



RESEARCH ARTICLE

Antagonist-mediated systemic resistance in mustard (*Brassica juncea* (L.) Czern.) against *Sclerotinia sclerotiorum*: Role of antioxidant enzymes and stress proteins

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Abstract

Sclerotinia rot caused by *Sclerotinia sclerotiorum* is a major constraint in mustard production. The present investigation evaluated the antagonistic potential of fungal and bacterial bioagents and their role in inducing biochemical defence responses in mustard cultivars RH-30 (*Brassica juncea* (L.) Czern.) and HC-212 (*Brassica carinata* A. Braun). The investigation was conducted through both *in vitro* and greenhouse experiments. A total of fifteen *Trichoderma* isolates and eleven bacterial antagonists were evaluated against *S. sclerotiorum* using dual culture assays to determine their antagonistic efficacy. The most effective fungal and bacterial isolates were subsequently characterised through internal transcribed spacer (ITS) and 16S ribosomal RNA (16S rRNA) gene sequencing respectively. Under greenhouse conditions, selected antagonists were assessed for their ability to induce systemic resistance by analysing antioxidant metabolites, oxidative stress markers, defence enzymes and stress-responsive proteins following pathogen inoculation. Among the fungal antagonists, isolate HHT recorded the highest mycelial growth inhibition (77.33 %), followed by HST-2 (75.19 %), HRoT (74.89 %). Among bacterial antagonists, HRBa-3 exhibited the maximum inhibition (69.29 %). Molecular characterisation identified HHT as *Trichoderma asperellum* and HRBa-3 as *Bacillus subtilis*. Antagonist treated plants showed significantly enhanced antioxidative and defence-related biochemical responses following pathogen challenge. The cultivar HC-212 exhibited higher accumulation of total phenolics, stress-responsive proteins and greater activity of defence enzymes, including catalase (CAT), superoxide dismutase (SOD), polyphenol oxidase (PPO), peroxidase (POX), phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL), than RH-30. Conversely, lower levels of oxidative stress indicators such as malondialdehyde (MDA) and hydrogen peroxide (H₂O₂) were recorded, particularly under *T. asperellum* treatment. Correlation analysis revealed strong positive associations among biochemical defence markers, indicating a coordinated defence mechanism during pathogen stress. The study demonstrates that *T. asperellum* and *B. subtilis* are effective biocontrol agents capable of suppressing *S. sclerotiorum* and enhancing host defence mechanisms through induced systemic resistance. Therefore, these bioagents are recommended as environmentally sustainable components of integrated disease management strategies for the effective management of Sclerotinia stem rot in mustard.

Keywords: *Bacillus subtilis*; biocontrol agents; defence enzymes; Sclerotinia rot; *Trichoderma asperellum*

Introduction

Rapeseed-mustard is the third most important oilseed crop in India after soybean and sunflower, contributing 13.16 million tonnes or 33.24 % of total oilseed production (1). Indian mustard, a self-pollinated amphidiploid (2n = 36), is widely cultivated across northern and central India, with expanding cultivation in the south. Grown on 8.85 million hectares, it yields an average of 1428 kg/ha (1). Mustard oil is valued for its thermal stability, nutritional quality and health-promoting fatty acid profile (2). Despite productivity gains, yields are hampered by biotic and abiotic stresses, especially fungal diseases such as white rust, Alternaria blight and Sclerotinia stem rot. *Sclerotinia sclerotiorum* is the most destructive among all, causing yield losses up to 90 % (3).

Sclerotinia sclerotiorum is a cosmopolitan, necrotrophic pathogen infecting over 600 plant species, including major oilseeds such as soybean, sunflower, groundnut and mustard (4). It survives as sclerotia that persist in soil for up to 8 years and germinate either myceliogenically to infect stems or carpogenically to form apothecia that release airborne ascospores, often through senescent petals lodged at stem junctions (5). Environmental factors strongly influence its epidemiology. Apothecia formation requires moist soil and moderate temperatures (10–25 °C), with irrigation or rainfall favouring spore release. Ascospore survival is optimal at 22 °C and approximately 60 % relative humidity but reduced at high temperature and humidity (6). Pathogen invasion disrupts host

cell balance, triggering reactive oxygen species (ROS) accumulation. Plants respond with enzymatic (e.g., catalase (CAT), peroxidase (POX), polyphenol oxidase (PPO), superoxide dismutase (SOD)) and non-enzymatic (e.g., phenolics, soluble sugars) antioxidant defences, which are key indicators of resistance (7, 8).

Although chemical fungicides are commonly used for disease management, their repeated application raises environmental safety concerns and promotes the development of pathogen resistance. Furthermore, there is a limited availability of commercially resistant mustard cultivars. Thus, biological control offers a sustainable alternative. Although biocontrol agents such as *Trichoderma*, *Bacillus* and *Pseudomonas* spp. are widely recognised for their antagonistic activity against *S. sclerotiorum* through competition, antibiosis and mycoparasitism (9), relatively little attention has been given to their role in triggering systemic resistance in mustard. The biochemical basis of induced resistance, particularly the role of stress-responsive protein expression in contrasting mustard cultivars, is still not well understood. Induced systemic resistance (ISR) represents the plant's innate ability to reinforce its defence machinery when stimulated by beneficial microorganisms, allowing it to better withstand pathogen attack. Although biological agents have been explored for disease management in mustard, clear insights into how ISR functions at the biochemical and protein levels, especially during infection by *S. sclerotiorum* and across different cultivars, remain limited. Antioxidant enzymes and defence-related proteins were therefore selected as key indicators, as they play a central role in controlling oxidative stress, safeguarding cellular integrity and activating defence responses during pathogen challenge.

By linking antagonist activity with detailed antioxidant and protein profiling, this study goes beyond earlier reports and provides a clearer, cultivar-specific understanding of ISR-mediated resistance in mustard under controlled conditions. Addressing these gaps, the present study evaluates the ability of fungal (*Trichoderma asperellum*) and bacterial (*Bacillus subtilis*) antagonists to induce systemic resistance in mustard cultivars against *S. sclerotiorum*, with a focus on antioxidant metabolites, defence enzymes and stress-responsive proteins under controlled conditions.

Materials and Methods

Evaluation of fungal and bacterial antagonists under *in vitro* conditions

Fungal and bacterial antagonists were isolated from soil samples collected from different districts of Haryana using the serial dilution method. The antagonistic effect of bio-agents on the growth of *S. sclerotiorum* isolate SSR-1 was evaluated through dual culture assay (10). The colony diameter of the pathogen in the control plate was also recorded. Percent inhibition of mycelial growth over control was calculated using the formula described in previous research (11).

$$\text{Growth inhibition (\%)} = \frac{C - T}{C} \times 100$$

Where, C = radial growth of *S. sclerotiorum* in control (mm) and T = radial growth of *S. sclerotiorum* in treatment (mm)

Experimental design and treatments

The experiment was laid out in a completely randomised design (CRD) with 5 treatments and three replications per treatment, each consisting of 3 plants. Each replication consisted of one pot containing 3 plants, maintained under uniform watering and agronomic practices. The following treatments were applied: T₁, the most effective fungal antagonist (*Trichoderma* isolate HHT); T₂, the second most effective fungal antagonist (*Trichoderma* isolate HST-2); T₃, the first most effective bacterial antagonist (isolate HRBa 3); T₄, the second most effective bacterial antagonist (isolate HKrBa 3) and T₅, the control (diseased).

Plant material and growth conditions

Two mustard cultivars - *Brassica juncea* (L.) Czern. (RH-30) and *Brassica carinata* A. Braun (HC-212) were selected, based on their contrasting reactions to the pathogen, RH-30 being relatively susceptible and HC-212 exhibiting a more resistant response than RH-30. Healthy and uniform seeds were surface sterilised using 1 % sodium hypochlorite solution for 2 min, followed by three rinses with sterile distilled water to eliminate surface contaminants. Sterilised seeds were sown in earthen pots containing a pre-autoclaved soil: sand: well-decomposed farmyard manure (FYM) mixture in a 2 : 1 : 1 (w/w/w) ratio. Pots were maintained under screen house conditions during the rabi, 2024.

Pathogen inoculum preparation

The *S. sclerotiorum* isolate (SSR-1), originally obtained from infected sclerotia collected from the oilseed field of the Department of Genetics and Plant Breeding, Chaudhary Charan Singh Haryana Agricultural University, Hisar, was used for artificial inoculation. For inoculum preparation, actively growing mycelium of SSR-1 was cultured on potato dextrose agar (PDA) plates and incubated at 25 ± 2 °C for 5–7 days. At 35–40 days after sowing (DAS), plants were inoculated at the crown region using a 5 mm mycelial disc taken from the actively growing margins of PDA cultures. The inoculated sites were covered with sterile, moistened cotton and wrapped with parafilm to ensure favourable microclimatic conditions for infection.

