top dressing @ 2.5 kg/ha, while in control maximum incidence of 35.56 % was recorded (Table 5). The production of defense related

enzymes may be the reason for the minimum disease incidence in treated plants.

Several species of *Pseudomonas* and *Bacillus* have the ability to elicit ISR, but do so differentially in different plant species. The role of plant peroxidases have been extensively studied and many of their functions have been described such as scavenging of peroxidase, participation in lignifications, hormonal signaling and plant defense (Hiraga *et al*., 2001) [6]. The increased activity of defence related enzyme and phenol content were induced in treated plants compared to control. This is in confirmation with earlier workers.

Table 5: Effect of *B. subtilis* strain VB1 (talc formulation) on the incidence of wilt under glass house conditions

S. No.	Treatments	Germination (%)	Plant height (cm) 60 DAS	Wilt incidence (%) 60 DAS	Yield (g/pot)
1	ST with *Bacillus subtilis* VB1 @ 10g/kg of seeds	86.72^{b}	48.55^{c}	22.32^{bcd}	71.38^{b}
2	SA with *Bacillus subtilis* VB1 (Basal) @ 2.5kg/ha	74.00^{j}	49.66^{b}	24.43^{bc}	74.43^{b}
3	ST (10g/kg of seeds) + SA with *Bacillus subtilis* VB1 (Basal) @ 2.5kg/ha	89.97^{a}	52.18^{a}	20.39^{cd}	76.56^{ab}
4	ST (10g/kg of seeds) + SA with *Bacillus subtilis* VB1(Basal & top dressing) @ 2.5kg/ha	85.13^{c}	53.26^{a}	16.33^{e}	88.35^{a}
5	ST with *Pseudomonas fluorescens* Pf1 @ 10g/kg of seeds	79.00^{g}	47.33^{d}	23.87^{bc}	72.97^{b}
6	SA with *Pseudomonas fluorescens* Pf1 (Basal) @ 2.5kg/ha	83.65^{e}	49.74^{b}	26.46^{b}	73.67^{b}
7	ST (10g/kg of seeds) +SA with *Pseudomonas fluorescens* Pf1 (Basal) @2.5kg/ha	82.86^{ef}	53.55^{a}	20.57^{cd}	78.86^{ab}
8	ST (10g/kg of seeds) + SA with *Pseudomonas fluorescens* Pf1 (Basal & top dressing) @ 2.5kg/ha	86.12^{b}	52.80^{a}	19.32^{de}	85.67^{ab}
9	ST with Carbendazim @ 2g/kg of seeds	78.67^{h}	46.54^{e}	22.55^{bcd}	75.16^{b}
10	ST (2g/kg of seeds) + SD with Carbendazim @ 0.1%	84.00^{d}	49.64^{b}	20.34^{cd}	78.54^{ab}
11	Untreated pathogen inoculated control	76.00^{i}	47.55^{d}	35.56^{a}	50.45^{c}

ST – Seed treatment SA – Soil application SD – Soil drenching

Values are mean of three replications

Means followed by a common letter are not significantly different at 5 % level by DMRT.

High level expression of PO was reported in *P. fluorescens* (Pf1) treated tomato plants challenged with *F. oxysporum* f. sp. *lycopersici* (Ramamoorthy *et al*., 2002) [13]. Liang *et al*. (2011) [8] reported that *Bacillus megaterium* L8 strain increases the levels of SOD, POD, CAT, PPO and PAL activities in the treated roots and untreated leaves of cucumber seedlings were all significantly higher as compared with the control against cucumber damping off caused by *Pythium aphanidermatum*.

Devi *et al*. (2013) [4] studied the induction of systemic resistance to elucidate the role of *Pseudomonas fluorescens* and *Bacillus subtilis* in disease management of *Alternaria helianthi*. Sujatha and Ammani (2011) [16] studied the assay of pathogenesis related proteins including Phenylalanine ammonia lyase, Polyphenol oxidase and Peroxidase in the management of *Fusarium oxysporum and Rhizoctonia bataticola* in *Vigna mungo*.

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