

# Multifarious activity of bioformulated *Pseudomonas fluorescens* PS1 and biocontrol of *Sclerotinia sclerotiorum* in Indian rapeseed (*Brassica campestris* L.)

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**Abstract** PGPR strain *Pseudomonas fluorescens* PS1 was evaluated to formulate carrier based bioformulations. The viability of *P. fluorescens* PS1 was monitored at different time intervals during the period of storage at room temperature in different carriers such as soil, charcoal, sawdust and sawdust-soil. Sawdust-soil was found to be the most efficient carrier material for *P. fluorescens* PS1 followed by other carriers. After 1 year of storage, *P. fluorescens* PS1 was re-isolated and assayed for its antifungal activity against *Sclerotinia sclerotiorum* a phytopathogenic fungus causing stem blight in Indian mustard, *Brassica campestris*. Results of scanning electron microscopy exhibited that *P. fluorescens* PS1 caused morphological alteration in mycelia of *S. sclerotiorum*

as evident by hyphal perforation, and fragmented lysis. Seed bacterization of *B. campestris* with *P. fluorescens* PS1 induced enhanced seed germination, increased overall plant growth as well as reduced stem blight in mustard with improved yield. These findings demonstrate that *P. fluorescens* PS1 has significant potential to raise disease-free crops due to the presence of a wide array of PGP characteristics.

**Keywords** Sawdust-soil carrier · *Pseudomonas fluorescens* · Stem blight · *Sclerotinia sclerotiorum* · *Brassica campestris* L.

## Introduction

India is one of the largest rapeseed-mustard growing countries in the world, placed second after China in terms of rapeseed production. Demand is increasing worldwide for its edible oil that has lowest amount of undesirable saturated fatty acids and also contains adequate amounts of the two essential fatty acids, linoleic and linolenic, which are not present in many of the other edible oils. Brassica rapeseed-mustard is the second most important edible oilseed crop in India after groundnut, and accounts for nearly 30% of the total oilseeds produced in the country. However, rapeseed production and subsequent yield is severely limited because of soil borne infestation of *Sclerotinia sclerotiorum* (Lib.) de Bary, a pathogen that causes stem blight of *Brassica campestris*.

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Rhizospheric soil is a repertoire of microorganisms that includes beneficial rhizobacteria and pathogenic species that thrive in a nutrient enriched environment. Beneficial rhizobacteria are known to antagonize root pathogens through competition for nutrients and niche, aggressive colonization, parasitism, predation, production of lytic enzymes, antibiotics, bacteriocins, salicylic acid, release of volatiles, surfactants, rhamnolipids and induced systemic resistance (Haas and Defago 2005). Recent environmentally-friendly cultivation practices emphasize the need to maintain the microbial balance in the rhizosphere by application of plant growth promoting rhizobacteria (PGPR) and thereby, limit the density of soilborne pathogens to a minimum level for disease control (Adesemoye et al. 2009). Also, in disease control programs, the density of biocontrol agents in rhizosphere and efficient root colonization has a clear relationship with suppression of soilborne plant diseases. Among such beneficial rhizospheric microflora, species of *Pseudomonas* are well known for their plant growth promoting potential, aggressive rhizospheric colonization, and antagonistic abilities including the ability to produce lytic enzymes that digest chitin and  $\beta$ -1, 3-glucans as major components (Kumar et al. 2009; Arora et al. 2008a). Application to the rhizosphere is equally important and requires effective formulation.

The formulation of a bacterial inoculant is defined as a preparation containing one or more beneficial bacterial strains (or species), as well as organic and inorganic carrier materials. A good quality formulation promotes survival of bacteria, maintaining a viable population sufficient to exude growth promoting effects on plants. Carriers including alginate beads, charcoal, sand, sawdust and sugarcane-bagasse have been analyzed for production of bioinoculants using plant growth promoting fluorescent pseudomonads (FLPs) while antagonistic strains of FLPs have been formulated in talc, peat, vermiculite, lignite and kaolinite powder (Arora et al. 2008b; Vidhyasekaran et al. 1997). Formulation inadequacies are often the most common barriers to commercialization due to inefficient attributes during storage as well as non-reproducible results in the field. Hence, it is imperative to evaluate the survival of the immobilized bacteria in different carriers, and also their ability to retain attributes for plant growth promotion. *P. fluorescens* PS1, a previously isolated and characterized strain from sunflower rhizosphere has been

reported as a potential PGPR strain for growth promotion in oilseed crops (Bhatia et al. 2008). In the present study, cell viability of *P. fluorescens* PS1 was evaluated in various carrier materials, re-screened for PGP attributes and was evaluated for inhibitory action against *S. sclerotiorum*. Ability of *P. fluorescens* PS1 to produce various inhibitory metabolites was also determined, including extracellular release of cell wall degrading enzymes i.e. chitinases and  $\beta$ -1,3-glucanase. In addition, studies on establishment of introduced strain *P. fluorescens* PS1 in the root zone and performance of the formulation on growth of *B. campestris* were carried out in experimental field trials.

## Material and methods

### Strains and growth conditions

*Pseudomonas fluorescens* PS1 was procured from the culture collection of the Department of Botany and Microbiology, Gurukul Kangri University, Haridwar, Uttarakhand, India and re-isolated from sawdust-soil carrier after 1 year of storage (polythene bags) at room temperature by using a dilution plate technique. *P. fluorescens* PS1 was grown on tryptic soy broth agar medium (TSM) (HiMedia, India) at  $28 \pm 1^\circ\text{C}$  for 24 h at 150 rpm (pH 7.2) unless mentioned otherwise. The virulent fungal pathogen *S. sclerotiorum* was isolated from infected roots of *B. campestris* using a blotter technique (de Temp 1963) and pathogenicity was established on the host crop by artificial inoculation for corroborating Koch's postulates. The pathogen was maintained on potato dextrose agar (PDA) at  $4^\circ\text{C}$  for further studies.

