Indian Journal of Experimental Biology
Vol. 64, March 2026, pp. 254-266
DOI: 10.56042/ijeb.v64i03.20467

Enhanced immune responses induced by endophytic bacterial strains in Indian mustard against stem rot disease

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Received 25 June 2025;revised4 December 2025

The growing dependence on chemical fungicide for managing plant diseases has raised serious concerns due to their harmful impacts on human health and environment, creating an urgent need to explore safe, eco-friendly biocontrol alternatives. The present study evaluated the potential of bacterial endophytes *viz., Pseudomonas aeruginosa* (Accession no. OL413676) and *Serratia proteamaculans* (Accession no. OP595540) applied individually and as a consortium for managing *Sclerotinia sclerotiorum*, causal agent of stem rot in mustard through field-based pot experiments. The study further explored the role of these bacterial endophytes in boosting the defense responses through the activation of defense-responsive enzymes and metabolites. The results revealed that the dual application (seed treatment and foliar spray) of the bacterial consortium (T12) significantly boosted the activity of antioxidative and lytic enzymes compared to the fungal pathogen-inoculated control plants (T2). While most of the defense enzymes showed maximum induction at 120 hours post-inoculation (hpi), phenylalanine ammonia lyase (PAL) displayed its highest activity at 72 hpi, suggesting its potential role in early initiation of plant's defense response. Consistent with the defense enzymes activation, the T12 treatment also resulted in the increased accumulation of total phenols and lignin, showing peak accumulation at 72hpi, with lignin levels remaining elevated thereafter, possibly serving to restrict pathogen colonization. The endophytic consortium reduced the lesion length by 21.47% in PBR 357 and 16.91% in RLC3, suggesting it as an effective biocontrol agent. The findings indicate that these endophytic bacteria have the potential to activate the basal defense responses in Indian mustard, making them promising candidates for the management of *Sclerotinia sclerotiorum*.

Keywords: antioxidative enzymes, consortium, lytic enzymes, *Sclerotinia sclerotiorum, Serratia proteamaculans*

Brassica juncea, commonly known as Indian mustard, stands out as the predominant rapeseed-mustard crop in India, encompassing more than 90% of the total acreage[1]. It holds substantial economic importance owing to its applications as vegetables, seasonings, livestock feed, and a source of edible oil. In India, this crop has been documented susceptible to various diseases, including Alternaria leaf blight, white rust, Sclerotinia stem rot, downy mildew, and powdery mildew. Of particular concern is *Sclerotinia sclerotiorum*, a necrotrophic fungal pathogen recognized for its devastating effects, leading to considerable yield losses. It is prevalent with an incidence rate of 50-80% in different states of India[2]. Effective management of this pathogen is challenging due to its high dispersal capacity over long distances.

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Numerous efforts have been made to suppress *Sclerotinia sclerotiorum*, including the use of organic soil additives, soil sterilization, tillage and crop rotation. However, these treatments often prove ineffective to provide comprehensive pathogen control. The development of genetically resistant cultivars stands out as an environmental friendly and economical disease control[3]. Currently, no commercially available crop varieties exhibit total resistance. Breeding for resistance against Sclerotinia stem rot is challenging due to the polygenic nature of pathogen[4]. The integration of cultural practices with chemical control strategies is crucial for its effective management. Among these approaches, the application of fungicides emerges as a key method in effectively mitigating stem rot in rapeseed-mustard. However, the extensive use of fungicides have raised serious concerns, particularly about its toxicity to non-target organisms and the negative environmental impacts[5]. Consequently, efforts are underway to

develop environmental friendly and effective biocontrol strategies for the management of this disease.

Endophytes are prevalent in nearly all plant species, residing asymptomatically within the plant tissues. Among them, certain bacteria play a crucial role in plant defense mechanisms against pathogenic infections by producing secondary metabolites and antibiotics, which helps in inhibiting the germination of fungal ascospores and sclerotia. These naturally existing organisms also strengthen plant resistance against insects, pests, and other environmental factors[6]. Notably, *Pseudomonas brassicacearum* DF41 and *P. chlororaphis* PA23 have exhibited the ability to suppress *Sclerotinia sclerotiorum* in canola. This suppression is highly attributed to the production of pyrrolnitrin, hydrogen cyanide (HCN) and lipopeptides[7,8]. Additionally, *P. chlororaphis* PA23 has shown antifungal activity against *Sclerotinia sclerotiorum* in lettuce, with HCN production affecting the pathogen's growth[9]. In a dual culture assay, *Bacillus safensis* (TS46 bac4) and *B. australimaris* (SM2) inhibited the growth of *Sclerotinia sclerotiorum* by 57.97% and 52.89%, respectively[10].

The plant's defense against the pathogen is driven by the accumulation of signalling chemicals, such as ethylene, salicylic acid, and jasmonic acid, whose expression and regulation shape the effectiveness of induced systemic resistance (ISR) and systemic acquired resistance (SAR)[11]. Plants harboring endophytes exhibit an enhanced response to pathogens, characterized by the overexpression of jasmonate and ethylene genes, which in turn activates biocontrol mechanisms[12]. Therefore, protection of plants through induced resistance has tremendous potential in biocontrol of wide range of pathogens. Endophytes initiate ISR, as evidenced by the activity of enzymes such as peroxidase (POD), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), chitinase, β-1,3-glucanase, as well as the accumulation of phenols and lignin[13]. Concerning this aspect, our investigation aimed to elucidate the roles of microbial agents in triggering the synthesis of defensive enzymes and non-enzymatic metabolites, aiming to alleviate the progression of pathogenic lesions in Brassica plants. We affirm that the findings of our study are applicable not only to stem rot in *B. juncea* but also to other crops belonging to the Brassicaceae family. This research could serve as a foundation for further characterization and exploration of the interplay between gene regulations across various pathways during ISR mediated by endophytes.

Materials and methods

Collection of biocontrol agents, fungal pathogen and plant material

Two endophytic bacterial strains namely *Pseudomonas aeruginosa* (Accession no. OL413676) and *Serratia proteamaculans* (Accession no. OP595540) were sourced from the Division of Microbiology, IARI, New Delhi and Department of Microbiology, Punjab Agricultural University, Ludhiana, respectively. Both the bacterial strains were previously identified for their antagonistic effects against a local isolate of *Sclerotinia sclerotiorum* and the endophytic nature of these strains was confirmed using Scanning Electron Microscope which revealed the bacterial colonisation within treated roots of mustard plant, characterised by distinct rod-shaped structures[14]. Seeds of *B. juncea* varieties namely, PBR 357 (non-canola) and RLC 3(canola), susceptible to *Sclerotinia sclerotiorum* were procured from Department of Plant Breeding and Genetics, PAU, Ludhiana.

