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Biologically mediated systemic resistance against Marssonina leaf blotch of apple caused by *Marssonina coronaria* (Ellis & J.J. Davis) J.J. Davis

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Abstract
In order to determine the effect of biocontrol agents used for inducing resistance in plants against Marssonina leaf blotch caused by the fungus *Marssonina coronaria* (Ellis & J.J. Davis) J.J. Davis two polyhouse trials were conducted using scion bud wood of cultivars Starking Delicious, Tydeman's Early Worcester and Granny Smith apple. Three biocontrol agents, *Trichoderma viride, Pseudomonas fluorescens* and *Bacillus subtilis,* at three different dilution i.e. 10^{-3}, 10^{-4}, 10^{-5} for fungi and 10^{-3}, 10^{-5} and 10^{-7} for bacteria were applied on the test cultivars 48 hrs before inoculation. Distilled sterilized water was sprayed on control plants. All the biocontrol agents significantly lowered the disease intensity as compared to control. *Pseudomonas fluorescens* and *Bacillus subtilis* and *Trichoderma viride,* reduced disease severity by 15.50 – 33.50 per cent on the leaves Starking Delicious cultivar. Similarly, disease severity data in cultivar Tydeman's Early Worcester ranged from 5.33-16.66 per cent and on the leaves of cultivar Granny Smith it ranged from1.68-6.00 per cent against 28 and 9 per cent in Tydeman's Early Worcester and Granny Smith respectively. The activity of the defense enzymes peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) was found maximum in *Pseudomonas fluorescens* treated leaves in all the test cultivars. Thus biocontrol agents have potential for the control of Marssonina leaf blotch of apple in the field.

Keywords: Disease severity, *Trichoderma viride, Pseudomonas fluorescens, Bacillus subtilis,* peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL)

Introduction
Marssonina leaf blotch caused by the fungus *Marssonina coronaria* (Ellis & J.J. Davis) J.J. Davis causing premature leaf fall is relatively new threat to successful cultivation of apple in India. In India, this disease was first recorded in the year 1992 in Himachal Pradesh (Sharma *et al.,* 2004) and appeared in epiphytotic in the state in 1996 (Sharma, 2001) [22]. After it appearance, Marssonina Blotch appeared in epiphytotic form in Himachal Pradesh in 1996 and threatened the apple cultivation by causing premature leaf fall (Sharma and Kaul, 2000; Sharma, 2001) [21, 22] and it was so severe in some orchards that no foliage was left on the trees by the harvest time (Sharma and Gautam, 1997) [19]. It affects fruit size, colour, quality and fruit set adversely and reducing the tree vigour and fruit bearing capacity of the trees at the same time. Appearance of fruit spots on the produce makes it unsalelable in the market leading to direct economic loss to the growers. The disease can be kept at low levels by following protective fungicidal spray at short intervals during the growing season. This practice is in vogue in India also, particularly in Himachal Pradesh, where protective fungicidal spray programme is adopted every year to keep the disease under check (Sharma and Gautam, 1997; Sharma, 1999) [19, 20, 21]. But this approach may often lead to fungicide wastage particularly when weather conditions are not congenial for disease development. On the other hand, however, favourable weather conditions might result in high build up of the disease pressure making it compulsory to carry on fungicidal spray at short interval for effective disease control which will otherwise result in increased cost of production apart from added environmental pollution. Induced resistance to plant diseases has been a method used as an alternative to the fungicides against plant pathogens in the recent years. To induce systemic and local resistance against diseases, biotic preparations such as bacterial and fungal cell wall fragment, weakened or dead spore cultures, non-pathogenic strains and abiotic inducers such as UV, heavy metals, herbicides, ethylene and other chemicals are used. Biological control is a potential non-chemical means for plant disease management by reducing the harmful effects of a parasite or pathogen through the use of other living entities. The utilization of a plant's own defense mechanism is a fascinating arena of research which can be systemically activated upon exposure of plants to PGPR strains or infection by the plant pathogen.