Pathogenicity test

To confirm pathogenic nature of *S. sclerotiorum* and to prove Koch's postulates, pathogenicity of *S. sclerotiorum* was evaluated on mustard cultivar under screen house conditions. Five seeds were sown per pot in sterilised sandy loam soil. At 35–40 days of plant growth, artificial stem inoculation was carried out using the pathogen. Observations were recorded regularly for appearance and development of symptoms. After 5–7 days inoculated plants developed elongated, water-soaked lesions, which were later covered with dense, cottony white mycelial growth. Over time, these lesions girdled the stem, leading to wilting and eventual drying of the plants. The pathogen was successfully re-isolated from the infected tissue on PDA medium to confirm the identity of pathogen and compared with the original isolate.

Antagonist selection and application

The two most effective *Trichoderma* isolates, designated HHT and HST-2, were procured from soil samples collected from the Hisar and Sirsa districts of Haryana respectively. Similarly, two bacterial antagonists, HRBa 3 and HKrBa 3, were obtained from soil samples collected from the Rewari and Karnal districts. These isolates were selected based on their superior antagonistic activity against

S. sclerotiorum in prior *in vitro* screening assays. Most effective fungal and bacterial antagonists were characterised on molecular basis by using the primers ITS1 (5'TCCGTAGGTGAACCTGCCG3') and ITS4 (5'CTGTTGGTTTCTTTCTCCGC3') against *Trichoderma* isolate, HHT and primers 16s forward (GGATGAGCCCGCGCCTA) and 16s reverse (CGGTGTGTACAAGGCCCGG) against bacterial antagonist, HRBa 3.

For soil application, mass cultures of *Trichoderma* isolates were grown on a potato dextrose broth and incorporated into the potting mixture at a rate of 15 g/kg of soil. Bacterial antagonists were applied as aqueous cell suspensions at a final concentration of 1×10^6 colony-forming units CFU/mL. All antagonists were introduced into the soil 3 days prior to pathogen inoculation to allow sufficient time for colonisation and induction of resistance mechanisms.

Antioxidative response for induction of resistance

Leaf samples were collected at specific time intervals, namely 0, 2, 5, 10 and 15 days post-inoculation with the pathogen, from both control and treated plants. These samples were subsequently analysed for various biochemical parameters.

Determination of defence-related secondary metabolites

To assess defence-related secondary metabolites in mustard leaves, total phenolics, total soluble sugars (TSS), hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents were quantified. Leaf tissues (100 mg) were extracted with 80 % ethanol, heated at 80 °C for 1 hr and centrifuged at 4000 rpm for 20 min. The residue was re-extracted twice. Pooled supernatants were evaporated at 40 °C and the extract was reconstituted in 50 % ethanol. Total phenolics were estimated using the Folin-Ciocalteu method and absorbance was recorded at 650 nm. The TSS was determined by the phenol-sulphuric acid method (12), with absorbance measured at 490 nm. The H_2O_2 content was measured following previous studies (13) using trichloroacetic acid (TCA) extracts reacted with phosphate buffer and potassium iodide and absorbance was taken at 390 nm. Lipid peroxidation was assessed by estimating malondialdehyde (MDA) content through the thiobarbituric acid reactive substances (TBARS) method (14), in which extracts were reacted with 0.6 % thiobarbituric acid in 20 % TCA, heated and measured at 532 nm (corrected at 600 nm) using an extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$.

Determination of defence-related antioxidant enzymatic activities

Antioxidant enzyme activities including peroxidase (POX), superoxide dismutase (SOD), catalase (CAT), phenylalanine ammonia lyase (PAL), tyrosine ammonia lyase (TAL) and polyphenol oxidase (PPO) were assayed from fresh leaf extracts. One gram of mustard leaves were homogenised in 0.1 M phosphate buffer (pH 7.0) containing ethylenediaminetetraacetic acid (EDTA), β -mercaptoethanol and polyvinylpyrrolidone (PVP) to prevent phenolic oxidation and centrifuged at 12000 g for 30 min at 4 °C. The supernatant was used for enzymatic assays. Peroxidase (POX) activity was estimated by the o-dianisidine method (15), while superoxide dismutase (SOD) was assayed based on inhibition of nitroblue tetrazolium (NBT) photoreduction (16). Catalase (CAT) activity was determined according to previous research (17) by measuring the decomposition of H_2O_2 after termination with dichromate-acetic acid reagent. Polyphenol oxidase (PPO) activity was quantified using catechol as substrate

(18). Phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) activities were determined using phenylalanine and tyrosine respectively, as substrates in borate buffer, with reactions stopped by hydrochloric acid (HCl) and absorbance measured at 290 nm for PAL and 333 nm for TAL.

Protein expression analysis in antagonist pathogen treated plants

For protein expression studies, leaves of *B. juncea* (RH-30) and *B. carinata* (HC-212) treated with antagonists and *S. sclerotiorum* were sampled at 0, 2, 5 and 10 days after inoculation (DAI) and stored at -80 °C. Total protein content was quantified using the Bradford assay (19) at 595 nm with bovine serum albumin as standard. Protein extracts were prepared in tris buffer (pH 7.5) containing PVP, centrifuged at 10000 g and mixed with Laemmli's sample buffer (20), followed by heating at 100 °C for 3 min. Protein samples (20 μ L) were loaded onto polyacrylamide gels for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 and destained in methanol-acetic acid solution. Distinct protein banding patterns were analysed to evaluate differential expression in response to antagonist treatments.

Statistical analysis

The experimental data were analysed using OPSTAT (21) and R software (22). A two-way analysis of variance (ANOVA) was performed to evaluate the effects of treatments, varieties and days after inoculation and treatment means were compared using the critical difference (CD) test at a 5 % significance level. Correlation analysis was carried out in R to assess relationships among biochemical and enzymatic parameters, with significance tested at $p \leq 0.05$.

Results

The pathogenic nature of *S. sclerotiorum* was confirmed through artificial inoculation on mustard cultivar (RH-30) under controlled screen house conditions. Within 5–10 days post inoculation (DPI), the plants developed elongated water-soaked lesions at the collar region. These lesions gradually became encircled with a dense cottony white mycelial growth, eventually girdled the stem completely. Affected plants showed symptoms of wilting and subsequently dried out. Sclerotia were observed and collected from the artificially inoculated plants. To fulfil Koch's postulates, the pathogen was successfully re-isolated from the infected plant tissue. The re-isolated culture displayed identical morphological and cultural characteristics to the original isolate, thereby confirming *S. sclerotiorum* as the causal agent of the Sclerotinia rot disease in mustard (Fig. 1).

Evaluation of fungal and bacterial antagonists against *Sclerotinia sclerotiorum*

The dual culture assay was conducted to assess the antagonistic potential of fifteen *Trichoderma* isolates and eleven bacterial antagonists that were evaluated against *S. sclerotiorum* revealed clear and significant differences in their ability to inhibit the pathogen's mycelial growth. The highest percent mycelial growth inhibition (77.33 %) was recorded by isolate HHT which was significantly superior to all others against *S. sclerotiorum* which was isolated from Hisar. This was closely followed by HST-2 (75.19 %), HRoT (74.89 %), HJT (74.5 %) and HST-1 (73.99 %). These isolates

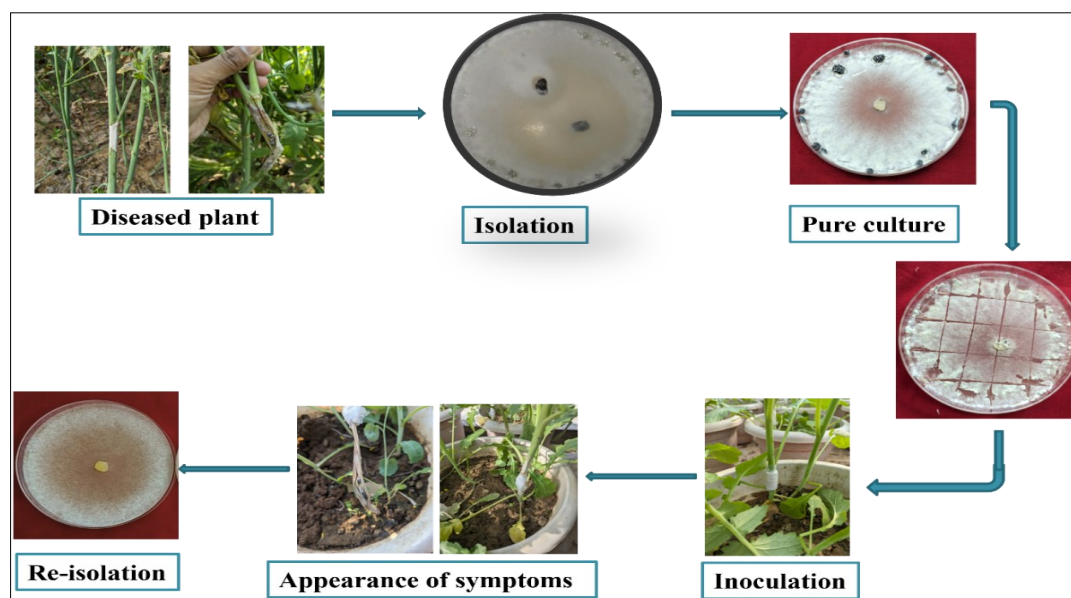


Fig. 1. Pathogenicity test of *Sclerotinia sclerotiorum* to causing Sclerotinia rot of mustard.