Isolation and purification of *Sclerotinia sclerotiorum*

A local isolate of *Sclerotinia sclerotiorum* maintained as sclerotia in the Oilseeds section, Pathology Laboratory was used for the study. The sclerotium was surface sterilized in 6% sodium hypochlorite for 3min, followed by 70% ethanol for 1min, and then rinsed twice with sterile distilled water for 1min each[15]. Each sclerotium was cut into halves, and the exposed side was placed onto Petri plates containing freshly poured potato dextrose agar (PDA) [composed of 200 g/L potatoes, 20 g/L dextrose (glucose), and 15 g/L agar]. A pure hyphal-tip culture was subsequently obtained through sub-culturing. The resulting pure culture was used to inoculate susceptible *Brassica juncea* (var. PBR357) plants.

Preparation of bacterial inoculum

To prepare the bacterial inoculum, 500 µl of bacterial cultures, previously grown at 28°C in nutrient broth for 24 h, were transferred into 100 mL of fresh nutrient broth. The nutrient broth composition included glucose at 1g/L, peptone at 15g/L, sodium chloride at 6g/L, and yeast extract at 3g/L. Subsequently, the cultures were incubated at 28°C with shaking at 180 rpm for 48 h. After the incubation period, the cultures were used as inoculums, with

optical density at 600 nm adjusted to 1.0, equivalent to approximately 10^9 colony forming units (CFU) per mL.

Preparation of charcoal-based bioinoculant

Charcoal was sterilized after being subjected to autoclaving at 121°C for 1h at a pressure of 15 psi. Following this, a bacterial inoculum (10^9 CFU per mL)was mixed with the sterilized charcoal in a 1:2 ratio (40 mL of inoculum to 80 g of sterilized charcoal) so as to attain an approximate concentration of 10^8 CFU per g in the resulting charcoal-based bio-inoculant.This charcoal-based inoculum maintained a count of 10^7 CFU per g for three months. The bacterial count was enumerated by serially diluting 10 g charcoal-based inoculum in normal saline up to 10^{-7} dilutions and plating 0.1 mL of the dilutions on nutrient agar. The Petriplates were incubated at 28°C for 48 h and colonies were counted. The CFU per gram charcoal-based inoculum was calculated by using the formula: (Number of colonies × Dilution factor)/sample volume.

Application techniques for endophytic microbes

Seed treatment

The surface sterilization of *B. juncea* seeds involved immersing them in a 20% sodium hypochlorite solution for 3 min, followed by three rinses with sterile de-ionized water. Subsequently, the seeds were treated with slurry containing the charcoal-based inoculum at a concentration of 25 mg per g of seeds.

Foliar spray

A hand-operated spray pump was used to evenly spray 15–25 mL of inoculant (bacteria grown in nutrient broth for 48 h and diluted to attain concentration of 10^7 CFU/mL) per plant, ensuring the uniform moistening of the leaves, at the 50% flowering stage. Control plants received a similar volume of sterile normal saline water solution without bacterial inoculum through spraying. The foliar application of the bacterial endophyte inoculum was carried out 48 h before the inoculation (48 hbi) with the fungal pathogen *Sclerotinia sclerotiorum*.

Artificial inoculation of mustard plants with the pathogenic fungus culture

Inoculation was performed at the third internode using the "bit-wrap method" described by Buchwaldt *et al*[16], wherein a 5-mm mycelial disc from an actively growing PDA culture was wrapped around the stem using moist, sterile cotton and parafilm. The pathogen was re-isolated from the infected tissue to confirm pathogenicity. The verified culture was then used to artificially inoculate upto ten *B. juncea* plants (PBR 357 and RLC3) in each test line at the onset of flowering, specifically when 50% of the plants had at least one fully open flower. The inoculated plants received regular irrigation through sprinklers at specified intervals to ensure adequate humidity. The lesion length was recorded with a ruler at three weeks after inoculation.

Experimental design

In pot experiments conducted during 2020-21, the role of endophytic bacteria in plant defense was assessed using *B. juncea* varieties PBR 357 (non-canola) and RLC 3 (canola). Brassica seeds treated with charcoal- based inoculum were planted in earthen pots filled with non- sterilized soil and the pots were kept in the field. Each treatment consisted of 7 pots, with each pot containing three plants. The plants were divided into 12 groups and treated as shown in Table1.Biochemical analysis of defense related enzymes and non - enzymatic metabolites were performed in stem tissues harvested from each treatment at four distinct time points: 48 hours before

Table1 — Treatment details for evaluating the role of endophytic bacterial inoculation in *B. juncea* plants

T1	Naturally free from disease
T2	Stem inoculation with pathogenic fungus culture alone
T3	Positive control - Spray with fungicide (Carbendazim 0.1%) + pathogenic fungus inoculation after 24 h of fungicide spray
T4	Seed treatment with *Pseudomonas aeruginosa* + pathogenic fungus inoculation
T5	Seed treatment with *Serratia proteamaculans* + pathogenic fungus inoculation
T6	Seed treatment with endophyte consortium + pathogenic fungus inoculation
T7	Foliar spray with *P. aeruginosa* + pathogenic fungus inoculation
T8	Foliar spray with *S. proteamaculans* + pathogenic fungus inoculation
T9	Foliar spray with endophyte consortium + pathogenic fungus inoculation
T10	Seed treatment-cum-foliar spray with *P. aeruginosa* + pathogenic fungus inoculation
T11	Seed treatment-cum-foliar spray with *S. proteamaculans* + pathogenic fungus inoculation
T12	Seed treatment-cum-foliar spray with endophyte consortium + pathogenic fungus inoculation

inoculation (hbi), as well as 72, 120 and 168 hours post inoculation (hpi).

Biochemical analysis of defense related enzymes

Extraction of Peroxidase (POD), Polyphenol oxidase (PPO) and Phenylalanine ammonia lyase (PAL)

0.2 g of stem tissue was ground in 2 mL of 0.1 M sodium phosphate buffer (pH of 7.5) and centrifuged at 10,000 rpm for 30 min at 4°C. The resulting supernatant was used for testing the activity of POD, PPO and PAL.

