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Research Article

Enhancing Resistance to *Sclerotinia* Stem Rot in *Brassica juncea* Through Seed Priming with Bio-Control Agents

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Abstract

Brassica juncea (Indian mustard), a vital oilseed crop, is highly susceptible to Sclerotinia stem rot caused by *Sclerotinia sclerotiorum*, a pathogen that severely affects crop yield and quality. This study evaluated the effect of seed priming with bio-control agents, including *Bacillus subtilis*, *Trichoderma viride*, and their combination, on two varieties of *B. juncea* (RH30 and Varuna) under field conditions. Pathogen inoculation was performed at the reproductive stage, and morphological, biochemical, and yield-related parameters were assessed at 10 and 20 Days After Inoculation (DAI). The results demonstrated that the combined application of *B. subtilis* and *T. viride* significantly improved plant height, root and shoot biomass, and stem diameter. Biochemical analysis revealed enhanced levels of secondary metabolites such as flavonoids, phenolics, and ascorbate, along with increased activity of antioxidant enzymes, including Catalase (CAT), Polyphenol Oxidase (PPO), and Peroxidase (POX). These changes correlated with reduced disease symptoms, such as shorter stem lesion lengths, fewer sclerotia, and decreased stem breakage percentages. Additionally, yield attributes such as the number of siliquae per plant, seed size, and thousand-seed weight were markedly improved in plants treated with the bio-control agents. The combined treatment outperformed individual applications of *B. subtilis* or *T. viride*, demonstrating its superior efficacy in reducing disease severity and enhancing yield. This study highlights the potential of integrating bio-control agents into crop management practices to improve resistance against *Sclerotinia* stem rot and boost productivity in *B. juncea*. These findings offer a sustainable alternative to chemical methods for managing biotic stresses in oilseed crops.

Keywords: *Brassica juncea;* Biocontrol Agents; Phytopathogen; Seed priming; *Sclerotinia* stem rot

Introduction

Brassica juncea (Czern) L., commonly known as "Indian mustard" (AABB, 2n = 36), is a significant oilseed crop. This amphidiploid species originated from the natural hybridization of *B. rapa* (AA, 2n = 20) and *B. nigra* (BB, 2n = 16). In India, it ranks as the second-largest oilseed crop after groundnut and holds the fourth position globally, following the European Union (34%), China (23%), and Canada (19%). Predominantly cultivated in the eastern, northern, and north-western regions of India, it is grown both as a pure crop and as an intercrop on marginal and sub-marginal soils. Globally, India's edible oil industry is ranked fourth, following the United States, China, and Brazil, contributing 10% of the world's oilseed production. Oilseeds in India are grown on approximately 14.8% of the cultivable land area, making it the second-largest cultivation area after cereals [34]. *Sclerotinia sclerotiorum*, the causative agent of *Sclerotinia* rot, is one of the most destructive pathogens affecting rapeseed-mustard crops [45]. Shifting climatic conditions and modern agricultural practices exacerbate the risk of Sclerotinia rot epidemics by increasing inoculum buildup (He et al., 2016). This soil-borne fungus has a broad host range, infecting over 600 flowering plant species, including vital oilseed crops such as soybean, peanut, sunflower, and rapeseed-mustard. It is considered a non-host-specific pathogen [32]. *S. sclerotiorum* employs both myceliogenic and carpogenic infection pathways. The sclerotia resting in soil can germinate myceliogenically, forming apothecia that release airborne ascospores. These ascospores infect plants by developing appressoria, which often target basal stems. Developing resistant cultivars is the most effective and sustainable strategy for managing this devastating pathogen [7]. However, the *B. juncea-S. sclerotiorum* interaction remains poorly understood, with limited knowledge on host-pathogen dynamics, defence mechanisms, and resistance strategies [44]. Resistance to this pathogen is rare in cultivated mustard, although wild Brassicaceae relatives exhibit high levels of resistance [10,31,43]. The transfer of resistance genes from wild relatives to cultivated species faces numerous pre- and post-fertilization barriers [15]. Nonetheless, a few sources of resistance have been identified within the cultivated Indian mustard gene pool [33].

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Bacillus species, known for their dual role in promoting plant growth and combating phytopathogens, hold immense potential as Plant Growth-Promoting Rhizobacteria (PGPR) and biocontrol agents [4]. *Bacillus* strains produce various secondary metabolites, including cyclic lipopeptides, which exhibit strong antibiotic properties [28]. Among them, *Bacillus subtilis* is one of the most researched biocontrol agents in sustainable agriculture. Its strains, predominantly rhizospheric or endophytic, have demonstrated significant antagonistic activity against pathogens, including *B. subtilis* strains effective against corn leaf blight [47].

Similarly, *Trichoderma* species, which constitute over 60% of registered bio fungicides, are highly effective biocontrol agents. They enhance plant growth by producing enzymes, antibiotics, and invading harmful fungi. Furthermore, *Trichoderma* improves nutrient uptake, assists plants in withstanding abiotic stresses such as drought and salinity, and enhances resistance to diseases. This fungus has shown both direct and indirect benefits on plant development, positioning it as a critical component of sustainable agriculture. Its ability to biologically manage soil pathogens while promoting plant growth highlights its significance in agricultural systems.

Despite the extensive benefits of *Bacillus* and *Trichoderma* as biocontrol agents, there are no reports on their combined application in *Brassica* species. This gap represents a promising area for future research to enhance disease management and sustainable production in these crops.

Materials and Methods

The seeds of Indian mustard (*Brassica juncea* L. Czern), varieties RH30 and Varuna, were obtained from the Oilseed Section of the Department of Genetics and Plant Breeding at CCS Haryana Agricultural University, Hisar.

In the experiment, *Brassica juncea* seeds were treated with various biocontrol agents, including *Bacillus subtilis*, *Trichoderma* spp., a combination of *Trichoderma* spp. and *Bacillus subtilis*, and a control group without any treatment. The study was carried out under field conditions in the Oilseed Section of the Department of Genetics and Plant Breeding, CCS Haryana Agricultural University, Hisar. Disease incidence was monitored in both treated and untreated *Brassica* plants.