Selected PGPR, mainly fluorescent *Pseudomonas* spp, have been demonstrated to control plant diseases effectively by suppressing pathogens and deleterious microorganisms through siderophore-mediated competition for iron, or antibiosis (Schippers, 1992; Thomashow and Weller, 1995). Recently, research on mechanisms of biological control by PGPR revealed that some PGPR strains protect plants against pathogen infection through induction of systemic resistance, without provoking any symptoms themselves. Keeping in view the economic losses caused by the disease, present study was carried out to evaluate the effect of biocontrol agents as induced resistance compound against Marssonina leaf blotch pathogen.

Materials and Methods

Experimental material which included scion bud wood of apple cultivars Starking Delicious, Tydeman's Early Worcester and Granny Smith were collected form from Department of Fruit Science, UHF, Nauni and Regional Horticultural Research Station, Mashobra. Bud sticks of each cultivar thus procured were grafted on apple seedling rootstock and planted in earthen pots containing a substrate, composed of one part good orchard soil, one part sand and one part farm yard manure and they were maintained in the polyhouse for further use in the experiments. These plants were watered regularly and once a month supplied with hand full of farm yard manure. As the plant had put on 5-7 leaves, a tag was tied on the petiole of the smallest unfolding leaf on each shoot for later reference. Usually the tagged leaf and the immediate 5-6 leaves below were sprayed, inoculated and employed for induced resistance studies.

Induced resistance studies by application of biocontrol agents

Pseudomonas fluorescens and *Bacillus subtilis* were grown at 24°C for 48 h on nutrient broth with shaking. Cultures were centrifuged and then pellets were resuspended in sterile distilled water and centrifuged again. The resulting pellets were dispersed in sterile distilled water, and the concentration of the bacteria was adjusted to 10^3, 10^5 and 10^7 colony forming units (cfu) ml^{-1} using a haemocytometer. *Trichoderma viride* was multiplied on potato dextrose agar in petridishes for 15 days by incubating the dishes at 25°C. The petridishes were flooded with sterilized distilled water and the surface of the fungal colonies was scratched using an inoculation needle. The water containing the fungal spores was decanted and filtered through muslin cloth and the filtrate was retained. The strength of the bio-agent spores in the filtrate was adjusted to 10^3, 10^4 and 10^5 spores /ml using a haemocytometer.

Suspension of three biocontrol agents viz., *Pseudomonas fluorescens, Bacillus subtilis* and *Trichoderma viride*, at three different concentrations (10^3, 10^5 and 10^7 cfu/ml for bacteria and 10^3, 10^4 and 10^5 spores / ml for fungus) was sprayed on to the test plants and sterilized distilled water was sprayed on control plants 48 hours before inoculation with the pathogen *Marssonina coronaria.* Disease severity and percent disease control was recorded 10 days after inoculation according to the rating system from 0-5 with slight modification as given by Filajdic and Sutton, 1991 (Table 1). The experiment was laid in Factorial CRD with three replications. The experiment was repeated in the 2nd year separately for each treatment as in earlier experiments.

Table 1: Scale (0-5) for disease severity

Numerical ratings	Description
0	No symptom,
1	0-3% leaf area covered with lesions, no yellowing and no defoliation,
2	4-6% leaf area covered with lesions, yellowing and/or defoliation,
3	7-12% leaf area covered with lesions, no yellowing and no defoliation,
4	13-25% leaf area covered with lesions, yellowing and/or defoliation
5	26-50% leaf area covered with lesions, yellowing and/or defoliation

Per cent disease intensity was calculated by using the following formula as suggested by McKinney (1923)

$$\text{Disease index (\%)} = \frac{\text{Sum of all the disease ratings}}{\text{Total number of ratings} \times \text{Maximum disease grade}} \times 100$$

Per cent disease control was also calculated by the formula as I=C-T/C×100 Where, I = Per cent disease control, C = Per cent incidence/severity in untreated control, T = Per cent incidence /severity in the treatment.