Peroxidase (POD) (EC 1.11.1.7)

POD activity was assessed using spectrophotometer based method outlined by Shannon *et al*.[17] About 3 mL of 0.05M guaiacol dissolved in 0.1M sodium phosphate buffer (pH 6.5) was combined with 100 µL enzyme extract in a cuvette. Subsequently, 0.1 mL of freshly prepared 0.8M H_2O_2 was added to the cuvette. The reaction mixture, excluding H_2O_2, was used as a blank reference. Changes in absorbance rate were monitored at 470 nm at 30 s intervals for 3 min. POD activity was measured and expressed as $\Delta A\ min^{-1}g^{-1}$ FW (ΔA= change in absorbance)

Polyphenol oxidase (PPO)(EC 1.10.3.1)

PPO activity was evaluated following the protocol outlined by Zauberman *et al*.[18]. The reaction mixture was prepared by adding 1mL of 0.1M sodium phosphate buffer (pH 6.8), 0.5mL of 100 mM 4 methyl catechol and 0.5mL enzyme extract to a cuvette. Enzyme activity was assessed by measuring the rate of change in absorbance at 410 nm at 30 s interval for 3 min. PPO activity was expressed in $\Delta A\ min^{-1}g^{-1}$FW (ΔA= change in absorbance)

Phenylalanine Ammonia Lyase (PAL) (EC 4.1.3.5)

PAL activity in stems of Brassica plants was determined according to the method of Burrell and Rees[19]. Briefly, the reaction containing 300 µL of crude enzyme extract and 2.7 mL of 0.03 M phenylalanine prepared in 0.05 M sodium borate buffer (pH 8.8) was incubated at 37°C for 1 h. Subsequently, the reaction was stopped by adding 0.3 mL of 1 N HCl. Control samples were prepared without incubation for each sample. The samples were assessed for absorbance at a 290 nm wavelength. PAL activity was then quantified and reported as µg of t-cinnamic acid formed $h^{-1}g^{-1}$ FW.

Extraction of chitinases and glucanases

Sample weighing 0.2 g was homogenized in a 2 mL of 0.1M sodium acetate buffer (pH 4.8). Following centrifugation at 10,000 rpm for 15 min at 4°C, the resulting supernatant was employed for chitinase and β-1, 3 glucanase assay.

Chitinase

Chitinase activity (EC 3.2.1.14) was determined by following the method described by Boller and Mauch[20]. The enzyme extract (200 µL) was combined with 200 µL of chitin (0.5%) in 0.1 mL of 0.1 M sodium acetate buffer (pH 4.8) to create the reaction mixture. This mixture underwent incubation in a water bath at 37°C for 1 h. The reaction was terminated by heating the mixture to 100°C for 5 min and adding 0.1 mL of potassium tetraborate. Subsequently, 3 mL of diluted dimethyl amino benzaldehyde (DMAB) reagent was added, and the mixture was incubated at 37°C for 20 min. The absorbance of all samples was measured at 544 nm wavelength. Chitinase activity was determined and expressed as µ moles NAG released $min^{-1}g^{-1}$FW.

β- 1,3 glucanases (EC 3.2.1.6)

β-1,3 glucanases was assayed according to the protocol outlined by Kauffmann *et al*.[21] Briefly, the reaction was prepared by combining 500 µL of 4% laminarin solution with 500 µL of the enzyme extract, and this mixture was then incubated at a temperature of 40°C for 1 h. The reaction was terminated by adding 375 µL of dintrosalicylic acid reagent (DNSA) and subsequently heating the mixture in a boiling water bath for 5-15 min. The resulting solution exhibited a red-brown color, which was stabilized by the addition of 1 mL of 40% potassium sodium tartarate and subsequent vortexing. The measurement of the absorbance of released glucose was performed at a wavelength of 575 nm. Enzyme activity was quantified and expressed as µ moles glucose released $min^{-1}g^{-1}$FW.

Biochemical analysis of non-enzymatic metabolites

Total phenols

Stem tissues harvested from different treatments were quantified following Swain and Hillis[22] method. Initially, 0.5 g of the tissue was homogenized in 10 mL of 80% methanol and heated at 80°C for 2 h. The resulting mixture was filtered, adjusted to 10 mL with 80% methanol, and used for phenolic compound determination. In a test tube, 0.5 mL of the extract was dried, re-dissolved in 6.5 mL of distilled water, and mixed with 0.5 mL of freshly prepared

Folin-Ciocalteau reagent and 1 mL of sodium carbonate solution. After 1h incubation in darkness, absorbance was measured at 760 nm, with a blank reference. The phenol content was quantified and expressed in mg g^{-1}DW.

Lignin content

Lignin content was determined following the protocol outlined by Lee *et al.*[23]. Initially, the lyophilized stem sample was ground into a fine powder. Approximately 20 mg of the finely crushed dried stem tissue was then homogenized in 2 mL of 95% ethanol. This resulting extract underwent centrifugation at 10,000 rpm for 30 min. The resulting pellet was subjected to three washes with 95% ethanol followed by two washes with a mixture of ethanol and hexane in a 1:2 ratio. Subsequently, the pellet was dried overnight at 45°C.The dried pellet was then washed once with 0.5 mL of acetyl bromide in acetic acid (in a 1:3 ratio). Following this, 0.5 mL of acetyl bromide in acetic acid (1:3 ratio) was added to the pellet, and the mixture was incubated at 70°C for 30 min. After incubation, the reaction mixture was allowed to cool to room temperature (22 ± 2°C). In a Falcon tube, 0.1 mL of the resulting extract was combined with 0.18 mL of 2M NaOH and 0.2 mL of 7.5M hydroxylamine-HCl. The volume was adjusted to 10 mL with acetic acid, and the mixture under went centrifugation at 10,000 rpm for 20 min. The absorbance of the supernatant was measured at 280 nm, using acetic acid as a blank reference. The lignin content was quantified and expressed as mg g^{-1}DW.

Statistical Analysis

The experiment was conducted in triplicates and data was analyzed statistically using SPSS 22.0 software on the basis of mean values with One Factor Analysis and Tukey's Test performed at $P<0.05$.Spearman's rank correlation coefficients were calculated to assess the relationship between lesion length and the measured biochemical parameters using OPSTAT statistical software (CCS HAU, Hisar, India). Statistical significance was evaluated at P value ≤ 0.05.

Results

Endophyte-mediated activation of antioxidative enzymes

The application of bacterial endophytes both *via* seed treatment and foliar spray, on mustard plants infected with *Sclerotinia sclerotiorum* led to a significant increase in the activity of antioxidative enzymes across different time intervals (48 hbi, 72, 120 and 168 hpi) in comparison to the plants solely inoculated with the fungal pathogen *Sclerotinia sclerotiorum* (T2) (Fig. 1). Three control groups were included: one group consisted of plants subjected solely to stem inoculation with the fungus culture (T2);second group received no treatment and was naturally free from disease (T1) while in the third group, plants were sprayed with fungicide (T3).