Four different treatments were given to each genotype of *Brassica juncea* (RH30 and VARUNA) against sclerotinia stem rot diseases

T_1: Control (without seed priming) + *Sclerotinia sclerotiorum*

T_2: Seeds priming with *Trichoderma species* + *Sclerotinia sclerotiorum*

T_3: Seeds priming with *Bacillius subtilis* + *Sclerotinia sclerotiorum*

T_4: Seeds priming with (*Trichoderma* + *Bacillius subtilis)* + *Sclerotinia sclerotiorum*

Sampling was carried out at the reproductive stage (80 days) following the inoculation of *Sclerotinia sclerotiorum* fungus in all *Brassica juncea* plants. Observations were recorded 10 and 20 days after inoculation.

Morphological Parameters

Plant Height

The height of the plants was measured using a meter scale, extending from the soil surface to the tip of the apical shoot at both observation intervals. The average plant height was then calculated.

Stem Diameter

The diameter of the stem was measured using a vernier caliper.

Fresh and Dry Weights of Roots and Shoots

The fresh weights of roots and shoots were recorded immediately after they were collected from pots and the field using a weighing scale. To determine the dry weight, the samples were dried in an oven at 80°C for 48 hours.

Biochemical Parameters

Flavonoid Content

The total flavonoid content of the sample was determined using the aluminum chloride colorimetric method. Quercetin served as the standard for constructing the calibration curve. A stock solution of quercetin was prepared by dissolving 5.0 mg of quercetin in 1.0 mL of methanol. Serial dilutions of the stock solution were made using methanol to achieve concentrations ranging from 5 to 200 µg/mL. To each 0.6 mL of diluted quercetin standard solution, 1.5 mL of 2% aluminum chloride was added. The mixture was allowed to rest at room temperature for 60 minutes. Absorbance was measured using a UV-Vis spectrophotometer at 420 nm against a blank. The calibration curve was used to calculate the total flavonoid content in the test samples.

Total Phenolic Content

The method described by Singleton and Rossi [39] was adapted with minor modifications to estimate the total phenolic content of the extracts using the Folin-Ciocalteu reagent. Absorbance measurements of the samples and standards were taken at 765 nm using a spectrophotometer, with the reagent blank serving as a reference. For the test, 1 mL of the sample was mixed with an equal volume of Folin-Ciocalteu reagent, followed by the addition of 2 mL of water. After a 5-minute incubation, 10 mL of a saturated sodium carbonate solution (20% w/v in water) was added to the mixture. The reaction was left in the dark for 30 minutes before being centrifuged. The absorbance of the resulting blue color in the samples was measured at 765 nm.

Total Sugar Content

A 100 mg sample of leaf tissue was homogenized with 5 mL of 80% methanol. The mixture was incubated in a water bath at 85°C for 1 hour. After incubation, the tubes were centrifuged at 4000 rpm for 30 minutes. The supernatant was carefully transferred into separate test tubes and heated at 100°C until the volume was reduced to 1 mL. The volume was then adjusted to 5 mL using 80% methanol. To 0.5 mL of the extract, 2 mL of 2% phenol was added, and the mixture was incubated at room temperature for 5 minutes. Subsequently, 5 mL of H_2SO_4 was added, and the solution was allowed to incubate for 20 minutes. Absorbance was measured at 630 nm using 80% methanol as the blank. A standard curve was prepared using glucose at various concentrations.

Ascorbate Content

The total ascorbate content was determined using the method outlined by Mohammad et al. [25]. A 100 mg sample of leaf tissue was extracted with 6% trichloroacetic acid. From this extract, 4 mL was mixed with 2 mL of 2% dinitrophenylhydrazine solution, followed by the addition of a drop of 10% thiourea in 70% ethanol. The mixture was boiled in a water bath for 15 minutes and then allowed to cool to room temperature. Subsequently, 5 mL of 80% H_2SO_4 was added to the mixture while keeping it at 0°C. The absorbance of the resulting hydrazine complex was measured at 530 nm.

Anthocyanin Content

Total anthocyanin content was measured by homogenizing 200 mg of leaf tissue with 3 mL of acidified methanol. The homogenate was transferred to a test tube and stored at 4°C for two days. After incubation, the mixture was centrifuged at 12,000 rpm for 15 minutes, and the absorbance of the supernatant was recorded at 530 nm and 657 nm.

H_2O_2 Content

The hydrogen peroxide content in fresh leaves (µmol/g) was determined based on the method by Velikova et al. (2000). Fresh mustard leaf tissue (0.2 g) was ground in 3 mL of 0.1% (w/v) Trichloroacetic Acid (TCA) and centrifuged at 10,000 rpm for 30 minutes at 4°C. The reaction mixture consisted of 750 µL of the supernatant, 750 µL of 10 mM potassium phosphate buffer, and 1.5 mL of 1 M potassium iodide (KI) reagent. The reaction was incubated in the dark for 1 hour, and the absorbance was recorded at 390 nm.

Glutathione Content

Total thiol content (GSH + GSSG) was estimated using the method of Ellman [8]. Leaf tissue (200 mg) was extracted with 1.0 mL of 5% sulfosalicylic acid, and the resulting supernatant was used for the analysis. The reaction mixture included 3.0 mL of sodium phosphate buffer (0.1 M, pH 7.4, 5 mM EDTA) and 0.6 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). After incubation for 15 minutes at room temperature, the absorbance was measured at 412 nm. A blank without the extract was used to adjust for DTNB absorbance. The total -SH content was calculated using an extinction coefficient of 13.6 $mM^{-1}cm^{-1}$ as described by Peter et al. (1988).

Enzyme Assays

For enzyme extract preparation, stem tissue (0.2 g) was homogenized in 3 mL of 0.1 M potassium phosphate buffer (pH 7.0) and centrifuged at 10,000 rpm for 15 minutes at 4°C. The resulting supernatant was collected and used for the enzymatic assays of Peroxidase (POX), Catalase (CAT), and Polyphenol Oxidase (PPO).

Catalase (CAT)

Catalase activity was measured following the method of Aebi et al. [1] with minor modifications. The reaction mixture (3 mL) contained 0.1 mL of enzyme extract, 1.5 mL of 50 mM phosphate buffer (pH 7.0), 1 mL of 75 mM H_2O_2, and 0.4 mL of distilled water. The activity of catalase was determined by monitoring the decrease in absorbance of H_2O_2 at 240 nm (extinction coefficient: 39.4 mM^{-1} cm^{-1}). Results were expressed as U min^{-1} g^{-1} Fresh Eeight (FW).