### Physico-chemical properties of carrier used

Solid carriers such as soil, charcoal, sawdust and sawdust-soil (1:1, w/w) were taken as supporting materials for the growth of *P. fluorescens* PS1. Equal amounts of each solid carrier was mixed with distilled water and stirred thoroughly to form a slurry or paste and the pH was determined (Page et al. 1982). Water holding capacity of a carrier was determined on a dry weight basis. Water was added to 100 g of oven dried carrier material with continuous stirring until the carrier became saturated. The slurry was transferred in a measuring cylinder with a sieve (0.25 mm)

covered drain hole at the bottom. The water was allowed to drain overnight from the carrier under normal conditions. After drying, the weight of the left-over carrier material in the cylinder was measured, and percent water holding capacity was recorded. To analyze inherent moisture capacity, 10 g solid carrier was placed into an oven at 70°C for 24 h. The carrier material was weighed and kept again in the oven for 24 h to determine the end point of moisture loss. Moisture content was calculated by the following formula:  $M = [(W_1 - W_2)/W_2] \times 100$ ; where, M = moisture content (%),  $W_1$  = weight of carrier before drying, and  $W_2$  = weight of carrier after drying.

### Bio-inoculant preparation

*P. fluorescens* PS1 was grown in tryptic soy broth (TSB) at 28±1°C for 24 h at 150 rpm (pH 7.2). The broth culture of ~10<sup>8</sup> cells/ml was utilized for inoculant preparation. The solid carrier materials were ground separately and air dried before mixing and curing followed by double sterilization (121°C for 20 min). The sterile carrier materials (40 g each) were packed in recommended (50–70 µm thick) low density polythene bags of flexible sheets to protect from loss of moisture. The bags were sealed leaving about 25% airspace to give proper aeration to the inoculants. *P. fluorescens* PS1 (inoculum) was mixed thoroughly with supporting carrier material under aseptic condition, sealed and stored at room temperature. The initial count in each carrier-based preparation was made so as to obtain 10<sup>8</sup> cells/g at the time of storage.

### Viability studies

The viability of *P. fluorescens* PS1 cells was determined in four bio-inoculant preparations, including soil (sand 56%, silt 22%, clay 17%, texture—sandy loam, colour—yellow), sawdust (from *Shorea robusta*), charcoal and combination of sawdust and charcoal (1:1). The samples (1 g each) were collected from bioformulations after different time intervals up to 360 days under aseptic conditions. Suitable dilutions were spread plated on TSM, amended with antibiotics (rifampicin and streptomycin—50 µg/ml each), and incubated at 28±1°C for 48 h. The bacterial population (CFU/g) was enumerated. The

experiment was conducted in triplicate, and one bag of each carrier from each replicate was investigated after every 30 days interval.

### In vitro antagonistic activity of *P. fluorescens* PS1 against *S. sclerotiorum*

Antagonistic activity of *P. fluorescens* PS1 against *S. sclerotiorum* was evaluated on PDA plates by dual culture technique. Five days old mycelial disc (5 mm) was placed in the centre of modified PDA medium, supplemented with 2% sucrose. An exponentially growing culture (10<sup>8</sup> CFU/ml) was spotted 2 cm juxtaposed from the fungal disc. The plates were incubated at 28±1°C for 5 days. Growth inhibition was calculated by measuring the distance between the edges of the bacterial and fungal colonies by using the formula:  $100 \times [C - T]/C$ , where, C is the radial growth of fungus in control and T is the same in dual culture. For preparation of SEM samples, the mycelia were collected with the help of a sterile forcep from the zone of interaction, which were fixed overnight using 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.3) and washed thrice in phosphate buffer (10 min each). Thereafter, samples were dehydrated through 70, 80, 90 and 100% ethanol (5 min each) and re-suspended thrice in 100% ethanol at room temperature. Ethanol was then replaced by liquid CO<sub>2</sub> and the samples were air dried. The samples were then mounted on stubs and coated with gold. These coated specimens were observed at 15 kV in a LEO 485 VP scanning electron microscope and photomicrographs were recorded.

### Bioassay for lytic enzymes

Ability of *P. fluorescens* PS1 for extracellular chitinase activity was studied using a solid chitin minimal medium (CMM, 0.5% colloidal chitin) while β-1,3-glucanase activity was studied using laminarin as carbon source in minimal medium (Dunne et al. 1997). *P. fluorescens* PS1 was spot inoculated on these plates and incubated at 30±2°C for 4–5 days. These plates were examined for development of clear zones around colonies up to 7 days. The size of the clear zone (CZ) and colony size (CS) were measured in case of chitinase and CZ/CS ratio was determined, while growth on laminarin as the carbon source was recorded for β-1,3-glucanase activity.

