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Influence of Microbial Seed Priming on Inducing Systemic Disease Resistance in French Bean (*Phaseolus vulgaris* L.)

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Plant disease control by ecofriendly approaches are gaining importance to overcome the ill effects of chemical pesticides especially in food grains and vegetables which are consumed directly. One such approach is biopriming /microbial seed priming, which is an emerging technology with ability of beneficial microorganisms to induce resistance in plants. The present study was taken up to evaluate the impact of some strains of plant growth promoting rhizobacteria (PGPRs) *viz.*, *Rhizobium* sp. (Rhi. sp) *Bacillus megaterium* (B. m), *Pseudomonas fluorescens* (P. f) and *Trichoderma harzianum* (T. h) individually and in combinations for their ability to induce pathogenesis-related proteins like peroxidase (PO), phenyl alanine ammonia lyase (PAL), polyphenol oxidase (PPO), β-1,3- glucanase and chitinase against *Fusarium* sp. associated with French bean plants. In the plants that were raised using microbial (PGPR and biocontrol agents) primed seeds, an effective reduction in disease incidence was observed. These plants also developed local and systemic resistance and increased activity of PO, PAL, PPO, β-1,3-glucanase and chitinase against *Fusarium* sp. was observed and several fold increase in the accumulation of defense enzyme activities was recorded. The combined inoculation treatments showed greater protection than single inoculation. On the other hand, result of microbial primed seed of French bean suggests that enhanced activities of defense enzymes may contribute to protection of French bean plants against *Fusarium* sp.

Key words: Microbial seed priming, Peroxidase, Phenylalanine ammonia lyase, Phenylalanine ammonialyase, Polyphenol oxidase , β-1, 3-glucanase and Chitinases.

French bean (*Phaseolus vulgaris* L.), is a legume belonging to family Fabaceae and is also known as kidney bean, garden bean, snap bean and string bean. It is an important vegetable crop which serves as the major source of protein in the diet. French bean is also rich in lipids which are needed by the body to absorb vitamin A. These legumes vegetables are affected by several soil-borne pathogenic fungi including various species of *Pythium*, *Fusarium* and *Rhizoctonia*, causing pre or post-emergence damping off, foot rot and wilt diseases (Agrios, 1997; Allen *et al.*, 1998), which leads to severe reduction in yield. Plant disease control by ecofriendly approaches are gaining importance to overcome the ill effects of chemical pesticides especially in food grains and vegetables which are consumed directly. One such approach is biopriming /microbial seed priming, which is an emerging technology of seed treatment that integrates biological and physiological aspects of disease control which is being used as an alternative method for controlling many seed and soil-borne plant pathogens and enhancing germination and yield of crop plants (El-Mohamedy, 2004).

Plant growth promoting rhizobacteria (PGPR) and biocontrol agents are used to reduce the disease incidence through mechanism of competition for ecological niche/substrate, production of siderophores, hydrogen cyanide and fungal cell wall lysing enzymes such as chitinases and β-1,3-glucanases which degrade chitin and glucan present in the cell wall of fungi (Glick and Bashan 1997; Wang *et al.*, 2000; Attia *et al.*, 2005;

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Saravanakumar *et al*., 2007) and degradation of toxin produced by pathogen (Duffy and Defago, 1997).

Microbial seed priming is a promising technology which can develop plant defence mechanism or systemic resistance in plants against plant pathogens. Present study was conducted to evaluate the triggering of plant defence mechanism by microbial seed priming with biocontrol agents and compatible PGPRs and their effect was evaluated by recording the activity of different enzyme like peroxidase (PO), phenylalanine ammonialyase (PAL), polyphenol oxidase (PP), β-1, 3-glucanase and Chitinases.