Peroxidase (POD)

A progressive rise in the POD activity was observed in the stems of both mustard varieties, PBR 357 and RLC3, upto 120 hpi, followed by a subsequent decline as depicted in (Fig.1A). The maximum POD activity was recorded in treatments T12 (seed treatment and foliar spray with a bacterial consortium) and T10 (seed treatment and foliar spray with *P. aeruginosa*), exhibiting 2.11 and 2.05 fold increase over the control (T2) in PBR 357. In case of canola-quality mustard variety RLC3, significant enhancements of 1.84, 1.82, and 1.74 fold were recorded with T10 (combined seed treatment and foliar spray with *P. aeruginosa*), T9 (foliar spray alone with the endophytic consortium) and T12 (seed treatment and foliar spray with a bacterial consortium) respectively in comparison to plants solely inoculated with the fungal pathogen *Sclerotinia sclerotiorum* (T2).

Polyphenol oxidase (PPO)

Similar to the pattern observed in POD activity, there was a gradual increase in PPO activity in the stem of both mustard varieties, PBR 357 and RLC3, from 48 hbi to 120 hpi, followed by a decline thereafter (Fig.1B). Notably, PPO activity was significantly higher in PBR 357 when treated with T11 (seed treatment and foliar spray with *S. proteamaculans*), T12 (seed treatment and foliar spray with the endophytic consortium) and T10 (combined seed treatment and foliar spray with *P. aeruginosa*), resulting in an increase of 2.16, 1.86 and 1.73 fold, respectively over the control (T2). RLC3 demonstrated the highest fold change of 1.78, 1.76, 1.35 and 1.34 in PPO activity with T11 (seed treatment and foliar spray with *S. proteamaculans*), T10 (seed treatment and foliar spray with *P. aeruginosa*), T9 (foliar spray alone with the endophytic consortium) and T12 (seed treatment and foliar spray with a bacterial consortium) respectively,

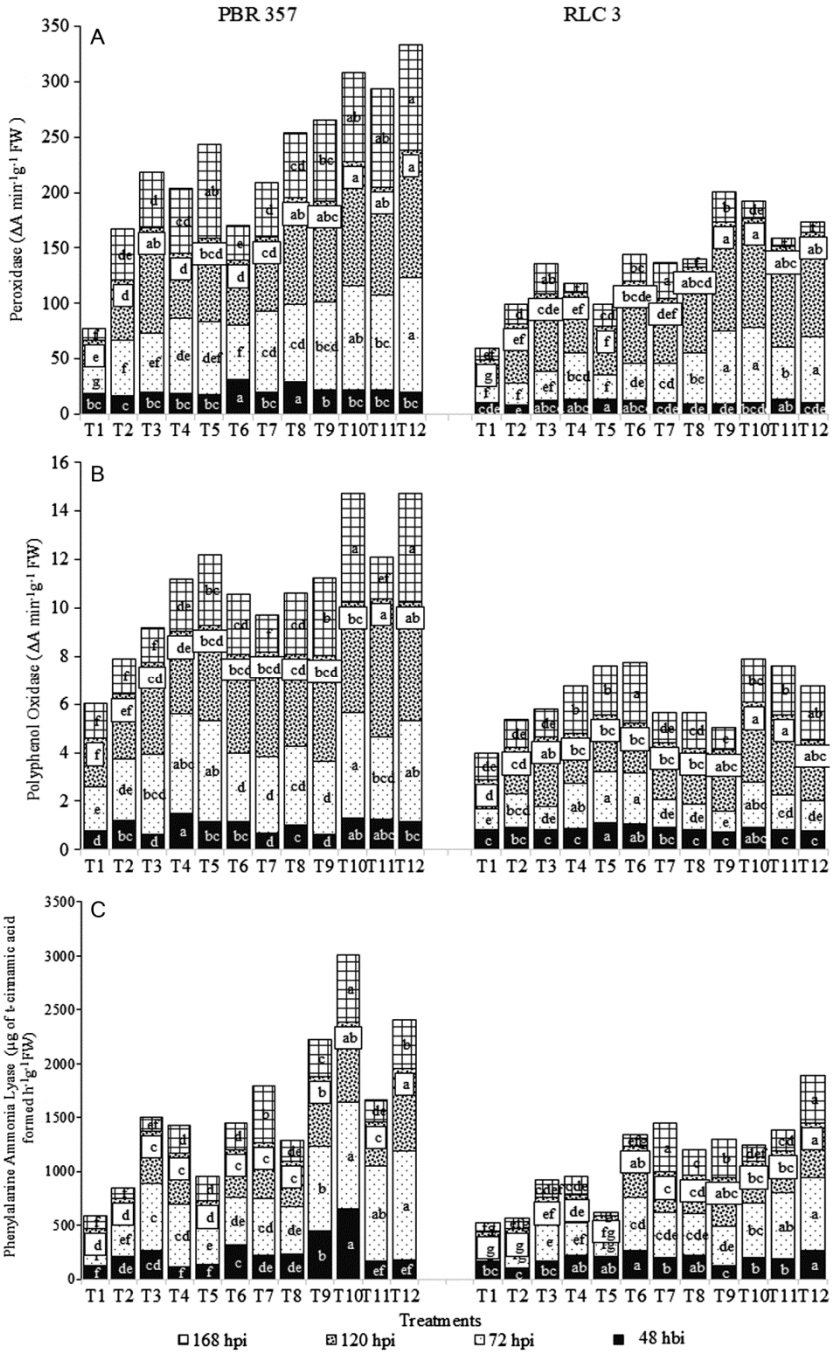

Fig. 1 — Effect of endophytes on induction of defense-related antioxidative enzymes: Peroxidase (A), Polyphenol oxidase (B), Phenylalanine ammonia lyase (C), in *Brassica juncea* varieties (PBR 357 and RLC3) following challenge with *Sclerotinia sclerotiorum.* T1- Naturally free from disease, T2-Stem inoculation with pathogenic fungus culture alone,T3-Spray with fungicide (Carbendazim)+ pathogenic fungus inoculation after 24 h of fungicide spray, T4-Seed treatment with *Pseudomonas aeruginosa* + pathogenic fungus inoculation, T5-Seed treatment with *Serratia proteamaculans*+ pathogenic fungus inoculation, T6-Seed treatment with endophyte consortium+ pathogenic fungus inoculation, T7-Foliar spray with *P. aeruginosa* + pathogenic fungus inoculation,T8-Foliar spray with *S. proteamaculans*+ pathogenic fungus inoculation, T9-Foliar spray with endophyte consortium+ pathogenic fungus inoculation, T10-Seed treatment cum Foliar Spray with *P. aeruginosa* + pathogenic fungus inoculation, T11-Seed treatment-cum-foliar Spray with *S. proteamaculans*+ pathogenic fungus inoculation, T12- Seed treatment-cum-Foliar Spray with endophyte consortium + pathogenic fungus inoculation. Data represent mean ± SE of three replicates. Different letters indicate significant difference between treatments at respective time intervals of 48 hbi, 72, 120, and 168 hpi at $P< 0.05$ as determined by Tukey's HSD test.

in comparison to plants solely inoculated with fungal pathogen (T2). Despite T11 being identified as the most effective treatment for both mustard varieties, significant decrease in its activity was recorded at 168hpi in PBR357.