Extractions and estimation of enzymes for polyphenol oxidase and peroxidase activity

0.5 g sample was homogenized in 5 ml of 0.1 M potassium phosphate buffer (pH 7.5) containing 2% (w/v) polyvinylpyrrolidone (PVP) and 0.25% (v/v) Triton X. The homogenate was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatants were used as crude enzyme extracts to assay the enzymatic activities.

The Polyphenol oxidase activity was determined spectrophotometrically. The assay mixture contained 1.95 ml of 0.1 M potassium phosphate buffer (pH 7.5), 1 ml of catechol (0.025 M) and 50 µl diluted enzyme extract. The enzyme activity was expressed as change in absorbance at 420 nm was recorded at 30 s intervals for 3 min. The enzymatic activity was expressed as the change in the absorbance of the reaction mixture min^{-1} g^{-1} on a fresh weight basis

Peroxidase activity was assayed spectrophotometrically. The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol, 0.5 ml of enzyme extract, and 0.5 ml of 1 per cent hydrogen peroxide. The reaction mixture was incubated at room temperature (28±1°C) for 30 minutes. Change in absorbance at 420 nm was recorded at 30 s intervals for 3 min. The enzymatic activity was expressed as the change in the absorbance of the reaction mixture min^{-1} g^{-1} on a fresh weight basis (Hammerschmidt *et al*., 1982).

Extraction and estimation of phenylalanine ammonia lyase (PAL) activity

One gram of the leaf sample was homogenized in 3 ml of ice-cold 0.1M sodium borate buffer, (pH 7.0) containing 1.4 mM of 2-mercaptoethanol and 0.1g of insoluble

polyvinylpyrrolidone. The extract was filtered through cheese cloth and the filtrate was centrifuged at 15000 g for 15 minutes. The supernatant was used as enzyme source. Phenylalanine ammonia lyase activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid (Dickerson *et al*., 1984). Sample containing 0.4 ml of enzyme extract was incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 minutes at 30ºC. The amount of trans-cinnamic acid formed from L- phenylalanine was measured spectrophotometrically at 290 nm. Enzyme activity was expressed as µg of trans-cinnamic acid (in µ mol quantities) min^{-1} g^{-1} freshweight.