were statistically at par with one another, indicating similarly high antagonistic efficacy. On the other hand, isolates like HFrT (67.92 %), HGT (68.79 %) and HMT-1 (68.87 %) exhibited the lowest inhibition, with significantly lower performance compared to the top group as shown in Table 1 and Fig. 2 (a). Among all the eleven bacterial isolates tested, HRBa 3 stood out as the most effective in inhibiting the fungal growth of both *S. sclerotiorum* isolate SSR-1. It showed the highest mycelial inhibition, recording 69.29 % against SSR-1 isolate. These values were significantly higher than those of any other treatment and clearly indicated the superior antagonistic potential of this isolate. A second group of isolates demonstrated moderate efficacy against the SSR-1 isolate, include HCdBa 1 (42.84 %), HCdBa 2 (37.09 %), HRBa 2 (34.85 %) and HHBa 1 (36.43 %). These isolates performed similarly to each other

(statistically at par) but remained significantly less effective than HRBa 3 as shown in Table 1 and Fig. 2 (b). Among all the tested fungal and bacterial antagonists most promising isolates under *in vitro* were used for greenhouse experiment.

Molecular identification of most effective bioagents

Molecular identification of *Trichoderma* isolates based on phylogenetic analysis

Trichoderma isolate, HHT exhibiting strong antagonistic activity under *in vitro* conditions was subjected to molecular characterisation to confirm their species identity. Phylogenetic analysis based on ITS region sequences clearly resolved the evolutionary relationships among the experimental isolate and reference *Trichoderma* species (Fig. 3). The maximum likelihood

Table 1. Effect of fungal and bacterial antagonists on mycelial growth inhibition of *Sclerotinia sclerotiorum* under *in vitro* conditions

Sl.No.	<i>Trichoderma</i> isolates	Location	Percent mycelial growth inhibition
1.	HRT*	Bawal, Rewari	72.23 ^d ± 0.278
2.	HGT	Gurugram	68.79 ^g ± 0.377
3.	HST-1	Sirsa	73.99 ^c ± 0.231
4.	HCdT	Charkhi Dadri	70.27 ^{ef} ± 0.641
5.	HHT	Hisar	77.33 ^a ± 0.308
6.	HST-2	Sirsa	75.19 ^b ± 0.235
7.	HRoT	Rohtak	74.89 ^{bc} ± 0.216
8.	HFrT	Faridabad	67.92 ^g ± 0.482
9.	HFtT	Fatehabad	71.14 ^e ± 0.011
10.	HJT	Jhajjar	74.5 ^{bc} ± 0.400
11.	HBhT	Bhiwani	70.8 ^{ef} ± 0.255
12.	HMT-1	Mahendergarh	68.87 ^g ± 0.125
13.	HMT-2	Mahendergarh	69.90 ^f ± 0.361
14.	HKT	Kaul, Kaithal	70.43 ^{ef} ± 0.279
15.	HKrT	CSSRI, Karnal	70.32 ^{ef} ± 0.281
C.D. ($p \leq 0.05$)			0.95
Bacterial isolates			
1.	HKrBa 1	Ucchani, Karnal	4.54 ^g ± 1.11
2.	HKrBa 2	Ucchani, Karnal	3.74 ^g ± 1.46
3.	HKrBa 3	Ucchani, Karnal	8.01 ^{fg} ± 1.22
4.	HCBa 1	Charkhi Dadri	42.84 ^b ± 0.17
5.	HCBa 2	Charkhi Dadri	37.09 ^c ± 0.85
6.	HRBa** 1	Bawal, Rewari	17.42 ^e ± 0.23
7.	HRBa 2	Bawal, Rewari	34.85 ^c ± 1.07
8.	HRBa 3	Bawal, Rewari	69.29 ^a ± 0.09
9.	HHBa 1	Hisar	36.43 ^c ± 0.87
10.	HHBa 2	Hisar	8.67 ^f ± 1.75
11.	HHBa 3	Hisar	24.53 ^d ± 2.76
C.D. ($p \leq 0.05$)			3.72

*HRT: where 1st letter (H) stands for Haryana, 2nd letter (R) stands for district (Rewari) and 3rd letter (T) stands for *Trichoderma*; **HRBa: where 1st letter (H) stands for Haryana, 2nd letter (R) stands for district (Rewari) and 3rd letter (Ba) stands for Bacterial antagonist; ;CSSRI: ICAR–Central Soil Salinity Research Institute.

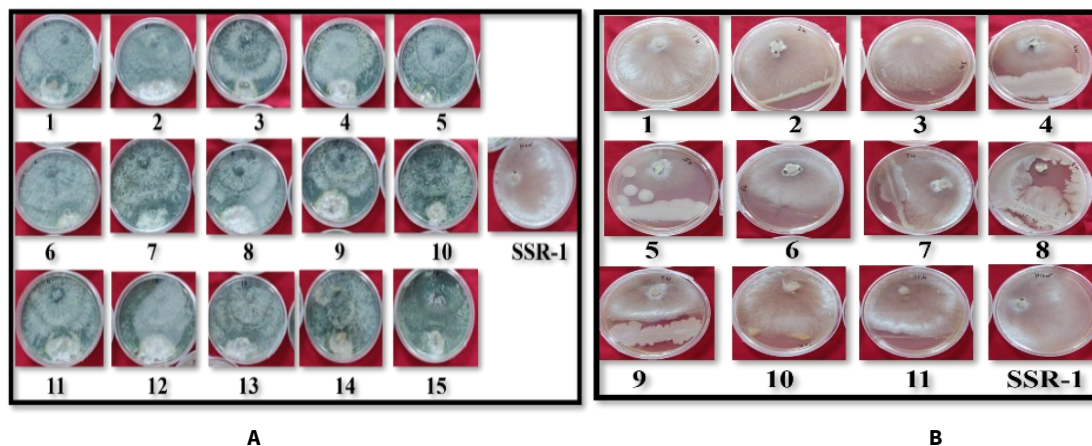


Fig. 2. Effect of different a) *Trichoderma* isolates and b) bacterial antagonists on mycelial growth inhibition of *Sclerotinia sclerotiorum*.

tree grouped the experimental isolate HHT (GenBank accession no. PP577651) within a well-supported *Trichoderma asperellum* clade, with a bootstrap value of 100 %, indicating a high degree of confidence in species assignment. The isolate clustered closely with reference strains of *T. asperellum*, including TDM11 (PP837620.1), BHU-BOT-RYRL16 (KR856222.1), strain G (KF723005.1), TNC52 (FJ004799.1), C2S505 (MN783048.1), SAGBF0005 (PZ142643.1), H531 (KY978581.1) and jz-3 (MG171157.1), demonstrating strong genetic similarity and confirming its taxonomic placement as *T. asperellum*. *Hypocrea lixii* CBS 132620 (NR111207.1) was used as the outgroup and formed a distinct lineage with strong bootstrap support (100 %), providing a reliable root for the phylogenetic tree. Short branch lengths among *T. asperellum* isolates suggest high sequence conservation within the species, whereas longer branch lengths separating different species reflect greater evolutionary divergence. The scale bar (0.01) represents approximately 1 % nucleotide sequence divergence, indicating the level of genetic distance among the analysed taxa and highlighting the effectiveness of ITS sequences for species discrimination within the genus *Trichoderma*. These results provided robust molecular evidence for the accurate identification of HHT as *T. asperellum* (GenBank accession no. PP577651).

Molecular identification of bacterial antagonists based on phylogenetic analysis

Bacterial isolates exhibiting strong antagonistic activity *in vitro* were subjected to molecular identification. Phylogenetic analysis based on 16S rRNA gene sequences resolved the evolutionary relationships among *Bacillus* species, including the experimental isolate HRBa3 and the reference outgroup *Bacillus cereus* ATCC 14579 (Fig. 4). The maximum likelihood tree showed that HRBa3 (accession no. PV750873) clustered tightly with well-characterised *Bacillus subtilis* strains such as WYY-10, NIOER132 and MF7, along with several other reference isolates, indicating high sequence similarity and strong genetic conservation within the species. In contrast, *B. cereus* formed a clearly separated lineage, confirming its suitability as an outgroup and highlighting its genetic divergence from the *B. subtilis* clade. These results provided robust molecular evidence for the accurate identification of HRBa3 as *Bacillus subtilis* (GenBank accession no. SUB15371155).