For quantitative estimation of extra-cellular chitinase, 50 ml of chitinase minimal broth was inoculated with PS1 and incubated at  $30\pm 2^\circ\text{C}$  (180 rpm, 96 h). Culture filtrate was collected by centrifugation at 8,000 rpm for 20 min and utilized for enzyme assay. The assay mixture consisted of 0.25 ml of 1 M sodium acetate buffer (pH 5.6), 0.1 ml of colloidal chitin (0.2%) and 0.1 ml of suitably diluted enzyme solution. The reaction mixture was incubated at  $37^\circ\text{C}$  for 30 min in a water bath. For  $\beta$ -1,3-glucanase estimation, 50 ml of minimal broth (0.2% laminarin) was inoculated with PS1, incubated at  $30\pm 2^\circ\text{C}$  (180 rpm, 96 h) and harvested (8,000 rpm, 20 min,  $4^\circ\text{C}$ ). The reaction mixture incubated at  $37^\circ\text{C}$  for 30 min consisted of 0.25 ml of phosphate buffer (pH 5.5) containing 0.1 ml laminarin (0.2%) and 0.1 ml of suitably diluted enzyme. The activities of chitinase and  $\beta$ -1,3-glucanase were determined by estimating the amount of reducing sugar spectrophotometrically (Shimadzu) by DNS method. The enzyme activities (units) were defined as formation of 1  $\mu\text{mole}/\text{min}$  of N-acetyl-D-glucosamine (GlcNAc) from colloidal chitin by chitinase and D-glucose from laminarin by  $\beta$ -1,3-glucanase using dinitrosalicylic acid (DNS). Cellulase production ability of PS1 was determined by inoculating it into CMC amended Czapek-mineral salt agar plates. Appearance of a halo around bacterial growth confirmed presence of cellulase.

#### Utilization of fungal biomass for bioassay of lytic enzymes

*S. sclerotiorum* was grown in PDB (Himedia, India) for 7 days at 150 rpm,  $28\pm 2^\circ\text{C}$ . The fungal biomass was harvested (6,000 rpm, 10 min,  $4^\circ\text{C}$ ), washed twice with sterile normal saline (0.87%) followed by distilled water, before being dried in an oven (NSW, India) at  $52^\circ\text{C}$  till constant weight was obtained. The dried fungal mycelium (10 g/l) was used as substitute of chitin/laminarin in minimal broth for quantitative assay. The enzymes chitinase and  $\beta$ -1,3-glucanase were assayed as described above.

#### Ability to produce antifungal metabolites

*P. fluorescens* PS1 was grown in KB broth for 3 days at  $28\pm 1^\circ\text{C}$  and harvested (10,000 g for 20 min,

0.45  $\mu\text{m}$  Millipore filter). The acidified (pH 6.0) cell-free culture supernatant was used for estimation of siderophores (Csáky 1948). For estimation of volatile hydrocyanic acid (HCN), the strain was grown in tryptic soy broth (TSB) amended with glycine and incubated at  $28\pm 1^\circ\text{C}$  (150 rpm, 24 h). Filter paper (Whatman No: 1) was cut into uniform strips of 5 cm long and 1 cm wide. These were saturated with 0.05% picric acid in 1% sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) and placed inside the conical flasks in a hanging position. After incubation at  $28\pm 1^\circ\text{C}$  for 24–72 h, a change in colour of filter paper from yellow to brown or deep red was indicative of HCN evolution. The colour was eluted by placing the filter paper in a sterilized test tube with 10 ml of double distilled water and the absorbance (625 nm) was measured (Meena et al. 2001). The ability of *P. fluorescens* PS1 to utilize oxalic acid was determined by inoculating the log phase growth culture on 'oxalic acid degradation selective medium' that had ammonium oxalate as the sole source of carbon (Dickman and Mitra 1992). The plates were incubated in the dark for about 7 days at  $30\pm 1^\circ\text{C}$  and observed for a clear zone around bacterial growth indicating the presence of oxalate oxidase degrading oxalic acid. The ability to release surfactant was checked by observing lysis of RBCs, resulting in a clear zone formation on blood agar plates. For this, a cell-free culture filtrate (CFCF) was collected by filtering exponential phase broth culture through a Millipore membrane filter assembly. A 100  $\mu\text{l}$  aliquot of CFCF was inoculated in wells punched in blood agar plates under sterile conditions. Appearance of a clear zone (if any) was indicative of surfactant production (Mulligan et al. 1984).

#### Preparation of fungal inoculum

Inoculum of *S. sclerotiorum* was prepared in the laboratory by growing it on oat (*Avena sativa*) grains. These grains (150 g) were soaked in distilled water overnight, autoclaved twice ( $121^\circ\text{C}$  for 1 h) and inoculated with two discs (5 mm diam) of fungal mycelia cut from margin of actively growing 5 days old culture. Flasks were incubated at  $28\pm 1^\circ\text{C}$  for 15 days. Oat grains (2.5 g oat grains) containing mycelial fragments of *S. sclerotiorum* were mixed with 4 kg sterilized soil in field plots before sowing the seeds.

## Seed bacterization and pot assay

A short term pot-trial assay was carried out by placing sterile soil (4 kg each) in earthen pots (25 cm diam). Pots were arranged in complete randomized block design and each treatment was replicated five times. Pots were kept on a polyhouse bench and watered when required. Seeds of *B. campestris* with uniform shape and size were surface sterilized with 95% ethanol for 30 s, and then washed with sterile distilled water (5–6 times). The seeds were dried overnight under sterile air stream. Seed bacterization was done as described by Bhatia et al. (2008). Seeds coated with *P. fluorescens* PS1 and non-coated seeds were sown in pots having sandy loam soil (77.3% sand, 13.6% silt, 11.7% clay, total organic C 0.0976%, pH 6.4, having 36% water holding capacity) in the following four sets of treatments.

T1: Soil infested with *S. sclerotiorum* + non-bacterized seeds; T2: Soil inoculated with seeds treated with PS1 in sawdust; T3: Soil infested with *S. sclerotiorum* + PS1 in sawdust-soil (50:50 w/w) and T4: Control (without *S. sclerotiorum* and PS1).

After 15 days of sowing, early seedling emergence was observed. The disease incidence (%) was calculated 90 days after sowing (DAS) using the formula: Disease incidence(%) =  $100 \times \text{Total number of disease seedlings}(T_N) / \text{Total number of emerged seedlings}(T_E)$ .