Phenylalanine ammonia lyase (PAL)

Brassica plants treated with microbial agents exhibited a notable increase in PAL activity in stems challenged with pathogen as compared to control (T2). Generally, the peak in PAL activity was observed at 72 hpi in both mustard varieties (Fig. 1C). Compared to plants solely inoculated with fungal pathogen (T2), those treated with T12 (seed treatment and foliar spray with a consortium) and T10 (seed treatment and foliar spray with *P. aeruginosa*) exhibited the highest PAL activity, with respective fold increase of 3.24 and 3.16 in PBR 357. Likewise, application of T12 (seed treatment and foliar spray with consortium) and subsequently T11 (seed treatment and foliar spray with *S. proteamaculans*) led to a significant elevation in PAL activity on canola quality mustard var. RLC 3, surpassing the control (T2) by 2.82 and 2.57 fold, respectively.

These promising treatments also outperformed the positive control (T3) (Spray with fungicide) in terms of antioxidant enzyme activities. Overall, PBR 357 exhibited a higher fold change compared to RLC 3.

Endophyte-mediated activation of lytic enzymes

The bacterial endophytes, introduced through both seed treatment and foliar spray resulted in a significant enhancement in lytic enzymes over different time points (48 hbi, 72, 120, and 168 hpi) compared to plants exclusively exposed to the *Sclerotinia sclerotiorum* fungal pathogen (T2) (Fig.2).

Chitinase

Both the mustard varieties showed a gradual surge in chitinase activity up to 120 hpi and later declined as illustrated in (Fig.2A). It was evident that among the array of endophytic treatments examined, T12 (seed treatment and foliar spray with consortium) and T10 (seed treatment and foliar spray with *P. aeruginosa*) showed a significant increase in chitinase activity. Specifically, these treatments demonstrated enhancements of 2.03 and 1.92 fold, respectively, compared to the control (T2) in PBR 357. Similarly, T12 (seed treatment and foliar spray with an endophytic consortium) along with T9 (foliar spray with a bacterial endophytic consortium) were identified as the most effective, exhibiting the highest chitinase activity with respective fold increase of 1.88 and 1.84 in canola qualitymustard variety RLC 3 compared to plants solely inoculated with fungus (T2).

β-1, 3-glucanase

β-1, 3-glucanase activity was in synchronization with chitinase activity, with both PBR357 and RLC 3

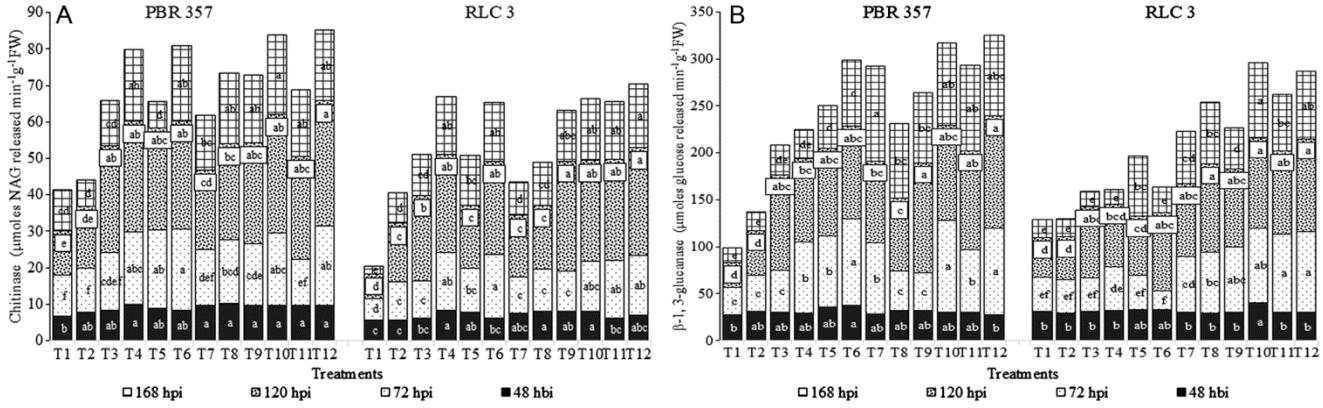

Fig. 2 — Effect of endophytes on induction of defense-related lytic enzymes: chitinase (A) and β-1,3-glucanase (B)in *Brassica juncea* varieties (PBR 357 and RLC3) following challenge with *Sclerotinia sclerotiorum.* T1- Naturally free from disease, T2-Stem inoculation with pathogenic fungus culture alone,T3-Spray with fungicide (Carbendazim)+ pathogenic fungus inoculation after 24 h of fungicide spray, T4-Seed treatment with *Pseudomonas aeruginosa* + pathogenic fungus inoculation, T5-Seed treatment with *Serratia proteamaculans*+ pathogenic fungus inoculation, T6-Seed treatment with endophyte consortium+ pathogenic fungus inoculation, T7-Foliar spray with *P. aeruginosa* + pathogenic fungus inoculation, T8-Foliar spray with *S. proteamaculans*+ pathogenic fungus inoculation, T9-Foliar spray with endophyte consortium+ pathogenic fungus inoculation, T10-Seed treatment cum Foliar Spray with *P. aeruginosa* + pathogenic fungus inoculation, T11-Seed treatment-cum-foliar Spray with *S. proteamaculans*+ pathogenic fungus inoculation, T12- Seed treatment-cum-Foliar Spray with endophyte consortium + pathogenic fungus inoculation. Data represent mean ± SE of three replicates. Different letters indicate significant difference between treatments at respective time intervals of 48 hbi, 72, 120, and 168 hpi at $P< 0.05$ as determined by Tukey's HSD test.

mustard varieties reaching peak enzyme activity at 120 hpi (Fig. 2B). Notable enhancement was observed in PBR 357 with T12 (seed treatment and foliar spray with an endophytic consortium) and T9 (foliar spray with a bacterial endophytic consortium), showing fold increase of 2.52 and 2.47, respectively, compared to the control (T2). Likewise, canola quality mustard variety RLC 3 exhibited 2.11, 2.07 and 2.07 fold higher β-1,3-glucanase activity with T12 (seed treatment and foliar spray with an endophytic consortium), T10 (seed treatment and foliar spray with *P. aeruginosa*) and T8 (Foliar spray with *S. proteamaculans* + pathogenic fungus inoculation) in comparison to control group (T2). The promising treatments demonstrated a significant rise in both chitinase and β-1,3-glucanase activity, surpassing even the positive control (spray with fungicide) (T3). Additionally, the findings imply a more pronounced induction in the lytic enzyme activities of PBR 357 relative to RLC 3.