Antioxidative response for induction of resistance against *Sclerotinia sclerotiorum* inciting *Sclerotinia* rot in mustard

A comprehensive analysis of antioxidative biochemical responses in the mustard cultivars RH-30 (*B. juncea*) and HC-212 (*B. carinata*) was carried out under different treatments and time intervals following inoculation with *S. sclerotiorum*. The parameters included total soluble sugar (TSS), total phenol (TP), malondialdehyde (MDA), hydrogen peroxide (H_2O_2)

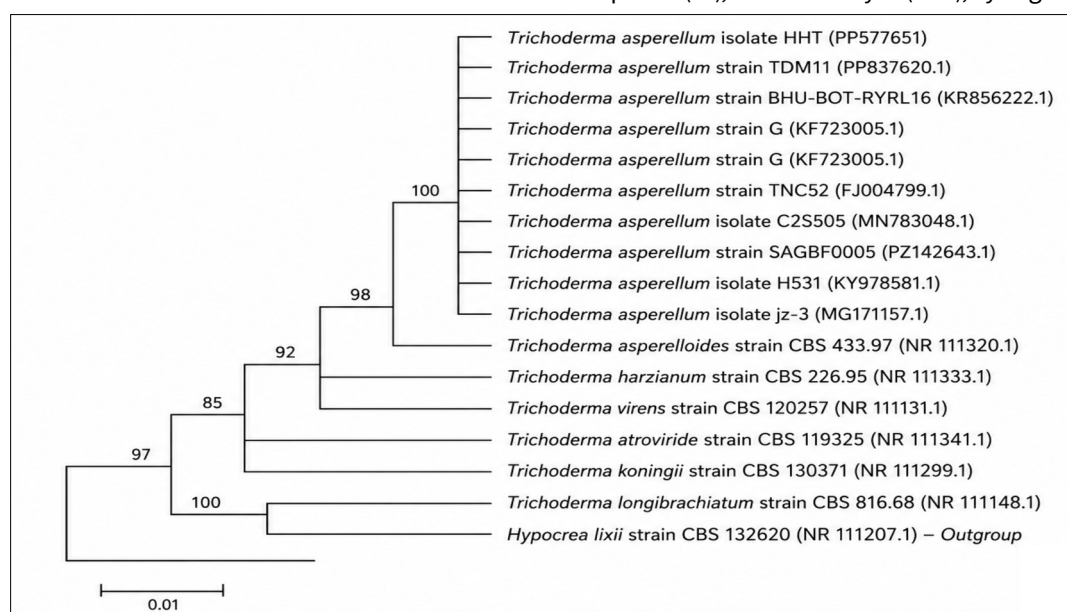


Fig. 3. Phylogenetic tree showing the relationship of *Trichoderma* isolates HHT with reference strains based on internal transcribed spacer region sequences.

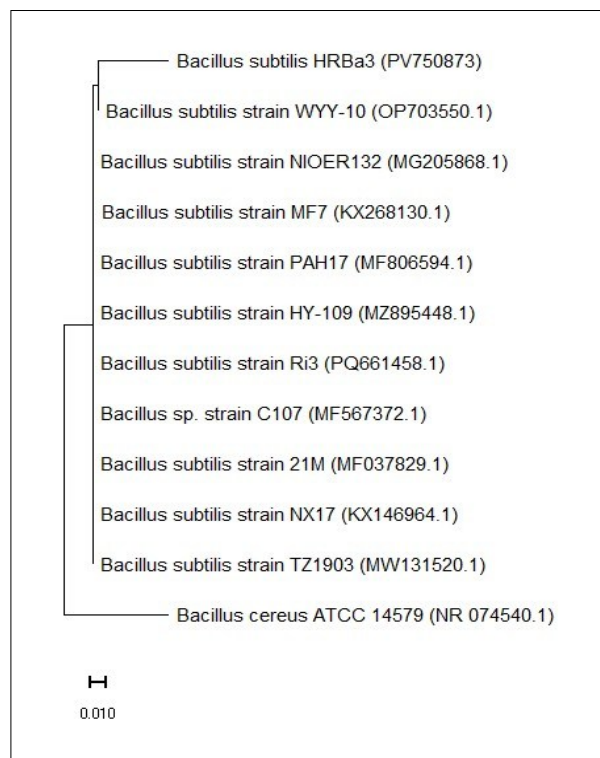


Fig. 4. Phylogenetic tree showing the relationship of bacterial isolates HRBa3 with reference strains based on 16S rRNA gene sequences. Numerical values: Pearson correlation coefficients (r -values); **Asterisks (*, **, ***): indicate statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$); Scatter plots: show relationships between each variable pair; Histograms with density plots: show distribution of each variable, Diagonal: distributions (histograms) of each individual variable.

and the activity of key defence enzymes like catalase (CAT), polyphenol oxidase (PPO), superoxide dismutase (SOD), peroxidase (POX), phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL). The findings are summarised in Table 2. The biochemical analysis of mustard cultivars RH-30 (*B. juncea*) and HC-212 (*B. carinata*) in response to *S. sclerotiorum* infection highlighted noticeable differences in how each cultivar coped with the pathogen over time and under different treatments.

Total soluble sugars not only serve as an immediate energy source but also participate in stress signalling, regulating reactive oxygen species (ROS) homeostasis by acting as both oxidants and antioxidants. In the present study, TSS levels gradually increased in both mustard cultivars up to 10 days post-inoculation (DPI), indicating enhanced mobilisation of carbohydrates to meet energy demands and osmotic adjustments under pathogen stress. Interestingly, RH-30 accumulated the highest TSS (5.38 mg glucose equivalents (GE) g^{-1} fresh weight (FW)) in the untreated control (T_0) at 10 DPI, suggesting that it relied more on sugar accumulation as a compensatory stress response rather than activating stronger antioxidative defences. Phenolics, on the other hand, play a pivotal role in structural and biochemical defence by contributing to lignin and suberin biosynthesis, thereby strengthening cell walls and hindering pathogen penetration. Total phenolic (TP) content was consistently higher in HC-212, reaching 4.77 mg catechol equivalents (CE) g^{-1} FW under T_1 (*Trichoderma* isolate HHT) at 10 DPI. This indicates that HC-212 effectively activated its phenylpropanoid pathway, facilitating the synthesis of antimicrobial phenolics that contribute to enhanced resistance against *S. sclerotiorum*. Malondialdehyde a reliable marker of lipid

peroxidation and cell membrane injury, progressively increased with disease development. At 10 DPI, RH-30 recorded higher MDA (2.22 nmol g^{-1} FW) than HC-212 (1.91 nmol g^{-1} FW) under the untreated control, reflecting greater oxidative damage in RH-30. By contrast, antagonist-treated plants maintained lower MDA levels, indicating reduced oxidative injury and better membrane stability.

A similar trend was evident for hydrogen peroxide (H_2O_2), a key ROS that serves dual roles as a damaging agent and a defence signal. Although both cultivars accumulated H_2O_2 upon infection, RH-30 showed a higher content (9.39 $\mu\text{mol g}^{-1}$ FW) than HC-212, suggesting a less efficient detoxification system. Controlled H_2O_2 accumulation in antagonist-treated plants indicates an active but tightly regulated signalling network rather than uncontrolled oxidative stress.

Enzymatic antioxidants showed clear cultivar differences, with HC-212 consistently outperforming RH-30. Catalase (CAT), which detoxifies H_2O_2 into water and oxygen, was significantly more active in HC-212, peaking at 7.33 $\mu\text{mol g}^{-1}$ FW under T_1 at 10 DPI. Similarly, polyphenol oxidase (PPO), involved in lignin formation and the conversion of phenolics into antimicrobial o-quinones, recorded higher activity in HC-212 (1.33 units g^{-1} FW) than RH-30 under the same treatment. Superoxide dismutase (SOD), the frontline ROS-scavenging enzyme, also showed stronger induction in HC-212 (2.93 units g^{-1} FW) compared to RH-30 (2.65 units g^{-1} FW). Peroxidase (POX), which reduces H_2O_2 and simultaneously contributes to cell wall lignification, followed the same pattern, with HC-212 reaching 0.82 units g^{-1} FW under T_1 at 10 DPI, slightly higher than RH-30 (0.76 units g^{-1} FW). Defence-related enzymes of the phenylpropanoid pathway were also more responsive in HC-212. Phenylalanine ammonia-lyase (PAL) activity peaked at 36.04 units g^{-1} FW and tyrosine ammonia-lyase (TAL) at 26.07 units g^{-1} FW in HC-212 under T_1 at 10 DPI, slightly higher than RH-30 (35.56 and 24.94 unit g^{-1} FW respectively). The lowest enzyme and metabolite levels were consistently recorded in untreated controls at 0 DPI, particularly in RH-30, highlighting its comparatively weaker basal defence.