## Field experiment

Sawdust:soil-based formulations of *P. fluorescens* PS1 were applied in the experimental field using the same number of treatments as above through bacterized seeds in 2007 and 2008. Bacterized seeds ( $\sim 10^8$  CFU/seed) were sown in sandy loam soil (74% sand, 14% silt and 12% clay, 0.035% total organic matter, pH 7.4, water holding capacity 35%) in a randomized plot design. The field experiments were conducted in Haridwar, Uttarakhand, India during the season October to February. Inter-row distance was maintained at 45 cm and within-row distance was kept at 35 cm. A total of ten plants were uprooted after time intervals of 30 DAS and germination index (15 DAS), total plant length (90 DAS), fresh and dry weight of seedlings (90 DAS), number of siliqua, grain yield/plant and grain yield/ha were recorded at the time of harvesting (120 DAS). Seeds treated with sterile sawdust-soil served as control.

## Competitive root colonization ability

The bacterial population densities in the rhizosphere of mustard plants, grown during field trials were analyzed on TSM supplemented with streptomycin and rifampicin (50 µg/ml each). The plants receiving the treatments ‘sawdust—soil + PS1’ and ‘sawdust—soil + PS1 + *S. sclerotiorum*’ were uprooted at intervals of 30 days to the time of crop harvest (120 DAS) and the bacterial population in rhizosphere was enumerated by viable counts. The loosely-adhered soil on the roots was removed and roots were cut into 1 cm long segments: 1 g of root segment was dipped in 10 ml of sterile distilled water, vortexed 4–5 times to release the attached bacteria into the sterile normal saline. A suitable dilution of the above suspension was poured into petri plates containing TSM amended with antibiotics, and incubated at  $28 \pm 1^\circ\text{C}$  for 48 h. The bacterial population (CFU/g of root segment) was enumerated. The growth of *P. fluorescens* PS1 was observed around the seeds. Further root exudates were prepared following Gamliel and Katan (1992). Contamination-free root exudates were mixed with 35 ml of tryptic soy broth (TSB) @ 15% (v/v) and inoculated with 0.1 ml exponential phase culture of *P. fluorescens* PS1 and incubated at  $28 \pm 1^\circ\text{C}$  for 24 h (150 rpm). The tubes were observed visually for turbidity against an inoculated control without root exudates.

## Statistical analysis

Statistical analysis was done using a two way analysis of variance (ANOVA) for individual parameters. All hypotheses were tested at the 1% confidence level. The standard error of the mean, variance, and ANOVA statistics were calculated using SPSS statistical software.

## Results

### Cell viability in different carriers

Sawdust-soil was found to be superior among the tested carriers, having sufficient water holding capacity, inherent moisture content and pH optima for the growth of *P. fluorescens* PS1 (pH 6.8, inherent moisture content 14.3%, water holding capacity 76%) (Table 1). The cell viability of *P. fluorescens*

**Table 1** Physico-chemical properties of carriers

Attributes	pH	Inherent moisture content (%)	Moisture content (%)	Water holding capacity (%)	Electrical conductivity (mho)
Sawdust-soil	6.8	14.3	9.6	76	nd
Soil	7.1	11.6	10	81	nd
Sawdust	7.2	8.14	8	74.3	6.2
Charcoal	7.0	6.8	3.4	71	5.6

nd not detected

PS1 in sawdust initially decreased to  $\sim 10^7$  CFU/g 30 DAS and increased thereafter. Soil proved the most inferior of the carriers tested as there was a drastic reduction in cell population. The cell population of  $\sim 10^8$  CFU/g initially decreased to  $\sim 10^6$  CFU/g 30 DAS in charcoal, which increased to  $\sim 10^7$  CFU/g up to 6 months (Fig. 1).

#### Plant growth promoting attributes and competitive root colonization

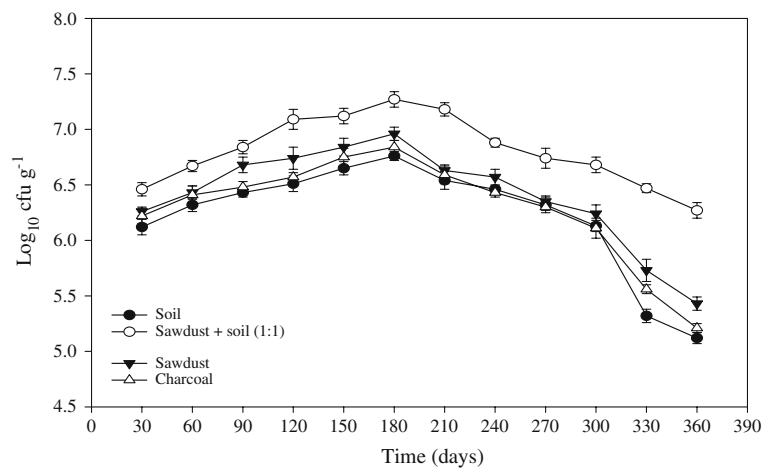
Re-isolated strain *P. fluorescens* PS1 exhibited plant growth-promoting (PGP) attributes such as production of siderophore and emission of volatiles (HCN). Siderophore production was evidenced by orange halos on CAS blue agar. HCN was observed as positive trait due to change in colour of filter paper from yellow to deep brown on incubation, as such there was no change in colour of filter paper when bacterial inoculum was not used on TSM plates (4.4 g/l). Surfactant-like activity was absent as no lytic zone was observed on blood agar plates. Degradation of oxalic acid was observed indicating the presence of oxalate oxidase (Table 2). This particular strain efficiently colonized the roots as

evident by high viable counts ( $10^9$ ) of *P. fluorescens* PS1 on antibiotic-amended plates. This was further confirmed by abundant growth of *P. fluorescens* PS1 colonies on and around the entire surface of emerging seeds of *B. campestris* as compared to controls and was reaffirmed by the turbidity observed with root exudates (Fig. 2). Utilization of root exudates suggested an inherent capability of the strain to aggressively colonize the mustard rhizosphere.