Results and Discussion

All the biocontrol agents at three different concentrations before pathogen inoculation significantly lowered disease intensity as compared to control (Table 2). None of the treated or control plants died as a result of Marssonina leaf blotch attack during the course of the two year study. Likewise none of the biocontrol agents evaluated was pathogenic to the test seedlings. Disease severity (%) of *Marssonina* leaf blotch on leaves was reduced by all biocontrol agents tested. Pooled data show that disease severity ranged from 15.50 – 33.50 per cent in bio control treatments in comparison to 48.00 per cent in control in highly susceptible cultivar Starking Delicious. Similar trend was recorded in disease severity data in cultivar Tydeman's Early Worcester ranging from 5.33-16.66 per cent in comparison to 28.00 per cent in control and in cultivar Granny Smith it ranged from 1.68-6.00 per cent in comparison to 9.00 per cent in the control. Pooled data were utilized to calculate per cent disease control. In cultivar Starking Delicious maximum disease control of 67.70% by *Pseudomonas fluorescens* at 10^{-5} dilution and minimum of 30.00 % by *Bacillus subtilis,* at 10 $^{-3}$ dilution Similarly in cultivar Tydeman's Early Worcester and Granny smith maximum disease control was found in *Pseudomonas fluorescens* at 10^{-5} dilution with 73.82 % and 85.2% and minimum in *Bacillus subtilis,* at 10 $^{-3}$ dilution (40 % and 42.77%). The study also showed that disease development is highest in cultivars Starking Delicious, moderate in Tydemen's Early Worcester and least in cultivar Granny Smith according to the field susceptibility. The present findings is in agreement with Alstrom (1991) [2] who demonstrated that *P. fluorescens* mediated ISR in bean against halo blight caused by *P.* syringae pv phaseolicola, Van Peer *et al.* (1991) [24] in carnation against fusarium wilt, and Wei *et al*. (1991) in cucumber against *Colletotrichum orbiculare* infection. More recently, PGPR-mediated induction of ISR has been reported for several other plant-pathogen system (Maurhofer *et al.,* 1994). Since the early 1990s, induction of systemic resistance by plant growth-promoting rhizobacteria (PGPR) has been investigated as a possible practical way to use induced resistance in agriculture. PGPR have been tested in the greenhouse and field for induced systemic resistance (ISR) to fungal (Chen *et al.,* 1995; Liu *et al.,* 1995 [10]; Pieterse *et al.,* 1996 [14]; Van Peer *et al.,* 1991 [24]; Wei *etal.,* 1991, 1996) [2, 26], bacterial (Alstrom, 1991 [2]; Liu *et al.,*1995 [10]; Park and Kloepper, 2000) [13], and viral (Maurhofer *et al.,* 1994; Raupach *et al.,* 1996) [11, 16] pathogens in various crops such as bean, carnation, cucumber, radish, tobacco, tomato, and *Arabidopsis.* Abdel-Kader *et al*. 2012 [1] reported that spraying vegetables, Cucumber, Cantaloupe, tomato and Pepper with the bio-agents, *T. harzianum, T. viride, B. subtilis, P. fluorescens* and *S. cerevisiae* was effectively able to reduce the foliar diseases comparing with untreated control. Similarly, Deore *et al*. 2004 evaluated culture filtrates of *T. viride*, *T. harzianum*, *T. hamatum, T. Iongiforum* and *T. lconlngll* for the management of powdery mildew of Cluster bean plants caused by *Leveilula taurica.* They found that culture filtrates of *Trichoderma* spp. either alone or in combination were found effective against powdery mildew.

Peroxidase and polyphenol oxidase mediate the oxidation of phenols and oxidized phenols which are highly toxic to the pathogen (Sequeira, 1983) [18]. In bioagents treated leaves activity of both peroxidase and polyphenol oxidase was higher in *Pseudomonas fluorescens* treated plants followed by *Trichoderma viride* and *Bacillus subtilis* in all the cultivars. Peroxidase has antifungal effects and has been implicated in the defence responses to pathogens in various crops. Increase in peroxidase activity has been shown to be associated with lignification, phenol oxidation and plant defence. Results of present study are also inconformity with those obtained by Anand *et al.* (2007) [3] who reported that the activities of the defence enzymes peroxidase (PO) and polyphenol oxidase (PPO), increased in the azoxystrobin and *P. fluorescens* treated cucumber plants. Activity of peroxidase was high in the resistant variety IIHR 517 A of chilli, and in the improved variety Pusa Jawala, as reported by Subhas Chander (1992) [23]. The higher peroxidase activity is correlated with disease resistance in many plants (Vidhyasekaran, 1988) [25].

Increased PAL activity was assayed in all the treatments and it also observed that the PAL activity increased with time lapse. In cultivars Granny Smith the activity was highest in *Pseudomonas fluorescens* treated plants (0.503.0.690, 0.950) followed by *Trichoderma viride* (0.490, 0.680, 0.920) and *Bacillus subtilis* (0.450, 0.640, 0.880) compared to water treated control plants. A similar trend was followed in cultivars Tydeman's Early Worcester and Starking Delicious and the activity was highest in *Pseudomonas fluorescens* treated plants followed by *Trichoderma viride* and *Bacillus subtilis.* Similarly, Ramamoorthy and Samiyappan (2001) [15] also reported that chilli seed treatment with *P. fluorescens* isolate Pf1 increased PAL activity, and that *Pseudomonas*-pretreated chilli leaves challenge inoculated with *C. capsici* showed an additional increase in PAL activity. However, Chen *et al*. (2000) [5] reported that high levels of PAL were induced in cucumber roots treated with *Pseudomonas corrugata* challenge inoculated with *Pythium aphanidermatum.* Sendhil Vel, (2003) [17] made a similar observation in grapevine plants inoculated with *Uncinula necator* and *Plasmopara viticola.*