MATERIALS AND METHODS

Prior to biopriming, the priming protocol was standardized by considering three parameters *viz*, different levels of temperature (5 °C, 15 °C, 25 °C), soaking hours (24 h and 32 h) in different concentration (-2 bars, -4 bars and -6 bars) of Poly ethylene glycol (PEG 6000) and the experiments were taken up under lab and greenhouse conditions. The experiments that gave best result at 25 °C and 32 h of soaking in -4 bar concentration of PEG 6000 was considered as standard for further biopriming studies. The primed seeds were soaked in sterile distilled water with tween 20 containing bacterial pellets and fungal spores singly and in combinations by following the procedure given by Raj *et al*. (2004). Unprimed seeds were kept as control. A green house experiment was taken up to evaluate the effect of microbial seed priming on biochemical parameters associated with disease resistance in French bean.

Microbial seed priming

Preparation of spore suspension

Bacterial suspensions were prepared by inoculating sterile broth with PGPRs and biocontrol agents *viz*., *Rhizobium* sp, *Bacillus megaterium*, *Pseudomonas fluorescens* and *Trichoderma harzianum* colonies grown on agar plates, which were incubated overnight on rotary shaker (25 °C and at 150 rpm). All spore suspensions were adjusted with sterile water to give a spore concentration of 10^6 –10^7 CFU/ml. The required volume (100 ml) of bacterial suspension was then centrifuged (12000 x g for 10 min) and the pellets were re-suspended in the sterile water pre determined for seed priming. Fungal isolates were grown on potato dextrose agar at 28 °C, following profuse sporulation. The spores were then harvested by adding sterile distilled water to the plates and scraping the spores into suspension. The suspension was filtered through a double layer of sterile glass wool and the spore suspension was adjusted with sterile water to give a spore concentration of 10^6 –10^7 CFU /ml.

Primed seeds were soaked in sterile distilled water containing bacterial pellet and fungal spores singly and in combination with the sticking agent (Tween 20) at the concentrations of 0.1 ml/L for bacteria and 0.5 ml/L for fungi. Flasks were kept in shaker at 150 rpm for 6 hrs. The bioprimed seeds were then air-dried on filter paper for 1 hr in a laminar flow hood, packed and stored in a refrigerator at 5 °C until required. Another group of surface sterilised seeds (using 3% sodium hypochloride for 5 min, then air-dried) were prepared as control treatments (Unprimed seeds) (Raj *et al*., 2004).

Procedure Preparation of Pathogen inoculum by broth drench method

Microbially primed seeds of French bean treated with different combinations of PGPRs and biocontrol agents were sown in plastic cups containing sterile coir pith in three replications for each treatment. The isolated *Fusarium* culture was mass multiplied in potato dextrose broth and crown region of the 15 days old plants was drenched

Treatments	Details
T_1	Control
T_2	Hydroprimed
T_3	PEG primed seeds
T_4	Microbial seed primed with *P. fluorescens* (P. f)
T_5	Microbial seed primed with *B. megaterium* (B. m)
T_6	Microbial seed primed with *Rhizobium* Sp.(Rhi.)
T_7	Microbial seed primed with *T. harzianum* (T. h)
T_8	Microbial seed primed with P. f + T. h
T_9	Microbial seed primed with B.m +T. h
T_{10}	Microbial seed primed with Rhi + T. h
T_{11}	Microbial seed primed with P. f + B. m + Rhi
T_{12}	Microbial seed primed with P. f + B. m + Rhi +T. h

with 15 ml of fungal biomass to French bean plants. Samples were taken at 3, 5 and 7 days after inoculation of pathogen for enzymatic estimation. Similar set of experiment was carried out in the sterile coirpith infested with *Fusarium* to record the disease incidence. Observations were recorded using standard protocols.

Per cent pre- and post- emergence damping off

Pre-emergence damping off (%) = [(GA-GT)/ GA] X 100

Where, GA - Germination percentage in absolute control

GT - Germination percentage in treatment

Post- emergence damping off (%) = [(GP-ND)/ GP] X 100

Where, GP - Germination percentage

ND - number of plants damped-off after emergence

Per cent disease incidence

PDI = (Number of infected plants/Total number of plants observed) X 100

Biological control efficacy (Guo *et al*, 2004).