#### Identification of pathogen and antagonistic performance of *P. fluorescens* PS1 re-isolated from sawdust-soil carrier

The pathogen was identified in the laboratory by observation of the ascoma which is apothecioid in shape and yellow-brown to tan in colour. Sclerotia formed by this fungus are large, irregular in shape, and dark-brown to black in color. This fungus can be partially identified on plant material by the production of a white fluffy mycelium. *P. fluorescens* PS1 strongly inhibited the mycelial growth of *S. sclerotiorum* in vitro (Fig. 3). SEM images from zone of interaction showed loss of integrity, hyphal perforation, fragmentation, and degradation of mycelia

**Fig. 1** Shelf life of biocontrol strain *P. fluorescens* PS1 in different carriers stored at room temperature. Bar represents standard error of mean



**Table 2** Comparative assessment of antagonistic ability of biocontrol agent *P. fluorescens* PS1

Characteristics	<i>P. fluorescens</i> PS1
Antagonism (%)	72 ( $\pm 0.23$ )
Growth on CMM (CZ/CS ratio)	+ (2.01)
Chitinase activity (units/ml)	0.87
Growth on CMM <sup>a</sup>	+
Chitinase activity <sup>a</sup> (units/ml)	0.69
Growth on MM with laminarin	+
$\beta$ -1, 3-glucanase activity (units/ml)	2.95
Growth on MM <sup>b</sup>	+
$\beta$ -1, 3-glucanase activity <sup>b</sup> (units/ml)	1.98
Siderophore ( $\mu$ g/ml) (OD at 525 nm)	31.6
HCN (OD at 625 nm)	0.09
Surfactant	–
Oxalate oxidase	+
Volatile organic compounds (VOC)	–
Cellulase	$\pm$

<sup>a</sup> Chitin replaced by fungal biomass

<sup>b</sup> Laminarin replaced by fungal biomass

All the PGP activities were performed thrice as independent experiments and values represents an average of three replicates in each independent trial. + Positive, – Negative,  $\pm$  Weak

(Fig. 4a–e). In vitro inhibition of the pathogen was linked to PS1 mediated extracellular enzymes chitinase and  $\beta$ -1,3-glucanase. PS1 formed clear zones (halos) around its spots on CMM as well as on modified CMM having fungal biomass as source of chitin. The CZ/CS ratio of PS1 was 2.013 with an enzymatic activity of 0.87 units/ml while it was 0.65 units/ml, when the strain was re-isolated from soil-sawdust bioformulation after a year of storage. Extracellular cell wall degrading enzyme activity of PS1 was further evidenced by production of  $\beta$ -1,3-glucanase,  $\beta$ -1,4-glucanase and utilization of fungal biomass as source of carbon to produce these enzymes. Cellulase activity of PS1 was low and did not form a large halo indicative of its production in vitro (Table 2).

#### Pot assay

In short-term pot trials, percentage seed germination was significantly increased in all the treatments. After 15 days of sowing, mustard seeds bacterized with *P. fluorescens* PS1 (sawdust-soil, w/w: 1/1) sown in *S.*

*sclerotiorum* infested soil (treatment 3) showed early seed germination which confirmed the nature of *P. fluorescens* PS1 as a good bio-priming agent. As shown in Table 3, the significant increase in seed germination was 87% (treatment 3) while it was 82% in non-infested soil (treatment 2) as compared to that of control (non-infested and/or non-bacterized) (treatment 4). At 90 DAS, a drastic decline in incidence of stem blight was observed in treatment 3 as compared to non-bacterized seeds (treatment 1) raised in fungal infested soil. At an early stage, plants raised in *S. sclerotiorum* infested soil showed clear stem blight symptoms. In such case, mycelial growth and sclerotia were clearly visible in the cortical region of the root and the collar region of infected plants; however, their apparent density was quite low. Diseased plants showed the blackening of stem at 90 DAS. Seed germination increased by 15% and 23% over the control in treatments 2 and 3 while it fell by 42% in the case of treatment 1, in which the pathogen was used alone (Table 3).

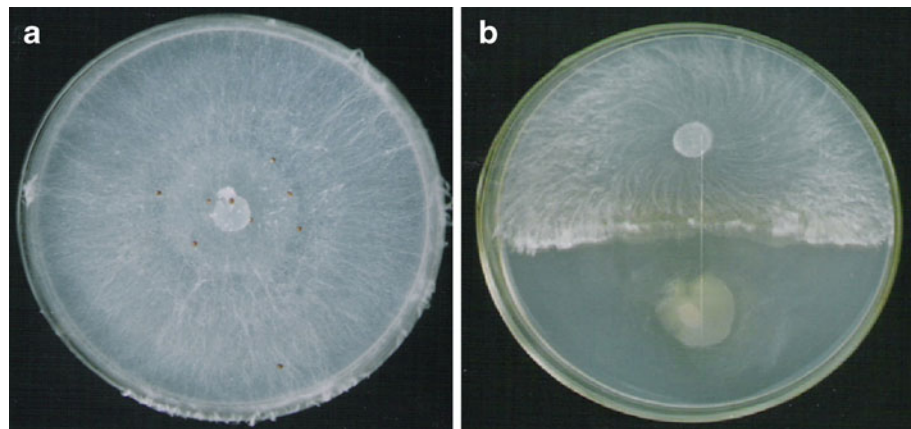
#### Field trials

In field trials, vegetative parameters such as seedling fresh weight, dry weight, shoot length and root length improved with application of *P. fluorescens* PS1 in treatment 3 (Tables 3, 4). Subsequently, increased seedling growth and the reproductive parameters number of siliqua and grain yield were recorded until 120 DAS. Number of siliqua per plant and grain yield per plant increased by 74% and 67% and 71% and