Polyphenoloxidase (PPO)

Polyphenol oxidase activity was determined based on the method of Gauillard et al. [11] with slight modifications. The reaction mixture consisted of 100 µL of enzyme extract, 1 mL of 0.1 M potassium phosphate buffer (pH 7.0), and 1 mL of 0.025 M catechol. The change in absorbance at 410 nm was recorded at intervals of 15 seconds for a duration of 2 minutes. The enzyme activity was calculated using the molar extinction coefficient of 2.9 mM^{-1} cm^{-1}.

Peroxidase (POX)

Peroxidase activity was assessed following a modified version of Maehly's (1954) method. The reaction mixture contained 50 µL of enzyme extract, 0.1 M potassium phosphate buffer (pH 7.0), 0.5 M H2O2, and 0.9 M guaiacol, making a total volume of 3.25 mL. Absorbance at 470 nm was recorded every 15 seconds for 2 minutes using a UV-Vis spectrophotometer. The enzyme activity was calculated based on the molar extinction coefficient of 26.6 mM^{-1} cm^{-1}.

Disease Parameters

Measurements included the length of stem lesions (cm), the percentage of stem breakage, and the number of sclerotia spores present in infected stems.

Yield and its Attributes

No. of siliquae per plant, thousand seed weight (g), seed size (mm), and seeds per plant were measured

Results

Morphological Parameters

Plant Height

Under biotic stress, an increase in total plant height was observed in both varieties by application og biocontrol agents (Figure 1). At 10 DAI, plant height increased by 1.03-fold in T3 compared to T0 in RH30, while Varuna showed a similar increase of 1.04-fold in T3 over T0. At 20 DAI, RH30 exhibited a 1.07-fold increase in T3 over T0, and Varuna showed a comparable rise of 1.01-fold in T3 relative to T0 (Figure 2).

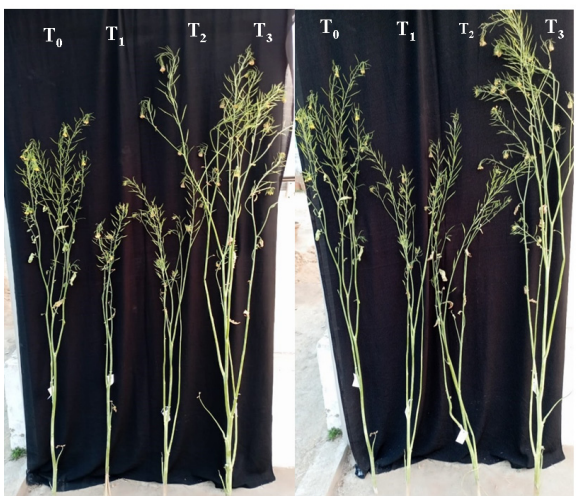

Figure 1: Morphology of plant height at 10 days after inoculation.

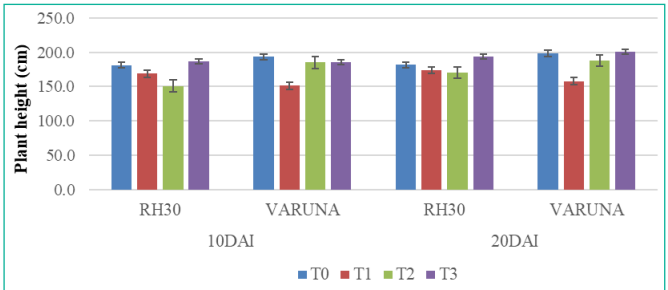

Figure 2: Effect of different bio-control agents on the plant height in two varieties at 10 DAI and 20 DAI against *Sclerotinia* stem rot disease.

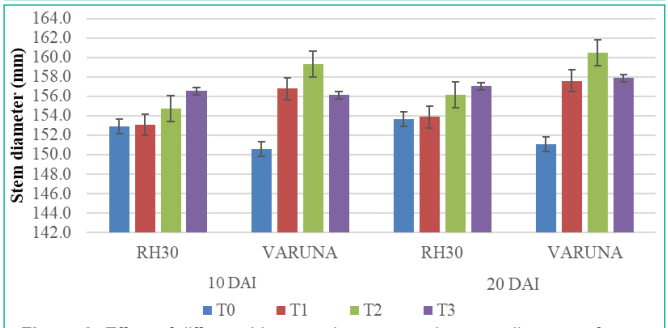

Figure 3: Effect of different bio-control agents on the stem diameter of two varieties of *Brassica juncea* at 10 DAI and 20 DAI against *Sclerotinia* stem rot disease.

Stem Diameter

The stem diameter increased in both varieties by application of biocontrol agents under biotic stress. At 10 DAI, RH30 showed an increase of (1.00-fold in T1, 1.01-fold in T2, and 1.02-fold in T3) compared to T2, while Varuna displayed a rise of (1.04-fold in T1, 1.05-fold in T2, and 1.03-fold in T3) over T2. At 20 DAI, the stem diameter in RH30 increased by (1.00-fold in T1, 1.02-fold in T2, and 1.02-fold in T3) relative to T0, and in Varuna, the increase was (1.04-fold in T1, 1.06-fold in T2, and 1.03-fold in T3) compared T0 (Figure 3).

Fresh Weight of Shoot and Root

Under biotic stress, the fresh weight of shoots and roots increased in both varieties by treatment of biocontrol agents. Shoots at 10 DAI, RH30 showed an increase of (1.05-fold in T1, 1.06-fold in T2, and 1.14-fold in T3) compared to T0, while Varuna exhibited a similar rise of (1.05-fold in T1, 1.07-fold in T2, and 1.19-fold in T3) over T0. At 20 DAI, the fresh shoot weight in RH30 increased by (1.07-fold in T1, 1.10-fold in T2, and 1.17-fold in T3) relative to T0, with Varuna showing a comparable increase of (1.07-fold in T1, 1.08-fold in T2, and 1.27-fold in T3) over T0 (Figure 4).