Together, these results demonstrated that both cultivars activated antioxidative and defence-related biochemical pathways upon *S. sclerotiorum* infection, but HC-212 exhibited a more efficient and sustained response, particularly under *Trichoderma* treatment (T_1). This suggests a relatively stronger resistance mechanism in HC-212. This cultivar's ability to better manage oxidative stress and activate key defence pathways position it as more resilient to *S. sclerotiorum* compared to RH-30. The enhanced defence may also be attributed to the differential modes of action of antagonists. *Trichoderma* spp. was well-known to trigger induced systemic resistance (ISR) through jasmonic acid (JA) and ethylene (ET) signalling pathways, leading to the priming of plants for faster and stronger defence upon pathogen attack. They also promote lignification, cell wall reinforcement and accumulation of secondary metabolites. In contrast, *Bacillus* spp. often activated both ISR and systemic acquired resistance (SAR), primarily through salicylic acid (SA) dependent pathways and are reported to enhance antioxidant enzyme activity and phenolic metabolism. The crosstalk between jasmonic acid (JA)/ethylene (ET) and salicylic acid (SA) signalling pathways allows plants to fine tune their responses depending on the type of antagonist, with *Trichoderma* favouring stronger structural and oxidative defences, while *Bacillus* enhances biochemical and systemic responses. The

Table 2. Effect of different treatments on various biochemical parameters of mustard cultivars RH-30 and HC-212 inoculated with *Sclerotinia sclerotiorum*

DPI	Treatments	Variety	TSS	TP	MDA	H ₂ O ₂	CAT	PPO	SOD	POX	PAL	TAL
0 DPI	T ₁	RH-30	1.19	2.73	0.64	1.89	0.97	0.54	0.89	0.25	13.52	6.63
		HC-212	0.63	3.04	0.52	1.82	1.38	0.60	0.99	0.31	15.48	8.60
	T ₂	RH-30	1.54	2.45	0.72	2.63	0.78	0.36	0.81	0.20	11.84	6.00
		HC-212	0.97	2.73	0.60	2.28	1.24	0.51	0.93	0.22	12.28	8.00
	T ₃	RH-30	1.32	2.63	0.70	2.14	0.91	0.47	0.85	0.21	12.80	6.44
		HC-212	0.81	2.89	0.54	2.05	1.31	0.54	0.95	0.26	13.04	8.23
	T ₄	RH-30	1.60	2.30	0.75	3.35	0.69	0.33	0.62	0.19	11.16	5.83
		HC-212	1.17	2.67	0.62	2.68	1.17	0.39	0.88	0.20	12.12	7.90
	T ₅	RH-30	2.25	1.73	0.88	3.82	0.53	0.24	0.29	0.14	9.08	5.00
		HC-212	1.57	2.05	0.67	3.55	0.72	0.27	0.49	0.15	11.48	6.53
2 DPI	T ₁	RH-30	2.16	3.05	1.34	3.76	2.11	0.78	1.20	0.50	20.32	17.50
		HC-212	1.56	3.25	0.85	3.29	2.83	0.91	1.49	0.56	26.80	20.20
	T ₂	RH-30	2.73	2.79	1.47	5.31	1.89	0.64	1.02	0.45	19.68	15.76
		HC-212	2.00	3.04	0.72	4.35	2.27	0.75	1.26	0.49	25.44	18.70
	T ₃	RH-30	2.24	3.03	1.39	4.34	1.96	0.73	1.06	0.46	21.56	16.83
		HC-212	1.72	3.14	1.13	3.68	2.66	0.79	1.41	0.52	25.88	19.87
	T ₄	RH-30	2.78	2.54	1.57	5.48	1.59	0.60	1.00	0.42	18.12	14.83
		HC-212	2.07	3.01	1.01	4.65	1.83	0.64	1.17	0.45	24.72	18.57
	T ₅	RH-30	3.40	2.12	1.78	6.27	1.28	0.59	0.84	0.33	13.08	11.00
		HC-212	2.69	2.79	1.60	5.54	2.00	0.61	1.00	0.38	14.28	12.63
5 DPI	T ₁	RH-30	2.76	3.61	1.49	5.32	6.30	1.12	1.87	0.69	30.16	20.30
		HC-212	2.24	3.88	1.24	4.68	6.72	1.21	2.63	0.73	33.04	21.33
	T ₂	RH-30	3.12	3.09	1.55	5.88	5.96	0.80	1.61	0.60	25.40	18.53
		HC-212	2.77	3.53	1.50	5.40	6.24	0.95	1.87	0.63	29.76	20.67
	T ₃	RH-30	2.91	3.24	1.52	5.60	6.11	1.09	1.79	0.64	28.52	19.10
		HC-212	2.58	3.71	1.31	3.86	6.46	1.13	2.10	0.71	31.96	20.90
	T ₄	RH-30	3.26	3.03	1.57	6.35	5.73	0.74	1.56	0.60	22.44	18.17
		HC-212	3.09	3.44	1.47	5.79	6.12	0.86	2.32	0.61	28.96	20.07
	T ₅	RH-30	5.00	2.80	1.96	7.62	3.31	0.67	1.32	0.38	16.16	13.23
		HC-212	4.08	3.24	1.70	6.89	3.72	0.72	1.67	0.40	23.08	15.00
10 DPI	T ₁	RH-30	3.84	4.18	1.83	6.92	6.89	1.22	2.65	0.76	35.56	24.03
		HC-212	3.24	4.77	1.65	6.35	7.33	1.33	2.93	0.82	36.04	26.07
	T ₂	RH-30	4.18	4.07	1.96	8.05	6.65	1.13	2.15	0.72	32.20	23.00
		HC-212	3.52	4.37	1.78	7.19	6.92	1.17	2.69	0.77	34.72	24.37
	T ₃	RH-30	4.02	4.21	1.91	7.57	6.80	1.18	2.47	0.75	33.92	23.47
		HC-212	3.40	4.58	1.76	6.72	7.12	1.22	2.82	0.79	35.40	25.20
	T ₄	RH-30	4.37	4.09	2.01	8.26	6.62	1.08	2.10	0.70	29.84	21.70
		HC-212	3.69	4.31	1.81	7.52	6.78	1.14	2.77	0.75	31.60	24.03
	T ₅	RH-30	5.38	3.70	2.22	9.39	4.22	0.89	1.78	0.47	23.84	16.17
		HC-212	4.99	3.97	1.91	8.81	4.59	1.00	1.95	0.49	26.84	16.66
15 DPI	T ₁	RH-30	3.34	3.48	1.60	5.87	5.84	1.12	2.41	0.73	29.40	23.70
		HC-212	2.60	3.73	1.55	4.69	6.27	1.15	2.63	0.82	31.80	23.87
	T ₂	RH-30	3.71	3.21	1.75	6.37	5.29	0.81	2.12	0.70	27.68	22.17
		HC-212	2.88	3.37	1.60	5.19	5.77	1.01	2.31	0.78	30.48	22.90
	T ₃	RH-30	3.41	3.41	1.73	6.15	5.76	0.99	2.22	0.71	28.60	23.00
		HC-212	2.64	3.54	1.57	4.95	6.16	1.08	2.41	0.82	30.80	23.37
	T ₄	RH-30	3.98	3.15	1.78	6.57	5.20	0.77	2.02	0.68	27.88	21.40
		HC-212	3.05	3.31	1.68	5.52	5.45	0.98	2.16	0.74	30.28	22.20
	T ₅	RH-30	4.24	3.04	1.81	7.59	3.46	0.64	1.67	0.32	23.08	15.00
		HC-212	3.34	3.12	1.73	6.18	4.23	0.80	1.89	0.44	24.00	16.27
C.D. (<i>p</i> ≤ 0.05)			0.13	0.15	0.06	0.22	0.19	0.04	0.07	0.02	1.10	0.80
SE(m) ±			0.05	0.05	0.02	0.08	0.07	0.01	0.03	0.01	0.39	0.28

*TSS (mg GE g⁻¹ FW): total soluble sugar; TP (mg CE g⁻¹ FW): total phenol; MDA (nmol g⁻¹ FW): malondialdehyde; H₂O₂ (μmol g⁻¹ FW): hydrogen peroxide; CAT (μmol g⁻¹ FW): catalase; PPO (units g⁻¹ FW): polyphenol oxidase; SOD (units g⁻¹ FW): superoxide dismutase; POX (units g⁻¹ FW): peroxidase; PAL (units g⁻¹ FW): phenylalanine ammonia lyase; TAL (units g⁻¹ FW): tyrosine ammonia lyase; DPI: days post inoculation.

observed superiority of T₁ in enhancing enzyme activities and phenolic content underscored its role in priming HC-212 for more robust resistance against *S. sclerotiorum*.

Correlation analysis of biochemical defence markers in mustard cultivars under *Sclerotinia sclerotiorum* infection

Correlation analysis revealed a highly coordinated network of biochemical and enzymatic defence responses in mustard cultivars RH-30 challenged with *Sclerotinia sclerotiorum* as mentioned in Fig. 5 and Supplementary Fig. 1. The strong associations among oxidative stress markers, antioxidant enzymes and phenylpropanoid pathway-related enzymes suggest that these defence components operate in a synchronised manner during resistance induction. Increased accumulation of soluble sugars was closely linked with oxidative stress responses,

indicating their role in energy supply, osmotic adjustment and signalling during pathogen challenge.

The positive relationships among H₂O₂, MDA and antioxidant enzymes demonstrate that pathogen-induced oxidative stress triggers an efficient ROS-scavenging system. Enhanced activities of CAT, SOD, PPO and POX likely contribute to maintaining cellular redox homeostasis while preventing excessive oxidative damage. Such coordinated antioxidant responses have been widely recognised as key determinants of disease resistance in *Brassica* and other crop species.