BCE = (DIPC- DIT/ DIPC) X 100

Where, DIPC – Disease incidence in pathogen control

DIT – Disease incidence in treatment group

Bioassay to study the defence related enzymes

The plant enzymes which are associated with induction of plant disease resistance like peroxidase, phenyl alanine ammonia lyase, polyphenol oxidase, β-1, 3-glucanase and chitinases were recorded on 3, 5 and 7 days after challenging of pathogen.

Estimation of peroxidase activity (Sadasivam and Manickam, 1992)

Enzyme activity was calculated and expressed as change in OD min^{-1} g of leaf $tissue^{-1}$.

Phenylalanine ammonialyase (PAL) (Dickerson *et al.*, 1984).

The enzyme activity was expressed as µmol of cinnamic acid min^{-1} $mg^{-1}$of protein

Estimation of poly phenol oxidase activity (Karthikeyan *et al.*, 2006).

The enzyme activity was expressed as changes in absorbance at 495 nm min^{-1} g^{-1} of fresh weight tissue.

Assay of β-1, 3-glucanase (Pan *et al.*, 1991).

The enzyme activity was expressed as nmol glucose released min^{-1} g^{-1} leaf tissue.

Chitinases assay

Leaf samples (1 g) were homogenized in 2 ml of 0.1 M sodium citrate buffer (pH 5.0). The homogenate was centrifuged at 16,000 g for 15 min at 4 °C and the supernatant was used in the enzyme assay. The colorimetric assay of chitinase was carried out as per Boller and Mauch (1988) using colloidal chitin as substrate. The enzyme activity was expressed as n mol GlcNAc equivalents min^{-1} g^{-1} of leaf tissue.

RESULTS AND DISCUSSION

The microbial primed seeds were evaluated for their biocontrol efficiency on French

Table 1. Effect of microbial seed priming with different treatments on Biocontrol efficiency when challenged with *Fusarium* sp. under green house condition.

Treatments	Germination Per-centage (%)	Pre-emergent damping off(%)	Post-emergent damping off (%)	Per-cent disease incidence (%)	Biocontrol Efficiency
T_1	37.50	48.35	42.07	89.42	-
T_2	41.28	37.19	40.82	77.69	13.09
T_3	42.35	28.42	23.52	57.58	34.16
T_4	76.87	21.86	16.49	37.37	57.12
T_5	78.50	20.31	17.08	35.36	59.24
T_6	71.32	22.49	23.65	45.68	48.21
T_7	79.86	19.23	14.01	33.47	62.79
T_8	84.38	11.03	20.31	30.51	64.91
T_9	83.25	9.30	11.80	20.67	76.24
T_{10}	82.58	10.78	12.69	23.07	73.98
T_{11}	94.31	5.03	5.78	10.45	88.02
T_{12}	96.57	4.96	5.02	9.36	88.64
SEm ±	0.76	0.53	0.78	0.38	0.35
CD @ 5%	2.22	1.56	2.29	1.12	1.04

bean crop challenged with fungal pathogen (*Fusarium)* under green house condition. Results obtained on percent germination, pre-emergence damping off, post-emergence damping off, per cent disease incidence and biocontrol efficiency are presented in Table 1.

The lowest germination per centage was recorded in T_1 control (37.50%) and highest in T_{12} (96.57%) followed by T_{11} (94.31%). Lowest pre-emergence and post-emergence damping off (%) was recorded in T_{12} (4.38 % and 5.02 % respectively) which was on par with T_{11} (5.27 % and 5.78 % respectively) and T_7 (19.23 % and 14.01%) *i.e.* microbial primed seed only with *Trichoderma harzianum* also showed lower pre-emergence and post-emergence damping off among all the individually used bioprimed treatments, followed by T_5 (20.31%) with *Bacillus megaterium* in pre emergent damping off and in reduced post emergent damping off T_4 (16 %) bioprimed with *Pseudomonas fluorescens*.