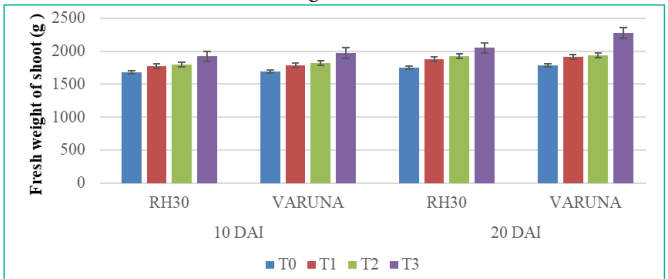

Figure 4: Effect of different bio-control agents on the fresh weight of shoot of two varieties of *Brassica juncea* at 10 DAI and 20 DAI against *Sclerotinia* stem rot disease.

Roots at 10 DAI, RH30 showed an increase of (1.03-fold in T1, 1.16-fold in T2, and 1.97-fold in T3) compared to T0, while Varuna exhibited a rise of (1.09-fold in T1, 1.15-fold in T2, and 1.74-fold in T3) over T0. At 20 DAI, the fresh root weight in RH30 increased by (1.03-fold in T1, 1.15-fold in T2, and 1.94-fold in T3) relative to T0, with a similar increase in Varuna of (1.09-fold in T1, 1.13-fold in T2, and 1.81-fold in T3) relative to T0 (Figure 5).

Dry Weight of Shoot and Root

The dry weight of shoots and roots increased in both varieties by application of biocontrol agents, under biotic stress. Shoots at 10 DAI, RH30 showed an increase of (1.03-fold in T1, 1.05-fold in T2, and 1.13-fold in T3) compared to T0, while Varuna exhibited a similar rise of (1.03-fold in T1, 1.13-fold in T2, and 1.14-fold in T3) over T0. At 20 DAI, the dry shoot weight in RH30 increased by (1.05-fold in T1, 1.05-fold in T2, and 1.14-fold in T3) relative to T0, with Varuna showing increase of (1.09-fold in T1, 1.14-fold in T2, and 1.16-fold in T3) over T0 (Figure 6).

Roots at 10 DAI, RH30 exhibited an increase of (1.07-fold in T1, 1.27-fold in T2, and 1.62-fold in T3) compared to T0, while Varuna showed a similar rise of (1.16-fold in T1, 1.23-fold in T2, and 1.50-fold in T3) over T0. At 20 DAI, the dry root weight in RH30 increased

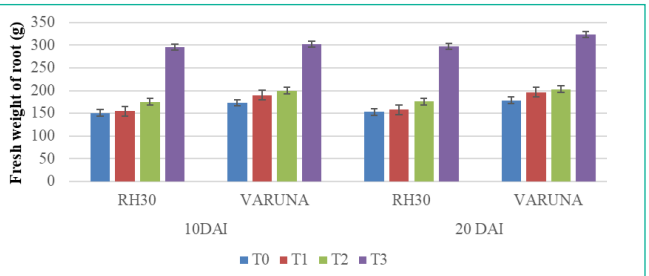

Figure 5: Effect of different bio-control agents on the fresh weight of root of two varieties of *Brassica juncea* at 10 DAI and 20 DAI against *Sclerotinia* stem rot disease.

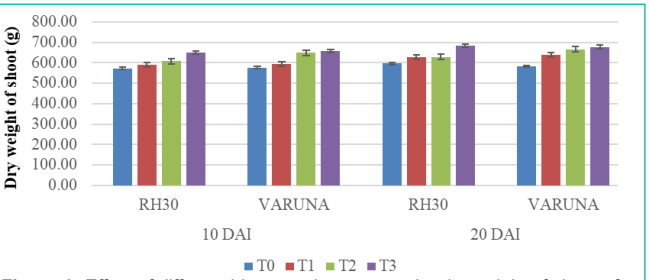

Figure 6: Effect of different bio-control agents on the dry weight of shoot of two varieties of *Brassica juncea* at 10 DAI and 20 DAI against *Sclerotinia* stem rot diseases.

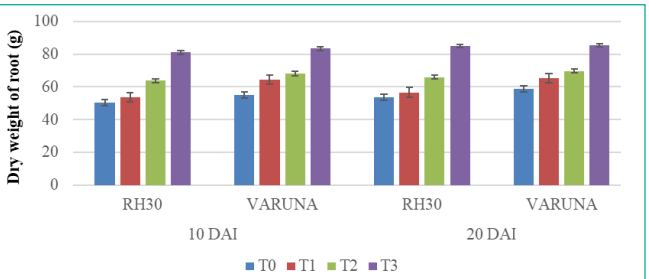

Figure 7: Effect of different bio-control agents on the dry weight of root of two varieties of *Brassica juncea* at 10 DAI and 20 DAI against *Sclerotinia* stem rot diseases.

by (1.05-fold in T1, 1.22-fold in T2, and 1.58-fold in T3) relative to T0, with Varuna demonstrating increase of (1.10-fold in T1, 1.17-fold in T2, and 1.45-fold in T3) compared to T0 (Figure 7).

Biochemical Parameters

Flavonoid Content

Application of biocontrol agents increased the total flavonoid content in both varieties, under biotic content. At 10 DAI, RH30 showed an increase of (1.05-fold in T1, 1.10-fold in T2, and 1.30-fold in T3) compared to T0, while Varuna exhibited a similar rise of (1.13-fold in T1, 1.10-fold in T2, and 1.20-fold in T3) over T0. At 20 DAI, the flavonoid content in RH30 increased by (1.10-fold in T1, 1.16-fold in T2, and 1.49-fold in T3) relative to T0, with Varuna showing a comparable increase of (1.20-fold in T1, 1.27-fold in T2, and 1.48-fold in T3) over T0 (Figure 8).

Total Phenolic Content

Under biotic stress, the total phenolic content increased in both varieties by application of biocontrol agents. At 10 DAI, RH30 displayed an increase of (1.06-fold in T1, 1.15-fold in T2, and 1.49-fold in T3) compared to T0, while Varuna exhibited a similar rise of (1.31-fold in T1, 1.37-fold in T2, and 1.49-fold in T3) over T0. At 20 DAI, the phenolic content in RH30 increased by (1.21-fold in T1, 1.17-fold in T2, and 1.49-fold in T3) relative to T0, with Varuna showing an increase of (1.10-fold in T1, 1.20-fold in T2, and 1.59-fold in T3) over T0 (Figure 9).