**Fig. 2** Abundant growth of *P. fluorescens* PS1 colonies around entire surface of emerging seeds of *B. campestris*

**Fig. 3** In vitro antagonistic activity of *Pseudomonas fluorescens* PS1 against *Sclerotinia sclerotiorum* in dual culture. **a** *S. sclerotiorum* alone **b** *P. fluorescens* PS1 and *S. sclerotiorum* interaction



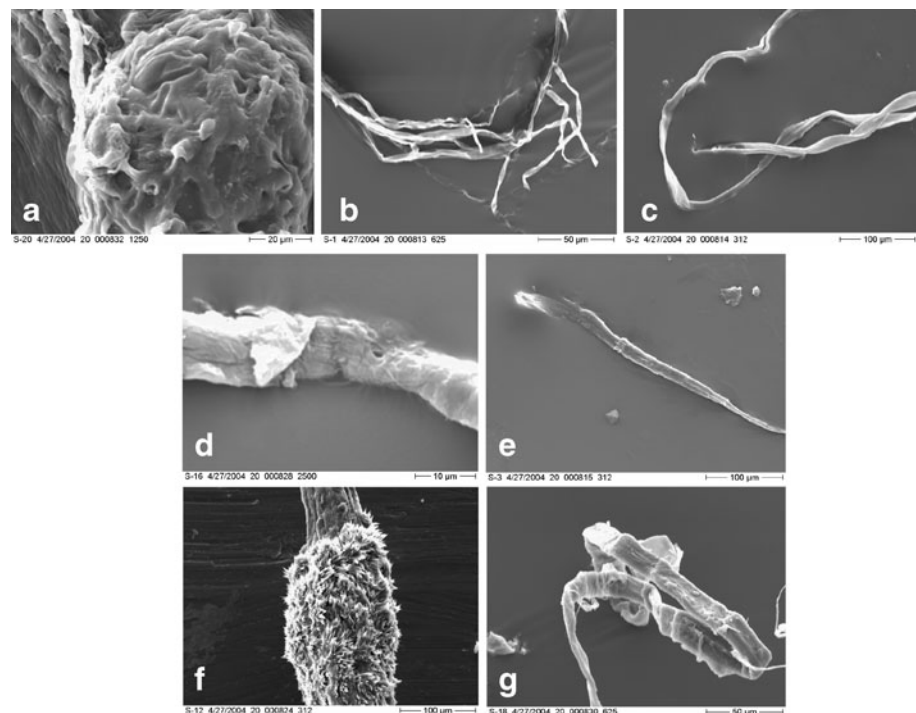
89% in treatment 3 and treatment 2, respectively as compared to the control (treatment 4). Grain yield per hectare was 72.54 and 84.2% in treatment 3 and treatment 2 respectively as compared to that of the control (treatment 4). All results were significant at 1% (LSD) (Tables 2 & 3).

#### Root colonization

The establishment of the antagonistic strain *P. fluorescens* PS1 under different sets of conditions from the

rhizosphere showed efficient and aggressive root colonization of 5.24 and 5.18 log CFU (Table 5) in non-infested and infested soils, respectively at 30 DAS. *P. fluorescens* PS1 maintained its populations in rhizosphere of *B. campestris* even after 120 DAS, 5.72 and 5.64 log CFU in non-infested and infested soils respectively. A positive correlation between root colonization and grain yield was observed up to 120 DAS. Root colonization increased up to 120 DAS and yield obtained was higher in the first year (876.3 kg/ha) compared to the subsequent year (721.2 kg/ha)

**Fig. 4** Scanning electron photomicrographs (SEM) showing deformities in fungal mycelia during interaction between *Sclerotinia sclerotiorum* and *Pseudomonas fluorescens* PS1. **a** Sclerotial degradation, **b** Twisting and coiling of mycelium, **c** Twisting and coiling of mycelium, **d** Pore formation in mycelium wall, **e** Breakage of Mycelium, **f** Unaffected sclerotia, **g** Unaffected hyphae





**Table 3** Effect of sawdust-soil (1:1) bioformulation of *P. fluorescens* PS1 on plant growth and yield parameters of *B. campestris*

Treatments	Seed germination (%) <sup>a</sup>	Stem blight (%) <sup>b</sup>	Shoot length (cm)	Root length (cm)	Fresh shoot weight (g)	Fresh root weight (g)	Siliqua Plant <sup>-1</sup>	Grain yield plant <sup>-1</sup> (g)	Grain yield hectare <sup>-1</sup> (Kg)
<i>S. sclerotiorum</i>	41.1	92.3	61.2	7.4	4.2	1.7	51.2	4.1	128.3
Sawdust-soil + PS1	82 <sup>c</sup>	1.2	144.6 <sup>c</sup>	19.2 <sup>c</sup>	45.1 <sup>c</sup>	11.3 <sup>c</sup>	191.3	22.3 <sup>c</sup>	924.2 <sup>c</sup>
<i>S. sclerotiorum</i> + sawdust-soil + PS1	87 <sup>c</sup>	2.6	143.7 <sup>c</sup>	19.1 <sup>c</sup>	49.2 <sup>c</sup>	9.9 <sup>c</sup>	198.6	18.2 <sup>c</sup>	986.6 <sup>c</sup>
Control	71	20.5	110.7	12.4	19.2	3.1	113.9	10.6	535.6
±SEM	0.41	–	7.69	3.4	26.3	2.4	1.7	8.9	26.7
CD at 1%	1.27	–	37.2	6.3	12.4	4.1	28.7	5.4	125.6