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Table 2: Efficacy of Biocontrol agents in controlling Marssonina blotch of apple caused by *Marssonina coronaria* under polyhouse during 2013-2014

Cultivars	Treatments	Dilution (cfu)	Per cent disease index			Per cent disease control
			2013	2014	Pooled	
Starking Delicious	*Pseudomonas fluorescens*	10^{-3}	29.00 (31.80)	28.00(30.52)	28.50(30.80)	40.62
		10^{-5}	18.00 (26.15)	19.00 (22.97)	18.50(22.60)	61.45
		10^{-7}	23.00(29.21)	26.00 (29.38)	24.50(28.51)	48.95
	Bacillus subtilis	10^{-3}	34.00 (33.16)	33.00 (33.23)	33.50(33.48)	30.20
		10^{-5}	20.00 (29.21)	20.33(25.93)	20.16 (25.82)	58.00
		10^{-7}	27.00 (30.96)	27.02 9.960	27.00 (29.96)	43.75
	Trichoderma viride	10^{-3}	29.00 (32.01)	27.00 (29.96)	28.00(30.52)	41.66
		10^{-4}	19.00 (27.58)	20.00(22.23)	19.50 (22.60)	59.37
		10^{-5}	24.00 (29.58)	22.00 (26.98)	23.00 (27.60)	52.08
	Control		47.00 (36.86)	49.00 (41.00)	48.00(40.50)	0.00
Tydeman's Early Worcester	*Pseudomonas fluorescens*	10^{-3}	16.00(25.63)	17.00 (23.68)	16.50(23.32)	46.52
		10^{-5}	7.66 (20.87)	7.00 (12.81)	5.33(13.23)	73.82
		10^{-7}	9.00 (22.36)	8.00 (16.21)	8.50 (16.71)	69.64
	Bacillus subtilis	10^{-3}	18.33(27.13)	15.00 (22.23)	16.66(23.32)	40.50
		10^{-5}	7.68 (22.88)	8.00 (16.21)	7.83(16.04)	64.8
		10^{-7}	11.00(25.07)	10.00 (18.13)	10.50(18.58)	55.35
	Trichoderma viride	10^{-3}	17.00 (26.15)	16.00 (22.97)	16.50(23.44)	41.07
		10^{-4}	6.00 (21.50)	7.00 (15.17)	6.59(14.6)	69.64
		10^{-5}	9.00 (23.81)	9.00 (17.20)	9.00(17.2)	67.85
	Control		28.00(30.5)	28.00(30.5)	28.00(30.5)	0.00
Granny Smith	*Pseudomonas fluorescens*	10^{-3}	5.00 (12.8)	5.68 (13.64)	5.33(13.23)	60.98
		10^{-5}	2.00(8.10)	1.90(7.42)	1.33(6.61)	83.33
		10^{-7}	1.60(7.39)	2.00 (6.61)	2.00 (7.01)	77.7
	Bacillus subtilis	10^{-3}	5.00(14.03)	5.20(13.00)	5.15(14.32)	42.77
		10^{-5}	4.00 (11.46)	4.33 (11.92)	4.16(11.69)	53.77
		10^{-7}	4.33(11.46)	4.50 (13.64)	4.41(11.69)	45.00
	Trichoderma viride	10^{-3}	4.00 (11.46)	4.00 (11.46)	4.00(11.46)	55.5
		10^{-4}	2.30 (8.10)	2.00 (8.10)	1.84(8.10)	76.00
		10^{-5}	2.33(8.75)	2.00 (8.10)	2.15(8.10)	76.11
	Control		9.00 (17.20)	9.00(17.20)	9.00(17.20)	0.00
$CD_{0.05}$	Varieties (V)		0.595	0.550	0.339	
	Treatments (T)		0.687	0.635	0.392	
	V X T		1.190	1.100	0.678	
	Concentrations(C)		0.595	0.550	0.339	
	V X C		1.030	0.953	0.587	
	T X C		1.190	1.100	0.678	
	V X T X C		2.060	1.906	1.175	