Total Sugar Content

The total sugar content showed a decline in both varieties after application of biocontrol agents, under biotic stress. At 10 DAI, RH30 exhibited reductions of (0.92-fold in T1, 0.74-fold in T2, and 0.63-fold in T3) compared to T0, while Varuna showed similar decreases of (0.98-fold in T1, 0.82-fold in T2, and 0.74-fold in T3) over T0. At 20 DAI, the sugar content in RH30 declined by (0.98-fold in T1, 0.81-fold in T2, and 0.85-fold in T3) relative to T0, and Varuna displaying reductions of (0.74-fold in T1, 0.71-fold in T2, and 0.54-fold in T3) compared to T0 (Figure 10).

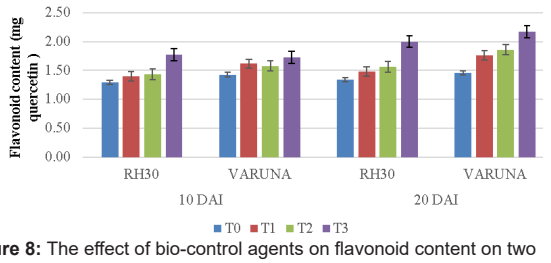

Figure 8: The effect of bio-control agents on flavonoid content on two varieties at 10 DAI and 20 DAI against *Sclerotinia* stem rot diseases.

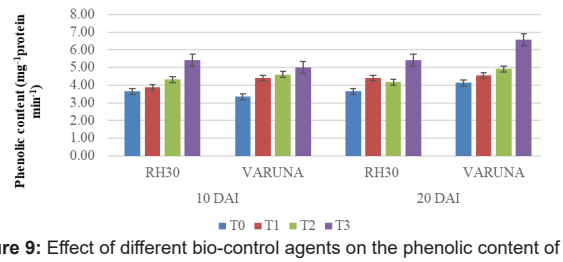

Figure 9: Effect of different bio-control agents on the phenolic content of two varieties of *Brassica juncea* at 10 DAI and 20 DAI against *Sclerotinia* stem rot disease.

Ascorbate Content

Under biotic stress, the total ascorbate content increased in both varieties after treatment with biocontrol agents. At 10 DAI, RH30 showed an increase of (1.23-fold in T1, 1.55-fold in T2, and 1.72-fold in T3) compared to T0, while Varuna exhibited a similar rise of (1.26-fold in T1, 1.39-fold in T2, and 1.67-fold in T3) over T0. At 20 DAI, the ascorbate content in RH30 increased by (1.07-fold in T1, 1.30-fold in T2, and 1.52-fold in T3) relative to T0, and an increase of (1.05-fold in T1, 1.14-fold in T2, and 1.68-fold in T3) over T0 in Varuna (Figure 11).

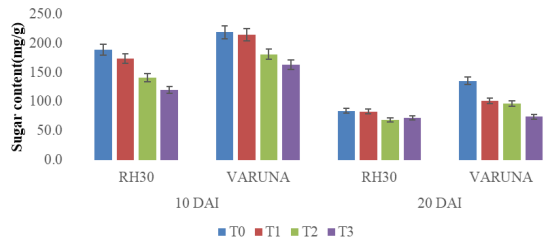

Figure 10: Effect of different bio-control agents on the total sugar content of two varieties of *Brassica juncea* at 10 DAI and 20 DAI against *Sclerotinia* stem rot disease.

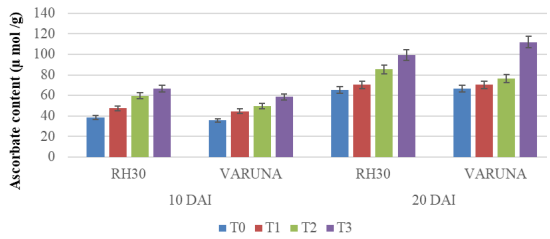

Figure 11: Effect of different bio-control agents on the total ascorbate content of two varieties of *Brassica juncea* at 10 DAI and 20 DAI against *Sclerotinia* stem rot disease.

Anthocyanin Content

The total anthocyanin content increased in both varieties after application of biocontrol agents under biotic stress. At 10 DAI, RH30 exhibited an increase of (1.05-fold in T1, 1.59-fold in T2, and 1.9-fold in T3) compared to T0, while Varuna showed similar increases of (1.26-fold in T1, 1.3-fold in T2, and 1.53-fold in T3) over T0. At 20 DAI, the anthocyanin content in RH30 increased by (1.08-fold in T1, 1.44-fold in T2, and 1.56-fold in T3) relative to T0, with Varuna showing increases of (1.1-fold in T1, 1.20-fold in T2, and 1.37-fold in T3) over T0 (Figure 12).

H_2O_2 Content

The total H_2O_2 content decreased by application of biocontrol agents in both varieties under biotic stress. At 10 DAI, RH30 exhibited

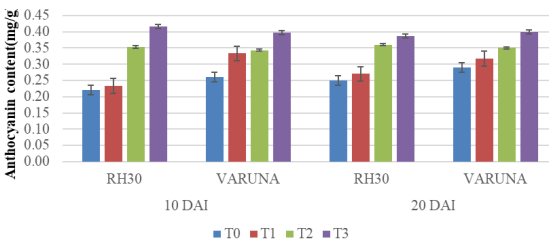

Figure 12: Effect of different bio-control agents on total anthocyanin contents in two varieties at 10 DAI and 20 DAI against sclerotinia stem rot disease.

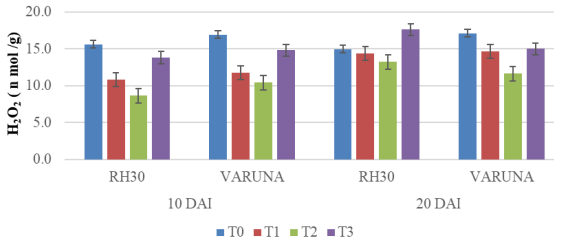

Figure 13: Effect of different bio-control agents on the H_2O_2 content of two varieties of *Brassica juncea* at 10 DAI and 20 DAI against *Sclerotinia* stem rot diseases.