Data average of two trials. Values are mean of three replicates. Analysis of variance (ANOVA) with two factors (variety x treatment)

<sup>a</sup> after 15 days sowing

<sup>b</sup> disease incidence after 90 days sowing

<sup>c</sup> significant at  $P>0.01$  level of ANOVA, other values significant at  $P>0.05$  level of ANOVA; SEM standard error of mean; CD critical difference

(Table 4). A higher yield in the first year is indicative of a more vigorous plant growth due to aggressive root colonization by *P. fluorescens* PS1. There was an increase in grain yield as root length increased by 54.83% (2007) and 32.03% (2008) over the respective controls, matching the increase in values of root colonization. With the increase in root colonization in *B. campestris* from 6.2 to 6.5 log CFU/g, an increase in grain yield from 825 to 875/ha was achieved in 2007, higher than the corresponding values of 5.7–6.2 log CFU/g and 725–750 grain yield/ha in 2008. Meanwhile in both trials, the population of indigenous bacteria increased linearly but in coexistence with the introduced strain *P. fluorescens* PS1, which was able to survive aggressively in the ecological niche comprising the root zone of *B. campestris*. A relationship between

grain yield ha<sup>-1</sup> and root colonization of *P. fluorescens* PS1 with sawdust-soil (1:1) bioformulation in the rhizosphere of *B. campestris* was observed in 2007 ( $y = -29.3 + 0.0406x$ ,  $r^2 = 0.805$ ) and 2008 ( $y = -77.0 + 0.115x$ ,  $r^2 = 0.707$ ), confirming that the benefits of seed priming bioagents were carried over to final yield.

## Discussion

Solid-based carriers provide the microenvironment and protect the transported microorganism from hostile conditions and maintain strains over an acceptable time period generally difficult to obtain (Smith 1992). Sometimes, carrier-based inoculants do

**Table 4** Comparative assessment of *P. fluorescens* PS1 (sawdust-soil (1:1) bioformulation) on growth and yield parameters of *B. campestris* under two field trials

Vegetative parameters	Year-2007					Year-2008				
	Sawdust-soil PS1	Control	SEM	CD1%	CD 5%	Sawdust-soil PS1	Control	SEM	CD 1%	CD 5%
Seed germination (%)	88 <sup>a</sup>	71	0.2	3.0	1.3	82 <sup>a</sup>	60	1.24	16.0	7.3
Seedling fresh weight (g)	49.2 <sup>a</sup>	19.2	0.8	11.4	5.2	42.7 <sup>a</sup>	14.6	0.40	5.2	2.4
Seedling dry weight (g)	26	14	0.3	7.3	1.5	21.5	12	0.5	6.8	3.1
Shoot length (cm)	144.6 <sup>a</sup>	86.4	0.4	6.3	2.9	128.3 <sup>a</sup>	63.7	0.3	4.1	1.9
Root length (cm)	19.2	12.4	0.2	3.5	1.6	13.6	10.3	0.3	5.0	2.3
Grain yield hectare <sup>-1</sup> (Kg)	876.3 <sup>a</sup>	510.4	0.6	38.7	14.0	721.2 <sup>a</sup>	496.7	4.1	52.7	24.2

Values are mean of ten randomly selected plant

<sup>a</sup> significant at  $P>0.01$  level of ANOVA, other values significant at  $P>0.05$  level of ANOVA; SEM standard error of mean; CD critical difference

**Table 5** Population densities of *P. fluorescens* PS1 and aerobic bacteria in the rhizosphere of *B. campestris* at different intervals

Treatments	30 DAS		60 DAS		90 DAS		120 DAS	
	PS1	AB	PS1	AB	PS1	AB	PS1	AB
Sawdust – soil + PS1	5.24±0.10	5.44±0.18	5.47±0.11	5.57±0.12	5.64±0.10	5.71±0.14	5.72±0.17	5.83±0.12
Sawdust– soil + PS1 + <i>S. sclerotiorum</i>	5.18±0.12	5.41±0.18	5.38±0.12	5.55±0.15	5.57±0.16	5.68±0.12	5.64±0.15	5.77±0.11

Data are from single representative experiment but samples were replicated three times

AB: Total viable population of aerobic bacteria

not impart the desirable benefits to the recommended crops in fields. Although, different countries including India have outlined certain norms for their recommendation as microbial bioinoculants, there are many limitations still to be addressed. Formulation plays a significant role in determining the efficacy of biological agents and carriers with pH close to neutral is one of the desirable properties among several others. Specific attempts have been made to develop antagonistic *P. fluorescens* strains for the management of soil-borne diseases, where a peat-based formulation was developed using fluorescent pseudomonads to control seedling disease in cotton (Hagedorn et al. 1993). In fact, formulations that transfer the growth promoting activities of a strain from laboratory to field would have a major impact in agriculture. Vidhyasekaran et al. (1997) used powder formulations of *P. fluorescens* to control pigeonpea wilt effectively. Talc formulations of *P. fluorescens* were effective even after 6 months of storage, while peat formulations were effective up to 60 days of storage (Vidhyasekaran et al. 1997). However, in the present study, the stability of PS1 was established even after 360 days of storage. Reed and Glick (2005) observed increased growth in canola by the application of ACC deaminase producing strains of *P. asplenii* immobilized in alginate beads.