Endophyte-mediated accumulation of non-enzymatic metabolites

Total phenols

The administration of bacterial endophytic treatments led to a substantial rise in the total phenol content within the fungal-inoculated stems of both *B. juncea* varieties when compared to the control (T2) (Fig. 3A), with the peak observed at 72 hpi. Notably, treatments T12 (involving seed treatment and foliar spray with a consortium), T7 (involving foliar spray with *P. aeruginosa*) and T11 (seed treatment and foliar spray with *S. proteamaculans*) resulted in the highest accumulation of total phenols in *B. juncea* var. PBR 357, showing respective fold increase of 3.91, 3.68 and 3.57. In case of RLC 3 stems, the greatest accumulation of total phenols occurred with the application of T10 (seed treatment and foliar spray with *P. aeruginosa*) and T11 (seed treatment and foliar spray with *S. proteamaculans*), resulting in fold increase of 3.14 and 3.12, respectively. Additionally, all effective treatments demonstrated a significantly greater increase in total phenols compared to even the positive control (T3). The accumulation of total phenols was notably higher in PBR 357 (non-canola mustard) compared to RLC 3 (canola mustard).

Lignin

The lignin content exhibited significant variation among different bacterial endophytic treatments in both *B. juncea* varieties compared to the control (T2) (Fig. 3B). Similar to phenols accumulation, there was a notable increase in lignin accumulation in the stems of mustard plants at 72 hpi, which generally remained consistent at subsequent time intervals of 120 and 168 hpi. Among the treatments, the stems of PBR 357 showed the highest lignin content following the application of T12 (seed treatment and foliar spray with a consortium), T10 (seed treatment and foliar spray with *P. aeruginosa*), and T11 (seed treatment

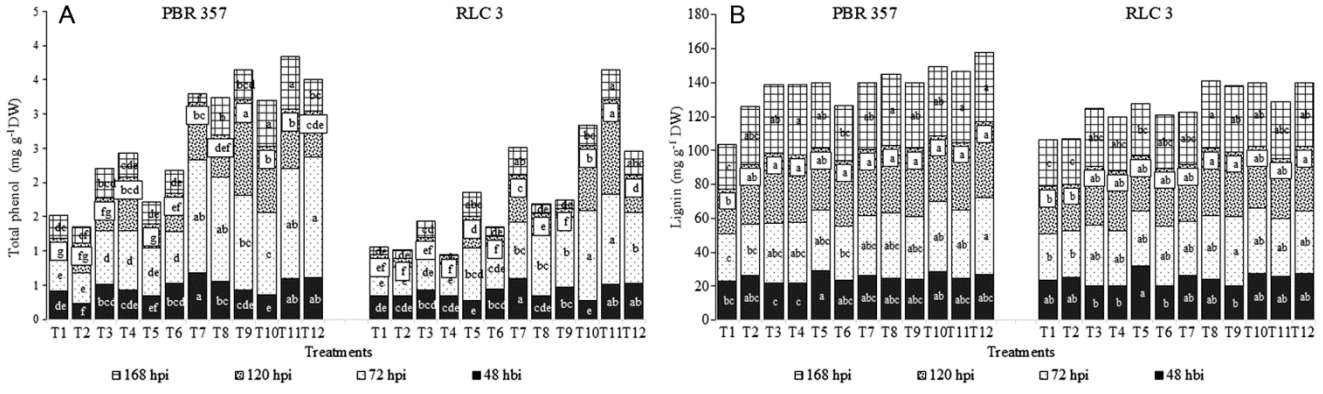

Fig. 3 — Effect of endophytes on accumulation of non-enzymatic metabolites: total phenols (A) and lignin content (B) in *Brassica juncea* varieties (PBR 357 and RLC3) following challenge with *Sclerotinia sclerotiorum.* T1- Naturally free from disease, T2 - Stem inoculation with pathogenic fungus culture alone,T3 - Spray with Fungicide (Carbendazim) + pathogenic fungus inoculation after 24 h of fungicide spray, T4 - Seed treatment with *Pseudomonas aeruginosa* + pathogenic fungus inoculation, T5 - Seed treatment with *Serratia proteamaculans* + pathogenic fungus inoculation, T6 - Seed treatment with endophyte consortium+ Fungus inoculation, T7 - Foliar spray with *P. aeruginosa* + Fungus inoculation,T8 - Foliar spray with *S. proteamaculans* + Fungus inoculation, T9 - Foliar spray with endophyte consortium + pathogenic fungus inoculation, T10 - Seed treatment cum Foliar Spray with *P. aeruginosa* + pathogenic fungus inoculation, T11 - Seed treatment-cum-Foliar Spray with *S. proteamaculans* + pathogenic fungus inoculation, T12 - Seed treatment-cum-Foliar Spray with endophyte consortium + pathogenic fungus inoculation. Data represent mean ± SE of three replicates. Different letters indicate significant difference between treatments at respective time intervals of 48 hbi, 72, 120, and 168 hpi at $P< 0.05$ as determined by Tukey's HSD test.

and foliar spray with *S. proteamaculans*), with fold increase of 1.51, 1.38, and 1.34, respectively, over the control (T2). In the case of canola mustard RLC 3, treatments T9 (foliar spray with a consortium), T12 (seed treatment and foliar spray with a consortium), and T8 (foliar spray with *S. proteamaculans*) resulted in the highest lignin accumulation, showing fold increase of 1.47, 1.37, and 1.34, respectively, compared to plants inoculated solely with fungus (T2). Most of the effective treatments exhibited higher lignin content compared to T3 (positive control).

Lesion length

The assessment of stem rot disease revealed that all endophytic treatments, administered through various methods, significantly reduced the lesion length in both *B. juncea* varieties compared to Brassica plants solely exposed to fungal pathogen (T2) (Fig. 4). However, the application of bacterial endophytes was not as effective as carbendazim (T3, positive control) in inhibiting lesion progression in both *B. juncea* varieties. The findings demonstrated that the positive control (T3) provided the most efficient disease control, resulting in the shortest lesion lengths of 6.13 cm in PBR 357 and 8.21 cm in RLC3. Among the endophytic treatments, the use of T12 (seed treatment and foliar spray with a consortium) and T11 (seed treatment and foliar spray with *S. proteamaculan*) led to a significant reduction in lesion length by 21.47% and 18.86%, respectively, compared to the control (T2) in PBR 357. In RLC 3, effective protection against stem rot was observed with T12 (seed treatment and foliar spray with a consortium) and T9 (foliar spray with a consortium), resulting in decrease of 16.91% and 14.94%, respectively, compared to pathogen-challenged control plants (T2).