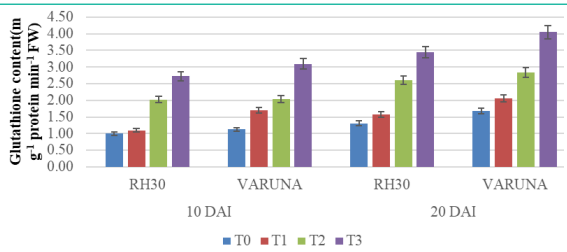

Figure 14: Effect of different bio-control agents on the glutathione content of two varieties of *Brassica juncea* at 10 DAI and 20 DAI against *Sclerotinia* stem rot diseases.

a reduction of (0.69-fold in T1, 0.55-fold in T2, and 0.88-fold in T3) compared to T0, while Varuna displayed similar decreases of (0.69-fold in T1, 0.61-fold in T2, and 0.87-fold in T3) over T0. At 20 DAI, RH30 recorded decreases of (0.95-fold in T1, 0.88-fold in T2, and 1.17-fold in T3) relative to T0, and Varuna showed reductions of (0.8-fold in T1, 0.67-fold in T2, and 0.87-fold in T3) over T0 (Figure 13).

Glutathione Content

Under biotic stress, the total glutathione content increased in both varieties by application of biocontrol agents. At 10 DAI, RH30 exhibited an increase of (1.10-fold in T1, 1.83-fold in T2, and 2.48-fold in T3) compared to T0, while Varuna showed similar increases of (1.50-fold in T1, 1.80-fold in T2, and 2.73-fold in T3) over T0. At 20 DAI, the glutathione content in RH30 increased by (1.19-fold in T1, 1.98-fold in T2, and 2.62-fold in T3) relative to T0, and, increase of (1.22-fold in T1, 1.69-fold in T2, and 2.41-fold in T3) over T0 in Varuna was recorded (Figure 14).

Enzyme Assays

Catalase (CAT)

Total catalase activity increased by application of biocontrol agents in both varieties under biotic stress. At 10 DAI, RH30 showed an increase of (1.72-fold in T1, 2.33-fold in T2, and 2.75-fold in T3) compared to T0, while Varuna exhibited increases of (1.45-fold in T1, 1.55-fold in T2, and 2.27-fold in T3) over T0. At 20 DAI, RH30 recorded increases of (1.32-fold in T1, 1.62-fold in T2, and 2.49-fold in T3) relative to T0, with Varuna showing comparable increases of (1.48-fold in T1, 2.27-fold in T2, and 2.87-fold in T3) over T0 (Figure 15).

Polyphenoloxidase (PPO)

Under biotic stress, total polyphenol oxidase activity increased in both varieties by application of biocontrol agents. At 10 DAI, RH30 exhibited an increase of (1.04-fold in T1, 1.33-fold in T2, and 1.53-fold in T3) compared to T0, while Varuna showed similar increases

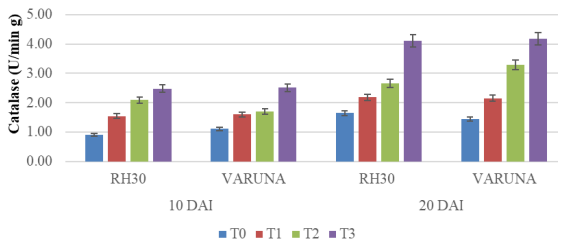

Figure 15: Effect of different bio-control agents on the total catalase activity of two varieties of *Brassica juncea* at 10 DAI and 20 DAI against *Sclerotinia* stem rot diseases.

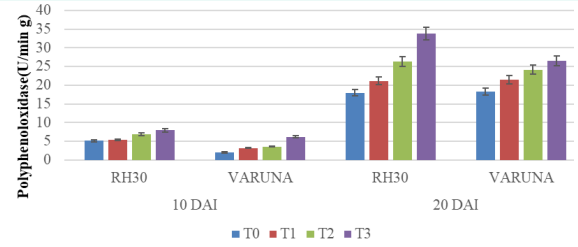

Figure 16: Effect of different bio-control agents on the total Polyphenoloxidase activity of two varieties of *Brassica juncea* at 10 DAI and 20 DAI against *Sclerotinia* stem rot disease.

of (1.59-fold in T1, 1.79-fold in T2, and 3.08-fold in T3) over T0. At 20 DAI, RH30 recorded increases of (1.19-fold in T1, 1.45-fold in T2, and 1.87-fold in T3) relative to T0, with Varuna displaying increase of (1.19-fold in T1, 1.31-fold in T2, and 1.44-fold in T3) compared to T0 (Figure 16).

Peroxidase (POX)

Peroxidase activity increased by application of biocontrol agents in both varieties under biotic stress. At 10 DAI, RH30 exhibited an increase of (1.44-fold in T1, 1.52-fold in T2, and 2.05-fold in T3) compared to T0, while Varuna showed similar increases of (1.64-fold in T1, 1.69-fold in T2, and 1.94-fold in T3) over T0. At 20 DAI, RH30 recorded increases of (1.25-fold in T1, 1.39-fold in T2, and 1.84-fold in T3) relative to T0, and, increase of (1.25-fold in T1, 1.49-fold in T2, and 1.52-fold in T3) was showed by Varuna over T0 (Figure 17).

Disease Parameters

The stem lesion length increased with the progression of disease infection at 10 days and 20 days post-inoculation. Both varieties exhibited an increase in stem lesion length. At 10 DAI, a reduction was observed (0.90-fold in T1, 1.03-fold in T2, and 0.67-fold in T3) compared to T0 in RH30, with a similar reduction (0.90-fold in T1, 0.72-fold in T2, and 0.71-fold in T3) compared to T0 in Varuna. At 20 DAI, a reduction was observed (0.87-fold in T1, 0.76-fold in T2, and 0.61-fold in T3) compared to T0 in RH30, and a similar decline

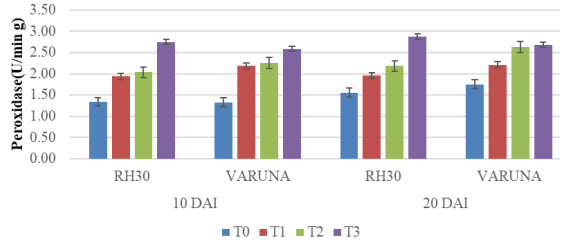

Figure 17: Effect of different bio-control agent on the total peroxidase activity of two varieties of *Brassica juncea* at 10 DAI and 20 DAI against *Sclerotinia* stem rot disease.