A notable outcome of the correlation analysis was the close association among PAL, TAL, PPO and POX, highlighting the central role of the phenylpropanoid pathway in defence regulation. Activation of this pathway promotes the synthesis of phenolic

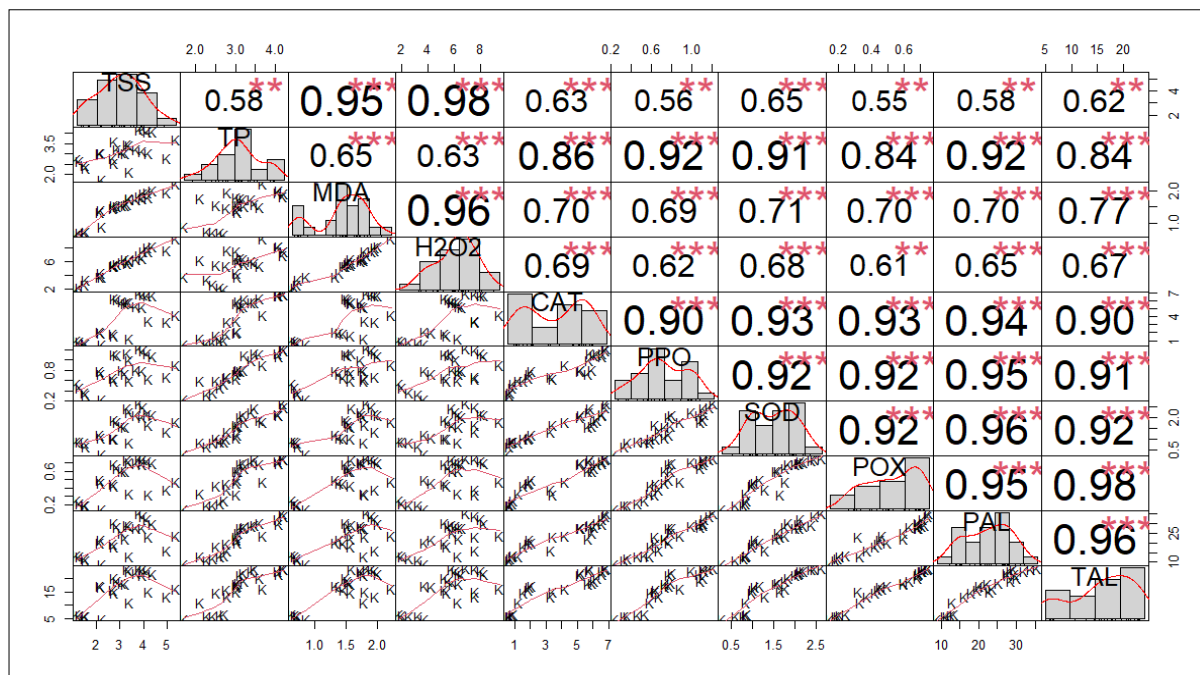


Fig. 5. Comparative analysis of biochemical defence responses in RH-30 mustard cultivar against *Sclerotinia sclerotiorum*. Numerical values: Pearson correlation coefficients (r -values); *Asterisks (*, **, ***) indicate statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$); Scatter plots: show relationships between each variable pair; Histograms with density plots: show distribution of each variable, Diagonal: distributions (histograms) of each individual variable.

compounds, lignin and other secondary metabolites that strengthen cell walls and restrict pathogen invasion. The strong linkage between phenolic content and defence-related enzymes further suggests that phenolics contribute not only to antioxidant protection but also to structural reinforcement and antimicrobial defence.

Similarly, correlation analysis in mustard cultivar HC-212 exposed to *Sclerotinia sclerotiorum* and treated with antagonists revealed a highly coordinated defence network (Fig. 6 and Supplementary Fig. 2). Strong positive associations were observed among oxidative stress markers, antioxidant enzymes and phenylpropanoid pathway-related enzymes, indicating a close linkage among key defence-related processes. Total soluble

sugars showed strong associations with MDA and H₂O₂, while oxidative stress markers were positively associated with antioxidant enzymes including CAT, SOD, PPO and POX. In addition, strong correlations among PAL, TAL, PPO and POX, along with their association with total phenol content, suggest coordinated variation between phenolic metabolism and antioxidant defence responses. These patterns indicate that multiple biochemical defence components responded in a synchronised manner during pathogen challenge and resistance induction.

Comparison of the two cultivars indicated that HC-212 exhibited a more integrated and coordinated defence network than RH-30. The stronger association among antioxidant and

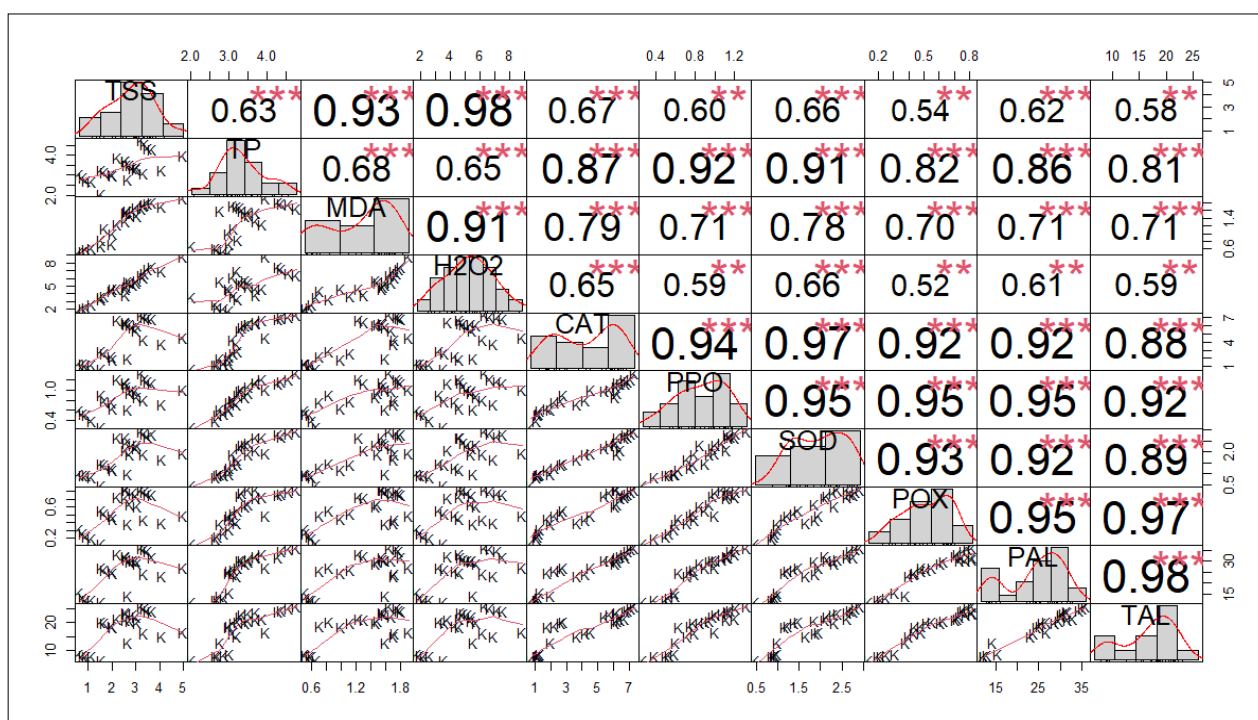


Fig. 6. Comparative analysis of biochemical defence responses in HC-212 mustard cultivars against *Sclerotinia sclerotiorum*.

phenylpropanoid-related components in HC-212 suggests a greater capacity to regulate oxidative stress and activate defence metabolism following pathogen attack. This enhanced biochemical coordination may explain its superior resistance response against *S. sclerotiorum*.

Overall, the findings support the concept that successful resistance against *S. sclerotiorum* involves the coordinated interaction of ROS generation, antioxidant detoxification and phenylpropanoid-mediated defence mechanisms. The superior biochemical integration observed in HC-212 highlights its potential value as a genetic resource for resistance breeding and for the development of sustainable disease management strategies.