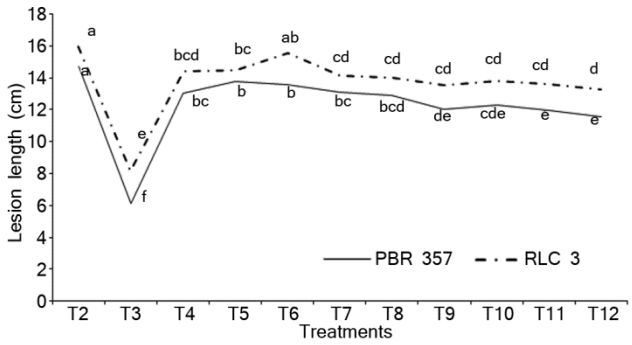

Fig. 4 — Effect of bacterial endophytes on lesion length in stem of *Brassica juncea* varieties, PBR 357 and RLC 3 following challenge with *Sclerotinia sclerotiorum.* T2-Stem inoculation with pathogenic fungus culture alone,T3-Spray with Fungicide (Carbendazim)+ pathogenic fungus inoculation after 24 h of fungicide spray, T4-Seed treatment with *Pseudomonas aeruginosa* + pathogenic fungus inoculation, T5-Seed treatment with *Serratia proteamaculans* + pathogenic fungus inoculation, T6-Seed treatment with endophyte consortium + pathogenic fungus inoculation, T7-Foliar spray with *P. aeruginosa* + pathogenic fungus inoculation,T8-Foliar spray with *S. proteamaculans* + pathogenic fungus inoculation, T9-Foliar spray with endophyte consortium + pathogenic fungus inoculation, T10-Seed treatment cum Foliar Spray with *P. aeruginosa* + pathogenic fungus inoculation, T11-Seed treatment cum Foliar Spray with *S. proteamaculans* + pathogenic fungus inoculation, T12- Seed treatment cum Foliar Spray with endophyte consortium + pathogenic fungus inoculation. Data represent mean lesion length of five plants. Different letters indicate significant difference between treatments at $P< 0.05$ as determined by Tukey's HSD test.

Correlation between biochemical traits and lesion development

Spearman's rank correlation revealed no statistically significant associations between lesion length and the measured defense-related biochemical traits (Table 2). However, a consistent negative trend was observed for lignin ($r = –0.231$) and total phenols ($r = –0.315$), indicating that treatments exhibiting higher lignifications and phenolic accumulation tends to develop comparatively shorter lesions. Although these trends did not achieve statistical significance, they suggest that structural defense components may contribute to restricting pathogen progression. In contrast, enzymatic antioxidants such as POD, PAL, chitinase, β-1,3-glucanase and PPO, showed weak or inconsistent correlations with lesion length, indicating that early fluctuations in inducible enzyme activities may not strongly aligned with the lesion development.

Discussion

In the preliminary investigation, we assessed the antifungal capabilities of two endophytic bacterial strains *viz., S. proteamaculans* and *P. aeruginosa*, using dual culture bioassay. The results revealed a strong inhibitory action of these strains on the phytopathogen *Sclerotinia sclerotiorum*. While numerous *in vitro* studies have explored the ability of bacterial endophytes to impede fungal growth directly, attributing this to the production of various compounds such as antibiotics, hydrolytic enzymes, siderophore, or hydrogen cyanide[24-26], limited studies have demonstrated into their biocontrol efficacy and mechanism in controlling fungal diseases in plants. Despite the growing interest in endophyte-mediated biocontrol, the mechanisms by which these microorganisms activate host defense pathways in *Brassica* species remain poorly understood.Hence, in

Table 2 — Spearman's rank correlation matrix between the disease lesion length and the measured defense-related biochemical traits

	POD	PAL	PPO	Chitinase	β-1,3 glucanases	Phenols	Lignin	Lesion length
POD	1.000							
PAL	0.762**	1.000						
Chitinase	0.622*	0.566 ^{NS}	1.000					
β-1,3 glucanases	0.811**	0.727**	0.594**	1.000				
PPO	0.270^{NS}	0.305 ^{NS}	0.648*	0.448 ^{NS}	1.000			
Phenols	0.685*	0.685*	0.371 ^{NS}	0.867**	0.333 ^{NS}	1.000		
Lignin	0.720**	0.490 ^{NS}	0.434 ^{NS}	0.888**	0.298 ^{NS}	0.727**	1.000	
Lesion length	-0.210 ^{NS}	-0.070 ^{NS}	-0.084 ^{NS}	-0.133 ^{NS}	0.340 ^{NS}	-0.315 ^{NS}	-0.231 ^{NS}	1.000

the present study we tried to elucidate the defense mechanism induced by endophytes, contributing to the mitigation of stem rot symptoms in *B. juncea* var. PBR 357 (non-canola mustard) and RLC 3 (canola mustard) when infected with *Sclerotinia sclerotiorum* under field-based pot trials.

Upon recognizing a pathogen, plants initiate a fundamental cellular process that involving the generation of reactive oxygen species (ROSs). This early oxidative burst also acts as a signalling cue that activates downstream defense pathways, including the induction of antioxidative and phenylpropanoid enzymes[27]. However, excessive ROSs production can be detrimental to plant cells, causing disruptions in their redox equilibrium, oxidizing lipids, and leading to the degradation of proteins, nucleic acids and chlorophyll[28]. Endophytes function to diminish levels of ROSs within plant cells by enhancing scavenging mechanisms, consequently promoting the efficacy of antioxidative system[29]. Our findings revealed a significant increase in antioxidative enzyme activities with POD and PPO reaching their peak levels at 120 hpi while PAL at 72 hpi in mustard plants subjected to seed treatment and foliar spray with *P. aeruginosa* and *S. proteamaculans* alone and in combination. This suggests that these bacterial endophytes may induce the activities of antioxidative enzymes, thus inhibiting the spread and proliferation of *Sclerotinia sclerotiorum* within the host tissues. Numerous studies have underscored the observation that bacterial endophytes stimulate the induction of PPO and POD, leading to heightened resistance in cauliflower, and brinjal upon encountering challenges posed by *Sclerotinia sclerotiorum*[30,31]. Defense enzymes, particularly PPO inhibit pathogen dissemination by facilitating the oxidation of phenolic compounds, leading to the creation of highly active o-quinones[32]. Due to their toxicity, o-quinones can directly harm pathogens or create a physical barrier that limits protein accessibility, potentially deterring pathogen attacks. During our investigation, we noticed that as the activity of PPO reached its highest point (120 hpi), there was a simultaneous decrease in phenol levels within the same timeframe. The observed decline in phenol content during peak PPO activity reflects their rapid conversion into quinones and other defense related oxidation products, rather than a depletion of the phenolic pool. This pattern indicates a functional link between elevated PPO activity and reduced phenols, suggesting that PPO mediated quinone formation contributed to plant's defense against phytopathogenic fungus.