Table 1: Effects of *sclerotinia* fungus disease and biocontrol agents on various disease parameters in different varieties of *Brassica juncea*.

Varieties	Treatment	10 DAI			20 DAI		
		Stem lesion length(cm)	Stem breakage (%)	Number of sclerotia spore	Stem lesion length(cm)	Stem breakage (%)	Number of sclerotia spore
RH30	T_0	10.33	58.33	21.33	21.33	69.00	22.33
	T_1	9.33	70.00	15.00	18.33	76.67	18.00
	T_2	9.00	71.67	18.00	16.00	76.67	21.00
	T_3	7.00	58.33	12.00	13.00	70.00	15.00
VARUNA	T_0	11.00	58.33	20.33	22.00	68.33	23.00
	T_1	10.00	68.33	16.00	16.67	80.67	20.00
	T_2	8.00	65.00	18.33	15.67	83.33	22.00
	T_3	7.33	55.00	10.33	12.33	65.00	15.00
CD at 5%		N/A	7.219	3.57	4.045	9.716	3.208

CD at 5% refers to Critical Difference at 5% level of significance

Table 2: Effects of *sclerotinia* fungus disease and biocontrol agents on various yield parameters in two varieties of *Brassica juncea*.

Varieties	Treatment	Number of seeds	Number of siliquae per plant	Seed size(mm)	Thousand seed weight(g)
RH30	T_0	1,610	153.33	1.41	3.23
	T_1	1,774	175.33	1.57	3.76
	T_2	2,790	278.33	1.68	4.74
	T_3	4,370	431.67	1.83	5.86
VARUNA	T_0	2,567	180	1.59	4.95
	T_1	3,173	314	1.67	3.79
	T_2	3,452	343.33	1.72	4.98
	T_3	4,654	465	1.88	5.15
CD at 5%		327.66	58.32	0.179	0.181

CD at 5% refers to Critical Difference at 5% level of significance

(0.74-fold in T1, 0.70-fold in T2, and 0.69-fold in T3) compared to T0 in Varuna (Table 1).

The stem breakage increased because of disease infection at 10 days and 20 days post-inoculation. Biocontrol agents' application decreased the stem breakage in both the varieties (Figure 18). At 10 DAI, in RH 30 a decrease was observed (0.8-fold in T1, 0.66-fold in T2, and 0.53-fold in T3) compared to T0, with a similar reduction (0.95-fold in T1, 0.78-fold in T2, and 0.53-fold in T3) compared to T0 in Varuna. At 20 DAI, a reduction was noted (0.81-fold in T1, 0.75-fold in T2, and 0.56-fold in T3) compared to T0 in RH30, and reduction of (0.93-fold in T1, 0.86-fold in T2, and 0.53-fold in T3) compared to T0 in Varuna (Table 1).

Under biotic stress, the number of sclerotia spores in infected stems were increased but showed a decline in both varieties by application of biocontrol agents. At 10 DAI, a reduction was recorded (0.71-fold in T1, 0.84-fold in T2, and 0.56-fold in T3) relative to T0 in RH30, while a comparable decrease (1.2-fold in T1, 0.78-fold in T2, and 0.53-fold in T3) was observed in Varuna. At 20 DAI, the spore count decreased further, with reductions of (0.81-fold in T1, 0.75-fold in T2, and 0.56-fold in T3) relative to T0 in RH30, and similar decreases (0.93-fold in T1, 0.86-fold in T2, and 0.53-fold in T3) were noted in Varuna (Table 2).

Yield and its Attributes

Yield attributes like siliquae per plant, seeds per plant, 1000-seeds weight and seed size we reduces by the disease infection and were improved by application of biocontrol agents. The number of siliquae per plant showed an increase of (1.43-fold in T1, 2.47-fold in T2, and 2.7-fold in T3) compared to T0 in RH30, along with increase of (1.44-fold in T1, 2.16-fold in T2, and 2.89-fold in T3) in Varuna. Seeds per plant displayed an increase of (1.19-fold in T1, 1.61-fold in T2, and 1.71-fold in T3) compared to T0 in RH30, and a similar increase (1.19-fold in T1, 1.72-fold in T2, and 1.75-fold in T3) was noted in Varuna. 1000-seeds weight recorded an increase of (1.16-fold in T1, 1.46-fold in T2, and 1.81-fold in T3) over T0 in RH30, with a comparable increase of (1.10-fold in T1, 1.44-fold in T2, and 1.73-fold in T3) in Varuna. For seed size an increase of (1.00-fold in T1, 1.05-fold in T2, and 1.05-fold in T3) compared to T0 was observed in RH30, and, increase of (1.00-fold in T1, 1.05-fold in T2, and 1.11-fold in T3) was observed in Varuna.

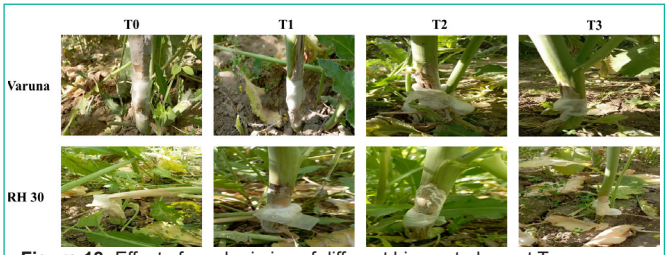

Figure 18: Effect of seed priming of different bio-control agent T_1 (*Trichoderma*), T_2 (*Bacillius subtilis*) and T_3 (*Trichoderma +Bacillius subtilis)*, T_0 (without seed priming) against the stem rot disease in *Brassica juncea*.

Discussion

Brassica juncea, a major oilseed crop in India, is extensively cultivated both as a pure crop and as an intercrop, particularly in marginal and sub-marginal soils of the eastern, northern, and northwestern states. With India being the second-most populous country globally and exhibiting a significant population growth rate [14,16], ensuring the health and productivity of such crops is crucial. *Sclerotinia sclerotiorum* is a major pathogen causing *sclerotinia* rot in *B. juncea*, necessitating effective control measures.