Protein expression analysis from antagonist-pathogen treated plant

The data presented in Table 3 and Supplementary Fig. 3 illustrated the effect of different antagonist treatments on total soluble protein (TSP) content in mustard cultivar RH-30 at different intervals (0, 2, 5 and 10 DPI) under *S. sclerotiorum* inoculation. In general, all treatments led to a gradual increase in total soluble protein content over time. Treatment T₁ (HHT) consistently led to the highest protein levels, reaching 12.46 mg bovine serum albumin (BSA) g⁻¹ FW at 10 DPI, with an overall averaging 9.34. Furthermore, T₃ (HRBa-3) showed strong performance, peaking at 12.06 mg BSA g⁻¹ FW and averaging 8.88 across all time points. On the other hand, the diseased control (T₅), which was infected but untreated, showed a slower increase in protein content and remained significantly lower at all time points. The healthy control (T₆) had the lowest protein levels throughout the study, likely because no stress or treatment triggered a defensive response. Statistical analysis confirmed that the differences between treatments, time points and their combinations were significant at the 5 % level, indicating that the observed changes in protein

content were meaningful and influenced by the treatments. Similar results were observed in cultivar HC-212, as presented in Table 4 and Supplementary Fig. 3. Here too, protein content increased over time in all treatments, with T₁ (HHT) again showing the most substantial effect. At 10 DPI, protein levels in T₁ reached 13.32 mg BSA g⁻¹ FW, with a mean value of 11.15. Following this, T₃ (HRBa 3) peaking at 12.75 and averaging 10.65. The T₂ and T₄ treatments also improved protein expression. Diseased control (T₅) showed moderate protein levels (mean: 7.46), while the healthy control (T₆) remained the lowest (mean: 5.47), like RH-30. Here too, statistical analysis indicated that treatment effects, sampling time and their interactions were all statistically significant at the 5 % level. In both cultivars, total soluble protein levels were significantly enhanced by the application of antagonists, especially in T₁ followed by T₃. Protein accumulation was higher in cultivar HC-212 as compared to RH-30 over time. These treatments likely stimulated the plant's defence mechanisms, leading to increased protein synthesis. Overall, the results highlighted the potential of biological antagonists in strengthening plant resistance to *S. sclerotiorum* by boosting protein expression as part of the defence response.

Protein expression profiling of different treatments in cultivar HC-212 and RH-30 against *Sclerotinia sclerotiorum*

To better understand how protein expression changes in response to *S. sclerotiorum* infection, equal amounts of total protein from each sample were separated using SDS-PAGE. The resulting gels, stained with Coomassie Brilliant Blue (CBB), revealed a clear pattern of distinct protein bands. The protein expression profiles, as visualised in the SDS-PAGE gel image (Fig. 7), revealed distinct patterns across different treatments and time points in both mustard cultivars, HC-212 and RH-30 after inoculation with *S. sclerotiorum*. Overall, an increase in band intensity was observed in both cultivars with increasing DPI, reflecting higher protein

Table 3. Effect of different treatments on total soluble protein (mg BSA g⁻¹ FW) in cultivar RH-30 against *Sclerotinia* rot of mustard

RH-30 (mg BSA g ⁻¹ FW)					
Treatments	0 DPI	2 DPI	5 DPI	10 DPI	Mean A
T ₁ (First most effective fungal antagonist i.e. HHT)	6.33	8.34	10.22	12.46	9.34
T ₂ (Second most effective fungal antagonist i.e. HST-2)	5.80	6.63	9.28	11.24	8.24
T ₃ (First most effective bacterial antagonist i.e. HRBa 3)	6.11	7.47	9.88	12.06	8.88
T ₄ (Second most effective bacterial antagonist i.e. HKrBa 3)	5.99	6.31	9.18	10.15	7.91
T ₅ (Diseased Control)	3.52	5.26	6.48	7.99	5.81
T ₆ (Healthy Control)	3.62	3.83	5.51	5.63	4.65
Mean B	5.23	6.31	8.43	9.92	
Factors	C.D. ($p \leq 0.05$)			SE(m) $\pm$	
Treatments (A)	0.178			0.062	
DPI (B)	0.145			0.051	
(A $\times$ B)	0.355			0.125	

DPI: days post-inoculation; BSA: bovine serum albumin; FW: fresh weight; C.D.: critical difference at $p \leq 0.05$; SE(m): standard error of mean.

Table 4. Effect of different treatments on total soluble protein (mg BSA g⁻¹ FW) in cultivar HC-212 against *Sclerotinia* rot of mustard

HC-212 (mg BSA g ⁻¹ FW)					
Treatments	0 DPI	2 DPI	5 DPI	10 DPI	Mean A
T ₁ (First most effective fungal antagonist i.e. HHT)	8.06	10.77	12.46	13.32	11.15
T ₂ (Second most effective fungal antagonist i.e. HST-2)	7.32	10.02	11.41	12.13	10.22
T ₃ (First most effective bacterial antagonist i.e. HRBa 3)	7.76	10.35	11.73	12.75	10.65
T ₄ (Second most effective bacterial antagonist i.e. HKrBa 3)	6.94	9.67	11.07	11.69	9.84
T ₅ (Diseased Control)	4.27	6.98	8.71	9.88	7.46
T ₆ (Healthy Control)	4.29	5.34	5.99	6.27	5.47
Mean B	6.44	8.86	10.23	11.01	
Factors	C.D. ($p \leq 0.05$)			SE(m) $\pm$	
Treatments (A)	0.184			0.062	
DPI (B)	0.15			0.051	
(A $\times$ B)	0.368			0.125	

DPI: days post-inoculation; BSA: bovine serum albumin; FW: fresh weight; C.D.: critical difference at $p \leq 0.05$; SE(m): standard error of mean.

accumulation as the infection progressed. However, cultivar HC-212 consistently showed stronger and more defined bands than RH-30, especially at 10 DPI, suggesting a more robust protein-based defence response. At 0 DPI, a protein band of approximately 55 kDa was visible in all treatments, but it appeared most intense in T₁ (HHT) in HC-212, followed by T₃ (HRBa 3). By 2 and 5 DPI, in addition to the 55 kDa bands, new bands around 35 kDa began to emerge. These were again more prominent in HC-212, especially under T₁, highlighting its continued and early activation of defence-related proteins. At 10 DPI, a broader range of protein bands was observed, including higher molecular weight bands (~75 kDa) and lower bands in the 25–35 kDa range. The intensity of these bands was significantly greater in HC-212 than in RH-30, particularly in antagonist-treated plants, suggesting enhanced synthesis of defence-related proteins in response to pathogen challenge. Importantly, plants treated with antagonists consistently exhibited more intense protein bands than those in the diseased control,

indicating greater protein accumulation under stress. This supports the idea that biocontrol treatments not only mitigate disease but also stimulate host defence mechanisms, leading to elevated expression of stress and pathogen-related proteins.

Discussion

Antagonistic potential of bioagents and molecular confirmation

In vitro evaluation revealed substantial variability among fungal and bacterial antagonists in their ability to inhibit the mycelial growth of *S. sclerotiorum*, emphasising the necessity of isolate-specific selection for effective biological control. Among the fungal antagonists, isolate HHT exhibited the highest inhibitory activity, followed by HST-2, HRoT and HJT, indicating superior antagonistic competence. The strong suppression of the pathogen by these isolates is likely attributable to the well-established mechanisms of