Presence of the desirable pH and other properties in soil, sawdust and charcoal proved to be suitable solid carriers for rhizobia and *Pseudomonas* (Arora et al. 2008b). Materials having high water holding capacity and good aeration have been considered as good carriers for bio-inoculants (Brockwell and Bottomley 1996). Among all carriers, the blended sawdust-soil gave the most promising results on residual cell viability, similar to that recommended for free living soil bacteria by the Bureau of Indian Standards: Specification for Inoculants (2000), and

proved the most suitable to be used as carrier material for *P. fluorescens* PS1. Soil and charcoal based carriers are, however, established and laterly have been used widely for rhizobial inoculant purposes. But in case of *P. fluorescens* PS1, these materials proved to be inferior carrier materials. A drastic reduction in cell populations of *P. fluorescens* PS1 was observed in soil, sawdust and charcoal. In charcoal, bacterial population remained at  $10^5$  CFU/g with prolonged incubation, which indicated that charcoal is an unsuitable carrier as the residual cell population was several times less than the recommended population ( $10^7$  CFU/g) as per Bureau of Indian Standard (BIS) norms (2002). Although sawdust was found to be an average carrier material, the viability of *P. fluorescens* PS1 initially decreased; however it was comparable to that of blended sawdust-soil after 30 days of further incubation. Sawdust-soil (w/w: 1/1) was the only carrier giving viable counts within the permissible limits. The cell population reached its maximum up to 6 month by  $\sim 10^9$  CFU/g and showed greater dissimilarity with other low-grade quality carriers. After 12 months, PS1 in sawdust-soil showed a reduction in CFU/g but appeared within the permissible limits.

All the carriers used were sterile because such forms maintain high populations and are known to have higher shelf-life as suggested by Roughley and Vincent (1967). The use of sterile carriers significantly reduced the threat to the quality of bio-inoculants arising from the presence of contaminants and other autochthonous microorganisms. However, a major obstacle is the involvement of high cost production that hinders their further development as commercial inoculants (Stephens and Rask 2000). Storage of the carrier materials was at room temperature and their effectiveness in terms of cell viability assessed; hence inoculants such as blended sawdust-soil, that are not

required to be kept at low temperature, have an added advantage in terms of cost and energy savings.

The sclerotial fungus *S. sclerotiorum*, survives in soil for a long time. Hence, the ability of *P. fluorescens* PS1 to inhibit the growth and development of *S. sclerotiorum* in vitro and under field conditions appeared to be an important attribute for biological control. Several *P. fluorescens* strains have been reported to control soil-borne diseases but field results have been inconsistent. Pseudomonads have exhibited a destructive effect on phytopathogens as evident by abnormal morphological structures, suppressed mycelial growth along with suppressed and arrested sclerotial development (Arora et al. 2008a; Gupta et al. 2006). Although no sign of *P. fluorescens* PS1 attachment to mycelia was observed in SEM studies, it restricted mycelial growth and caused sclerotial deformities due to direct involvement of extracellular antifungal compounds during antagonism. Degradation of fungal cell wall by lytic enzymes such as chitinase and  $\beta$ -1,3-glucanase has a major role to play in suppressing the growth of *S. sclerotiorum* but the possibility of involvement of other metabolites such as HCN, siderophores and oxalate oxidase can not be ruled out. Oxalate oxidase converts oxalic acid to carbon dioxide and hydrogen peroxide, helps in structural re-inforcement of plant cell walls leading to cell wall thickening and arresting the penetration of pathogen (Nagarajkumar et al. 2005). Degradation of oxalate by PS1 is an important attribute since necrotrophic pathogens like *S. sclerotiorum* produces oxalic acid, which is in turn instrumental in the infection process acting as a virulence factor (Schoonbeek et al. 2007). In the present study, growth inhibition of *S. sclerotiorum* may be attributed to the cumulative effect of all extracellular antifungal compounds including siderophores, HCN, chitinase,  $\beta$ -1,3-glucanase, and oxalate oxidase.

Enhancement in yield of several crops due to involvement of indirect biocontrol activities of fluorescent pseudomonads is well documented (Levenfors et al. 2008; Kumar et al. 2009). Several non-fluorescent and fluorescent strains have been found to increase yield in crops such as potato (Frommel et al. 1991), winter wheat (De frietas and Germida 1990), spring wheat (Kropp et al. 1996) and cereals (Seong et al. 1992; Validov et al. 2009). In the present study, application of *P. fluorescens* PS1 via a sawdust-soil-based bioformulation resulted in increased root-

length, shoot length, grain yield/plant, grain yield/ha, and these results are in strong agreement with previous findings of other researchers (Lifshitz et al. 1987; Kloepper et al. 1988; Bertrand et al. 2001). Further work is required to access the possibility of commercialization of this formulation, and application at large scale in *S. sclerotiorum* infested soil.

*P. fluorescens* PS1 colonized the rhizosphere successfully as evident by increased seedling emergence, vegetative plant growth parameters and protection against pathogen. Inadequate colonization by the introduced strain led to decreased biocontrol activity however reduction in disease can also be the outcome of altered microbe dynamics in the rhizosphere. Therefore, for any disease-suppressive mechanism to be effective, it is necessary that the biocontrol agent should be able to establish itself in the rhizosphere of host crop (Kloepper et al. 1988; Schippers et al. 1987). Improved capacity to compete for root exudates, synthesis of siderophore complexes, as also seen in our case, are in addition to antibiotic production, known mechanisms that help dominance in the rhizosphere (Gamliel and Katan 1992). The strain under consideration should be directly proportional to the population density in the ecological niche and co-exist with other indigenous aerobic culturable bacteria as also stated by Haas and Defago (2005).

In conclusion, a sawdust-soil combination of *P. fluorescens* PS1 inoculant efficiently controlled *S. sclerotiorum*, as well as promoted plant growth and yield, effectively increased seed bacterization and augmented root colonization in response to indirect plant growth promoting activities. Hence, the sawdust and soil based bioformulations of *P. fluorescens* PS1 could be good substitutes to traditional charcoal or peat based bio-inoculants and offer multifarious mechanisms for increased crop productivity.

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