Figures in parenthesis are arc sine transformed values

Table 3: Polyphenol oxidase activity in apple leaves after treatment with bioagents at different intervals

Cultivars	Treatments	Polyphenol oxidase activity (Change in absorbance /min/mg fresh wt.)		
		Sampling Interval (Hours)		
		48	72	96
Starking Delicious	*Pseudomonas fluorescens*	0.263	0.513	0.817
	Bacillus subtilis	0.230	0.490	0.780
	Trichoderma viride	0.250	0.513	0.807
	Control	0.230	0.437	0.750
Tydeman's Early Worcester	*Pseudomonas fluorescens*	0.377	0.810	0.960
	Bacillus subtilis	0.323	0.750	0.820
	Trichoderma viride	0.363	0.780	0.910
	Control	0.310	0.700	0.780
Granny Smith	*Pseudomonas fluorescens*	0.410	0.897	0.950
	Bacillus subtilis	0.390	0.820	0.890
	Trichoderma viride	0.403	0.840	0.930
	Control	0.360	0.790	0.810
$CD_{0.05}$		0.019	0.055	0.029

Table 4: Peroxidase activity in apples leaves after treatment with bioagents at different intervals

Cultivars	Treatments	Peroxidase activity (Change in absorbance/min/mg fresh wt.)		
		Sampling Interval (Hours)		
		48	72	96
Starking Delicious	*Pseudomonas fluorescens*	0.403	0.580	0.780
	Baccilus subtilis	0.387	0.520	0.760
	Trichoderma viride	0.400	0.570	0.763
	Control	0.360	0.490	0.633
Tydeman's Early Worcester	*Pseudomonas fluorescens*	0.490	0.597	0.880
	Baccilus subtilis	0.430	0.530	0.830
	Trichoderma viride	0.460	0.570	0.860
	Control	0.400	0.510	0.680
Granny Smith	*Pseudomonas fluorescens*	0.503	0.690	0.950
	Baccilus subtilis	0.450	0.640	0.880
	Trichoderma viride	0.490	0.680	0.920
	Control	0.410	0.610	0.720
$CD_{0.05}$		0.017	0.019	0.080

Table 5: PAL activity in apple leaves after treatment with bioagents at different intervals

Cultivars	Treatment	PAL activity (µmol trans-cinnamic acid $min^{-1}g^{-1}$)		
		Sampling Interval (Hours)		
		48	72	96
Starking Delicious	*Pseudomonas fluorescens*	0.403	0.580	0.780
	Bacillus subtilis	0.387	0.520	0.760
	Trichoderma viride	0.400	0.570	0.763
	Control	0.360	0.490	0.633
Tydeman's Early Worcester	*Pseudomonas fluorescens*	0.490	0.597	0.880
	Bacillus subtilis	0.430	0.530	0.830
	Trichoderma viride	0.460	0.570	0.860
	Control	0.400	0.510	0.680
Granny Smith	*Pseudomonas fluorescens*	0.503	0.690	0.950
	Bacillus subtilis	0.450	0.640	0.880
	Trichoderma viride	0.490	0.680	0.920
	Control	0.410	0.610	0.720
$CD_{0.05}$		0.015	0.052	0.220

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