The highest pre-emergence damping off (%) was recorded in T_1 (unprimed seeds) (48.35 %) followed by T_2 (hydroprimed seeds) (37.19%). The highest post-emergence damping off (%) was recorded in T_1 (unprimed seeds) (42.07%) and T_2 (hydroprimed seeds) (40.82%). The treatment T_{12} recorded the lowest per cent disease incidence percentage of (9.36 %) which was on par with T_{11} (10.45 %) and among all individual treatments T_7 (33.47 %) was the best. The highest per cent disease incidence was observed in controls T_1 (89.42 %) and T_2 (77.69). The highest biocontrol efficiency of 88.64 % was recorded in T_{12} which was on par with T_{11} (88.02 %) and lowest was observed in controls T_2 (hydroprimed seeds) (13.09 %) and T_1 (unprimed seeds) considered as untreated kept as absolute control for pathogen inoculation and hence biocontrol efficiency was recorded.

Effect of microbial seed priming on the activity of peroxidase, phenylalanine ammonialyase (PAL) and Poly phenol oxidase activity in French bean plants in the presence of fungal pathogens were measured in leaves at 3, 5, and 7 days of post inoculation. The results are presented in **Table 2.** Inoculation with microbially primed seeds and *Fusarium* resulted in significant increase in the activities of peroxidase, phenylalanine ammonialyase (PAL) and poly phenol oxidase activity (PPO) in leaves of French bean plants. Similar findings of increase in peroxidase proteins after application with biocontrol agents have been reported by several workers in different crops (Meena *et al*., 2000; Oostendorp *et al*., 2001). All enzyme activity peaked at 7 days in leaves of microbially primed seed treatments as compared to absolute control plants, while among the individual and different

Table 2. Effect of microbial seed priming on the activity of peroxidase (PO), phenylalanine ammonialyase (PAL) and poly phenol oxidase (PPO) activity in French bean plants in the presence of fungal pathogens

Treatments	Peroxidase activity (changes in absorbance $min^{-1} g^{-1}$)			Phenylalanine ammonia lyase activity(µmol of trans -cinnamic acid $min^{-1} g^{-1}$ of protein)			Poly phenol oxidase activity (changes in absorbance $min^{-1} g^{-1}$)		
	3 DAI	5DAI	7DAI	3 DAI	5DAI	7DAI	3 DAI	5DAI	7DAI
T_1	0.08	1.08	1.53	27.11	29.61	29.83	1.06	2.03	2.15
T_2	1.04	1.97	2.15	29.69	31.52	31.13	1.83	2.07	2.19
T_3	1.03	1.48	2.00	29.51	31.47	32.00	1.79	2.00	2.08
T_4	2.98	4.31	4.91	35.35	38.19	38.10	2.71	3.99	4.16
T_5	2.03	4.01	4.31	31.16	34.07	35.10	2.35	3.60	3.90
T_6	2.06	3.82	4.12	37.40	41.68	41.93	2.03	2.98	3.00
T_7	3.03	5.08	6.24	37.67	40.34	43.00	2.17	3.64	3.78
T_8	3.00	5.92	6.96	39.00	57.31	58.85	2.83	4.17	4.37
T_9	3.12	4.98	5.23	38.00	54.13	54.96	2.97	4.61	4.97
T_{10}	3.06	4.8	5.11	38.58	53.00	54.51	2.63	3.97	4.01
T_{11}	5.05	8.91	10.34	41.36	73.89	74.56	3.98	5.75	6.34
T_{12}	5.78	9.63	11.56	45.78	79.27	81.64	5.07	8.95	9.31
SEm ±	0.03	0.05	0.14	0.25	0.11	0.34	0.06	0.07	0.03
CD @ 5%	0.11	0.16	0.42	0.73	0.33	1.01	0.17	0.23	0.08

combinations of microbial seed priming treatments, the enzyme activity increased gradually with time. The peak activity of all three enzymes was found in treatment T_{12} were seed primed with P. f + B. m + Rhi + T. h shown at seven day after inoculation in all bioprimed treatments, in response to pathogen inoculation. The activity increased in treated plants compared with uninoculated plants of treatment T_1. The activity of PO increased by 7.5 folds, PPO