This study evaluated the impact of seed priming with bio-control agents on tolerance to sclerotinia stem rot in two varieties of *B. juncea* (RH30 and Varuna). Treatments included priming with *Bacillus subtilis*, *Trichoderma viride*, a combination of *B. subtilis* and *T. viride*, and a control. *Sclerotinia* infections were introduced at the reproductive stage (approximately 80 days) to both primed and non-primed plants. Results showed that the combined treatment of *T. viride* and *B. subtilis* led to greater plant height and higher fresh and dry weights of roots and shoots compared to individual treatments. Similar findings were reported by Sofy et al. [41], where the combined application of *Trichoderma* and biochar was more effective than their individual applications under varying NaCl concentrations.

Enhanced anthocyanin content was observed 10 and 20 Days After Inoculation (DAI) in both varieties, aligning with findings by Calla et al. [3], where anthocyanins functioned as robust secondary

metabolites and non-enzymatic antioxidants, directly inhibiting pathogens and protecting plants from reactive oxygen species (ROS) during biotic stress [21]. The inhibitory role of anthocyanins against *S. sclerotiorum* has also been documented in soybean and oilseed rape (Liu et al., 2019).

Ascorbate and glutathione levels increased at 10 and 20 DAI, consistent with Smirnoff and Wheeler (2000), who highlighted the role of ascorbic acid in detoxifying H_2O_2 through the ascorbate-glutathione cycle, an essential process for maintaining cellular redox balance. Glutathione's role in oxidative stress management and pathogen defense is also well-documented [27].

Total sugar content declined under all treatments during pathogen infection, similar to findings by Singh et al. (2022), where sugars contributed to ROS balance and participated in detoxification pathways like the oxidative pentose phosphate pathway [6]. This study also observed reduced genotypic variation in Total Soluble Sugars (TSS) post-infection, with sensitive genotypes showing greater declines, especially during advanced disease stages.

Polyphenol Oxidase (PPO) activity increased at 10 and 20 DAI in both varieties. PPO plays a vital role in defense by oxidizing phenolic compounds into reactive antimicrobial molecules like o-quinones, which act as a physical barrier against pathogens [5,42]. Enhanced PPO activity has been linked to constitutive resistance in several crops, including oilseed rape and sunflower [22].

Catalase (CAT) activity also increased in response to infection, aiding H_2O_2 detoxification near pathogen invasion sites. Genotypic variation significantly influenced CAT activity, which was higher in resistant genotypes before inoculation. This is consistent with Shetty et al. [36], who noted that increased CAT activity correlates with enhanced resistance to pathogens like *S. sclerotiorum*. Resistant genotypes maintained higher CAT activity post-inoculation, whereas sensitive genotypes experienced greater reductions, leading to oxidative bursts and cell death, promoting necrotrophic pathogen development [19,26].

Phenolic and flavonoid contents increased significantly in bio-control agent treatments, consistent with findings by Ahuja et al. [2]. Flavonoids and phenolic compounds play a crucial role in pathogen inhibition, acting as phytoalexins and ROS scavengers. This study also confirmed that Total Soluble Phenols (TSP) increased more significantly in resistant genotypes post-inoculation, providing enhanced resistance through antioxidant activity and inhibition of pathogen-derived enzymes [18,30,40].

The bio-control treatments reduced disease parameters such as stem lesion length, number of sclerotia, and stem breakage percentage. These results align with Sharma et al. [33], who demonstrated significant inhibition of *S. sclerotiorum* by *B. subtilis* and *T. viride*. Similar observations were reported by Mbazia et al. [24], where these bio-control agents reduced disease severity in faba bean. Moreover, *B. subtilis* strains like Em7 were found to significantly reduce stem rot frequency by 50–70%, as reported by Gao et al. [9].

Yield parameters, including the number of siliques per plant, seed weight, and seeds per plant, increased with seed priming treatments. These findings align with Haque et al. [12], who observed yield enhancements with biofertilizers. Similar results were reported by Pandey et al. [29] and Khatun et al. [17], emphasizing the positive effects of *Trichoderma* and *Bacillus* on plant growth and productivity. Bio-priming thus enhances yield while improving plant resilience to biotic and abiotic stresses, as corroborated by Singh et al. [38].

The present study highlights the efficacy of seed priming with bio-control agents like *Bacillus subtilis* and *Trichoderma viride* in enhancing the growth, defense mechanisms, and yield of *Brassica juncea* while reducing the severity of sclerotinia stem rot. These findings underscore the potential of integrating bio-control agents into sustainable agricultural practices for managing biotic stress in oilseed crops.

Conclusion

This study demonstrated the efficacy of seed priming with bio-control agents *Bacillus subtilis* and *Trichoderma viride*, individually and in combination, against *Sclerotinia* stem rot in *Brassica juncea*. The combined treatment proved most effective, enhancing morphological and biochemical traits, reducing disease severity, and improving yield parameters. These findings highlight the potential of bio-control agents as sustainable tools for managing biotic stress in oilseed crops while promoting plant growth and productivity. Integrating such eco-friendly strategies into agricultural practices could significantly contribute to sustainable crop production systems. Future research should focus on large-scale trials across diverse agro-climatic zones to validate these findings under varying environmental conditions. The molecular mechanisms underlying the enhanced defense responses observed in treated plants should also be explored to identify key genetic markers for resistance. Additionally, integrating bio-control agents into holistic crop management strategies, including crop rotation and nutrient optimization, could further improve disease resistance and productivity. Developing formulations with extended shelf life and compatibility with existing agricultural practices will be critical for their widespread adoption. Such efforts can contribute to the sustainable intensification of oilseed production, ensuring food and nutritional security in the face of increasing global challenges.

Author Statements

Author Contributions

Conceptualization, N.L. and R.S.; methodology, R.S. and A.R.S.; software, A.R.S. and R.S.; validation, N.L. and A.R.S.; formal analysis, R.S.; investigation, R.S. and. N.L.; data curation, R.S.; writing—original draft preparation, R.S., N.L. and A.R.S.; writing—review and editing, A.R.S. and N.L.; visualization, R.S.; supervision, N.L.; project administration, N.L. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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