There was a significant increase in the PAL activity in Brassica plants treated with the microbial agents. Incidentally, Tao *et al*.[33]documented that the beneficial endophyte *Burkholderia* ASV_550 enhanced plant's immune system by elevating PAL activity in tobacco thereby enhancing resistance against bacterial wilt disease. Furthermore, Tonnessen *et al.*[34] mentioned that the increase in PAL activity is essential for imparting resistance during stressful conditions and acts both as an inducible and constitutive enzyme against diverse pathogenic infections. PAL serves as a pivotal enzyme in the phenylpropanoid pathway, crucial for synthesizing secondary metabolites like phenols, lignin, and flavonoids.The link between elevated phenolic levels and enhanced plant resilience against pathogens has become a focal point of interest among researchers. Kolton *et al*.[35]explored this association, providing evidence for a direct correlation between heightened PAL activity and the accumulation of these bioactive compounds. Our own investigation corroborated these findings, revealing an upsurge in PAL activity and total phenols at the specific time point of 72 hpi. This observed escalation in enzymatic activity and phenolic accumulation holds profound implications. It signifies a reinforced defense mechanism against the

pathogen *Sclerotinia sclerotiorum*, particularly under the synergistic influence of biocontrol agents. These findings underscore the active and strategic role of endophytes in amplifying the phenylpropanoid pathway, thereby paving the way for novel approaches in fortifying plant defense strategies.

Our findings demonstrated that the application of endophytic bacterial isolates, either individually or in combination *via* seed treatment and foliar spray, resulted in a notable increase in the activities of lytic enzymes *viz.,* chitinases and β-1, 3-glucanases, in mustardplants at 120 hpi with *Sclerotinia sclerotiorum*. These enzymes play an essential role in hindering pathogen growth by targeting the key components of the pathogen cell walls.The elevated enzyme activity in the endophyte- treated plants indicates a possible priming effect, enabling the host to initiate a quicker or more enhanced defense response upon pathogen challenge.Various documented studies elucidated that certain bacterial endophytic strains possess the capability to elicit lytic enzyme activity, consequently bolstering resistance against a diverse array of pathogens[36,37]. Elevated lignin levels, a vital phenolic compound, are believed to serve a pivotal role in physically excluding pathogens and preventing their invasion into plants. The increased content of lignin may be associated with elevated plant resistance[38]. We also assessed the influence of endophytes on lignin levels in Brassica plants under pathogen-challenged conditions. Whether administered singly or in consortia, endophyte-treated plants showed increased lignin content. In line with these findings, Lastochkina *et al.*[39] reported the enhancement of lignin deposition in the roots of bean plants with the application of *B. subtilis*. In general, the lignin content persisted after 72 hpi, likely as a strategy to limit pathogen colonization by reinforcing cell wall barriers and impeding further pathogen ingress.

Brassica plants subjected to seed treatment and foliar spray with endophytic consortium exhibited a decrease in lesion length relative to plants solely inoculated with *Sclerotinia sclerotiorum.* The noted alleviation of disease symptoms may be ascribed to enhanced resistance within Brassica varieties against the infection, with the activation of the plant's defense mechanism. This mechanism is recognized as a pivotal indirect strategy employed by certain efficacious bioagents against plant pathogens[40]. Incidentally, Swathi *et al*.[41] employed various endophytic treatments and noted that the combination of seed treatment and foliar spray using consortia of *Pseudomonas fluorescens*, *Bacillus subtilis*, and *Pantoea dispersa* emerged as the most efficacious approach for managing bacterial leaf blight in rice. We noted that the fungicide triggered the production of defense enzymes, less prominently compared to the most effective treatments with bacterial endophytes. Nevertheless, the fungicide yielded a more substantial reduction in lesion length compared to the endophytes. This observed concordance is presumed to originate from the disparate mechanisms of action exhibited by fungicides and endophytes. Specifically, systemic fungicides such as carbendazim not only enhance defense enzyme production but also directly eradicate pathogens by mainly inhibiting nuclear division during fungal cell division, which enhances their overall efficacy[42].

Although the correlations were not statistically significant, the negative association between lesion length and lignin content suggests a potential protective role of lignification in restricting pathogen spread. Lignin-mediated strengthening of cell walls is a well-established barrier against necrotrophic pathogens, as reported in Brassica–Sclerotinia interactions and other host–pathogen systems. Studies have shown that increased lignin deposition limits the fungal hyphal penetration[43,44], supporting the trend observed in the present study. Similarly, the negative trend with total phenols aligns with literature indicating that phenolic accumulation contributes to structural reinforcement and oxidative defense. The lack of significance may be due to variation across treatments, timing of sampling, or the localized nature of lignin deposition. Nonetheless, these trends indicate that lignin and phenolic pathways may contribute to restricting lesion expansion and warrant further investigation.

Conclusion

Heavy dependence on chemical pesticides has led to environmental pollution, residue accumulation, and resistant pathogens. These issues threaten ecosystem and food system sustainability. Endophytes offer a safe and eco-friendly alternative for protecting plants from pathogens. In this study, the application of bacterial endophytes such *Pseudomonas. aeruginosa* and *Serratia proteamaculans* effectively enhanced the immune responses of Indian mustard against *Sclerotinia sclerotiorum.* The enhanced activities of antioxidative enzymes (POD, PAL, PPO) and lytic

enzymes (chitinases, β-1, 3-glucanases), along with elevated levels of defense-related metabolites (lignin and phenols) confirmed the ability of these bacterial strains in strengthening the defense system of mustard plants. A more intense effect was observed in PBR 357 than RLC 3, highlighting varietal disparities. This contributes to our knowledge of endophytes as inducers that significantly influence specific plant metabolites. It also offers insights into the systematic mechanisms by which host plants interact with their endophytes.

Author Contributions

S.S.conceived and designed the experiments. R.K., S.S., P.S.S. and P.V. executed the pot experiment.S.S., R.K., P.S.S., P.V., G.K. and A.S. helped in data interpretation. S.S and R.K. wrote the original manuscript. All the authors read and approved the final manuscript.

Declarations

Competing interests

The authors declare no conflict of interest (financial or non-financial).

Declaration of funding

The funding for this project was supported by All India Coordinated Research Project on Rapeseed-Mustard.

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