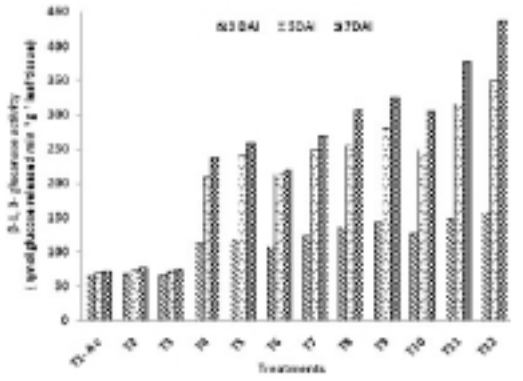

Fig. 1. Effect of microbial seed priming on β 1, 3-glucanase activity

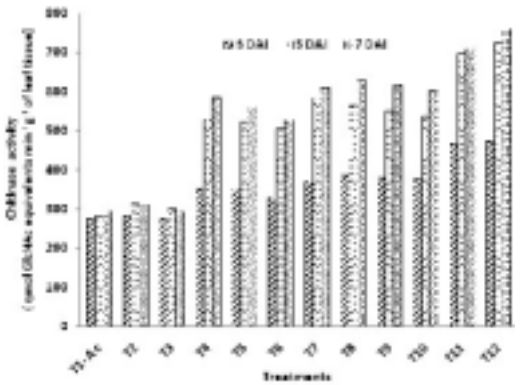

Fig. 2. Effect of microbial seed priming on chitinase activity

increased by 2.75 folds and PAL activity was raised by 4.33 folds in leaves of treatment T_{12} of French bean plants due to antagonists treatments and also due to inoculation with pathogen. The increase in enzyme activity lasted up to 7 days after inoculation, The maximum activity of the enzyme was observed in T_{12} (Bioprimed with P. f + B.m + Rhi + T. h) inoculated plants followed by T_{11} (Bioprimed with P. f + B. m + Rhi) inoculated plants. Similar work on PGPR are also involved in induced systemic resistance (ISR) against many diseases in a wide range of crops (Liu et al., 1995). The role of oxidative enzymes and of their metabolic products in the defence mechanisms of infected plants have also been studied by Waheed and Tehmina (2011). Ramamoorthy *et al.,* (2002) studied the Peroxidase activity in diseased plants and its effects on resistance or susceptibility in many host-pathogen interactions.

The results of β 1, 3- glucanase and chitinase activity was expressed as change in ·mol GlcNAc equivalents min^{-1} g^{-1} of leaf tissue and ·mol glucose released min^{-1} g^{-1} leaf tissue leaf tissue respectively. In general irrespective of the enzymes and bioprimed treatments, the enzyme activity was less at 3 DAI and increased at 5 DAI and 7 DAI (Fig. 1 and Fig. 2). Initially at 3 DAI T_{12} showed highest activity of both enzymes followed by T_{11} for all the bioprimed treatments in both enzymes. T_1 control showed the least activity of β 1, 3-glucanase and chitinase activity in *Fusarium* infested plants. Up to at 5 DAI significant increase has been taken compared to 3 DAI and gradual increase has taken over between 5 DAI to 7 DAI in T_{12} and also in other microbially primed treatments (Fig. 1 and Fig. 2). Among all single inoculatios of microbially primed seeds *T. harzianum* showed highest. In recent years, the use of PGPR as an inducer of systemic resistance in crop plants against different pathogens has been demonstrated under pots and field conditions (Viswanathan, 1999; Viswanathan and Samiyappan, 1999; Attia *et al.,* 2005).

CONCLUSION

In the present study microbial primed French bean seeds were evaluated for their biocontrol efficiency when challenged with *Fusarium* pathogen. The effective inhibition was observed in combinations of PGPRs and specific biocontrol agents, particularly in the seeds that were primed with the consortia of *Pseudomonas fluorescens*, *Bacillus megaterium*, *Rhizobium* sp. and *Trichoderma harzianum* with increased activity of PO, PAL, PPO, β-1,3- glucanase and chitinase enzymes which resulted in improved performance in terms of disease resistance, plant growth and yield of microbial primed French bean crop.

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