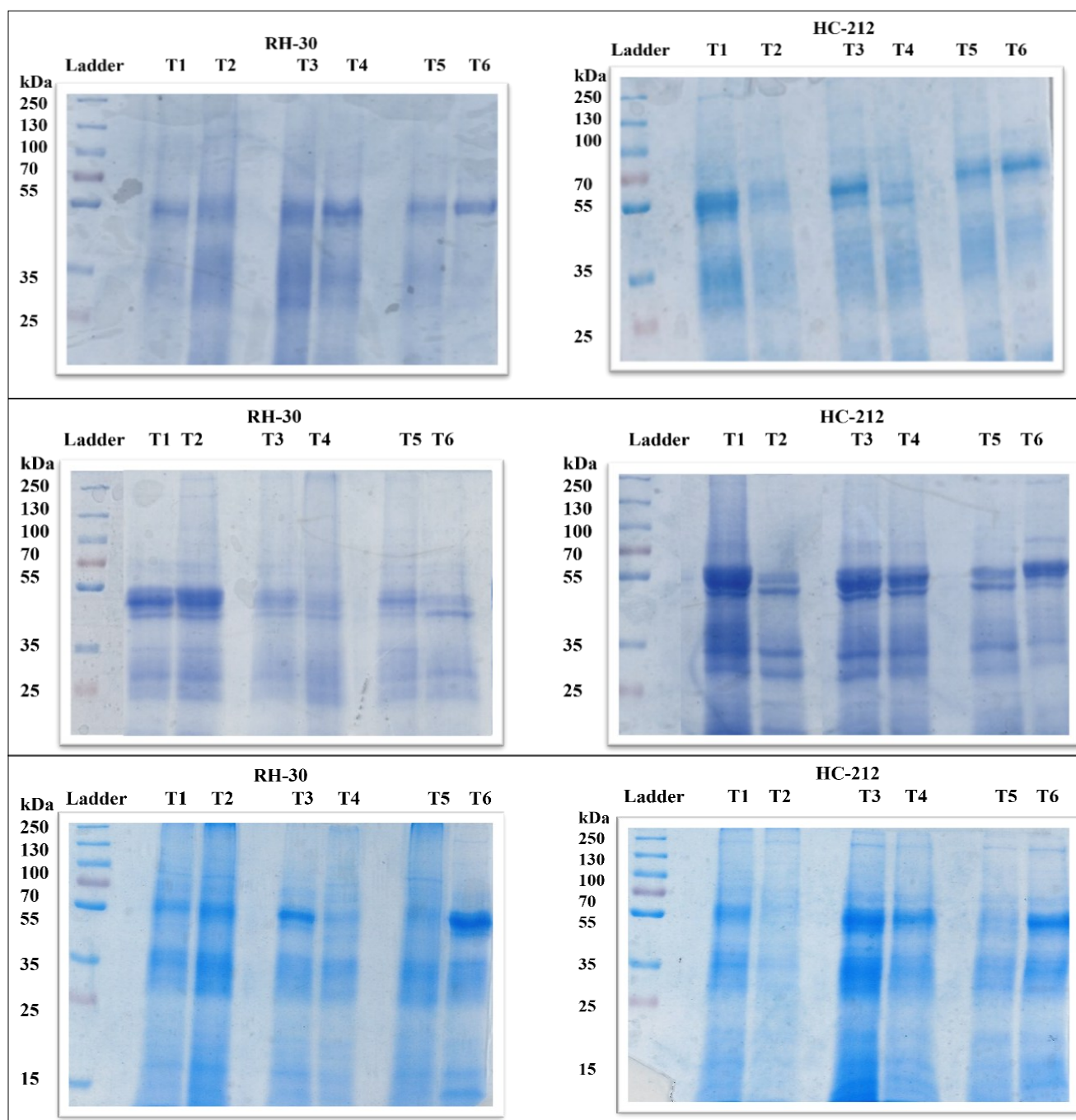


Fig. 7. Protein expression profile of various treatments in cultivar RH-30 and HC-212 against *Sclerotinia* rot of mustard at 0, 2, 5 and 10 days post inoculation by using SDS-PAGE. DPI: days post-inoculation

Trichoderma spp., including rapid rhizosphere colonisation, nutrient and space competition, antibiosis, mycoparasitism and secretion of cell wall-degrading enzymes such as chitinases and β -1,3-glucanases. Among the bacterial antagonists, isolate HRBa3 exhibited the strongest inhibitory effect against *S. sclerotiorum*, indicating its high antagonistic potential. Molecular characterisation corroborated the biological assays by identifying isolate HHT as *T. asperellum* and HRBa3 as *B. subtilis*. The close agreement between molecular identification and *in vitro* antagonistic performance reinforces the reliability of these isolates as promising candidates for sustainable management of Sclerotinia rot. The present findings are consistent with earlier reports demonstrating strong antagonism of *Trichoderma* and *Bacillus* species against *S. sclerotiorum*. More than 56 % mycelial inhibition and complete suppression of sclerotia by *Trichoderma hamatum* and *Trichoderma harzianum* has been reported (23). High antagonistic activity of *T. asperellum* isolates from lettuce rhizospheres, with inhibition up to 97.73 %, has also been documented, highlighting the broad biocontrol potential of *Trichoderma* spp. across crop systems (24). Similarly, *Trichoderma* isolates screened from mustard rhizosphere soils showed that isolates PBTMSR4 and PBTMSR8 reduced Sclerotinia stem rot incidence by up to 70 % and 60 % respectively, under field conditions (25).

Comparable inhibitory effects have also been reported for bacterial antagonists. Up to 82.5 % inhibition of *S. sclerotiorum* by *Bacillus* isolates has been documented (26), while 85.71 % inhibition was reported using *B. subtilis* strain LHS11 (9). Furthermore, *B. subtilis* strain BS-2301 has been shown to effectively suppress mycelial growth and reduce oxalic acid production, a key virulence determinant of *S. sclerotiorum* (27). In addition, *B. cereus* HF10 has been identified as a potent antagonist achieving 79 % mycelial inhibition following large-scale rhizospheric screening (28).

Antagonist-induced antioxidative defence, protein modulation and integrated resistance responses in mustard

Antagonist treatments markedly enhanced the activity of key antioxidant enzymes, including catalase, superoxide dismutase, polyphenol oxidase and peroxidase, reflecting an effective activation of host defence machinery in response to *S. sclerotiorum*. The cultivar HC-212 exhibited higher and more sustained induction of these enzymes compared with RH-30, indicating superior defensive competence. The coordinated upregulation of SOD and CAT suggests an efficient reactive oxygen species (ROS) detoxification system, thereby maintaining cellular redox homeostasis under pathogen stress. Elevated PPO and POX activities further point toward enhanced lignification, phenolic oxidation and oxidative cross-linking of cell wall polymers, which collectively contribute to the reinforcement of physical barriers and restriction of pathogen colonisation. Notably, enzymes of the phenylpropanoid pathway, phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL), showed the strongest induction in HC-212, especially under *Trichoderma*-based treatments. These enzymes function as key metabolic gateways for the biosynthesis of phenolics, lignin, flavonoids and other defence-related secondary metabolites. Their pronounced activation indicates a primed physiological state, enabling faster and more effective defence responses upon pathogen challenge. The present findings agree with earlier reports highlighting stronger antioxidant enzyme activity in resistant or moderately resistant *Brassica* genotypes.

Enhanced CAT, SOD and POX activities have been documented in resistant cultivars (29), while other studies similarly observed elevated defence enzyme levels in mustard genotypes exhibiting moderate resistance to *S. sclerotiorum* (30, 31). Furthermore, it has been confirmed that *Trichoderma* spp. and *B. subtilis* effectively stimulate antioxidant and defence-related enzymes, thereby strengthening host resistance (27, 32, 33).

The comparatively higher biochemical responsiveness of HC-212 observed in the present study suggests a more efficient induction of resistance by fungal antagonists than in RH-30. In addition to enzymatic defences, antagonist-treated plants exhibited significantly enhanced accumulation of total soluble proteins, further supporting the activation of defence-related metabolic processes. The higher protein content observed in HC-212, particularly under *Trichoderma* and *Bacillus* treatments, indicates increased synthesis of stress-responsive and defence-associated proteins. The SDS-PAGE profiling revealed the induction of distinct protein bands, especially within the 25–75 kDa range, which became more pronounced with pathogen challenge and antagonist application. These proteins likely correspond to pathogenesis-related (PR) proteins, ROS-detoxifying enzymes, molecular chaperones and proteins involved in signal transduction and protein folding. Correlation analysis provided deeper insight into the coordinated nature of host defence responses. Strong positive correlations among ROS-related parameters, antioxidant enzymes, phenylpropanoid pathway enzymes and protein accumulation demonstrated that mustard plants deploy an integrated and tightly regulated defence network rather than isolated biochemical responses. The consistently stronger and more extensive correlations observed in HC-212 suggest superior signalling efficiency and regulatory control, enabling this cultivar to balance ROS generation with detoxification while simultaneously confirming structural and biochemical defences. The enhanced and diversified protein expression patterns observed in HC-212 reflect a more robust molecular defence machinery, allowing effective containment of pathogen invasion. Numerous defence-related proteins have been identified in *Brassica napus* L. during *S. sclerotiorum* infection, many of which were differentially expressed between resistant and susceptible lines (34, 35). These findings are further supported by reports indicating the upregulation of peptidyl-prolyl cis-trans isomerase (PPIase), ethylene biosynthesis-related enzymes and proteins involved in ROS detoxification and cellular homeostasis as key components of induced resistance (32, 36).

Overall, the superior performance of HC-212 underscores the importance of host genotype in determining the success of ISR-based strategies. The synergistic interaction between effective bioagents and a responsive host genotype offers a sustainable and environmentally friendly alternative to chemical control for managing Sclerotinia rot of mustard.

Conclusion

Trichoderma asperellum (HHT) and *Bacillus subtilis* (HRBa3) were identified as highly effective antagonists of *Sclerotinia sclerotiorum*, combining strong *in vitro* inhibition with robust induction of resistance under greenhouse conditions. Both mustard cultivars activated antioxidant and defence pathways. However, HC-212 (*Bacillus carinata*) exhibited a consistently stronger response, marked by enhanced phenolic accumulation, efficient ROS detoxification and

pronounced activation of the phenylpropanoid pathway than RH-30 (*Bacillus juncea*). Increased accumulation of defence-related proteins, particularly under *Trichoderma* HHT treatment, further confirmed effective systemic resistance. Collectively, these findings highlight HC-212 as a more resilient host and underscore the potential of HHT and HRBa3 as eco-friendly biocontrol agents for integrated management of *Sclerotinia* stem rot in mustard.

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Authors' contributions

DK and NKY conceived and designed the study. DK, RP and AK conducted the experiments and collected the data. DK, P² and PV performed the data analysis. NKK, AK and P² assisted in laboratory work and compilation of experimental results. DK, P¹ prepared the manuscript. All authors have read and approved the final manuscript. [P¹ stands for Pooja & P² stands for Pratibha]

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that Artificial Intelligence (AI) tools were used only for language editing, grammar correction, sentence restructuring and improvement of manuscript readability. The AI tool used was ChatGPT (OpenAI). All scientific content, study design, data collection, data analysis, interpretation of results, conclusions and final manuscript revisions were performed, verified and approved by the authors. The authors take full responsibility for the accuracy, originality and integrity of the work presented in this manuscript.

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