Trehalose: A mycogenic cell wall elicitor elicit resistance against leaf spot disease of broccoli and acts as a plant growth regulator

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ABSTRACT

Elicitors are biochemicals, and the cell wall-derived elicitors from fungi can trigger defence mechanisms in plants by increasing the phytoalexin accumulation when they encounter the pathogens. The main objective of this research was to purify and characterize a cell wall elicitor from *Trichoderma atroviride (TaCWE)* and evaluate the seed priming effect of that elicitor for inducing systemic resistance in broccoli plants against leaf spot disease. Amongst the tested TaCWE concentrations of the seed priming (5, 10, & 25 mg ml^{-1}), 10.0 mg ml^{-1} showed significantly ($P < 0.05$) improved early emergence, the rate of germination at 94%, and observed seedling vigour of 2601. Also, elicitor (10 mg ml^{-1}) treatment alone induced 57% plant protection. On the contrary, the elicitor treated and pathogen inoculated plants induced a notable 72% protection against leaf spot disease of broccoli caused by *A. brassicicola*. Thus, the primed seeds with elicitor showed induced disease resistance and plant growth promotion. The prominent molecule present in the purified extracted cell wall elicitor is confirmed as trehalose. The AFM analysis indicated the trehalose length and width as 10.16 μm and 2.148 μm, respectively. FTIR chromatogram further confirmed trehalose in abundance with traces of carbon, hydrogen, nitrogen, oxygen, and LC-MS profile with a single peak eluted with a retention time of 3.78 min. The findings of this study contribute to understanding better the role of trehalose, a biogenic cell-wall elicitor that can induce systemic resistance against leaf spot disease and regulate plant growth in the broccoli plants.

1. Introduction

Elicitors are the biochemicals that trigger plants to produce phytoalexin or secondary metabolites excessively by modifying the physiology of the cell system. Elicitors can trigger defence in both host and non-host plants upon encountering potential pathogens [1–4]. Generally, elicitors are produced mainly by the microbial cell walls as structural components such as chitin, glucan, lipopolysaccharides (LPS) and flagellin that protects the plants by inducing defence responses against pathogen infection [5]. There are high-affinity binding sites specifically present for peptide, glycopeptide and oligosaccharide elicitors, which recognize the involvement of functional elicitor binding proteins [4,6]. All these elicitors belong to different families, including proteins, sugars, and lipids that produce a different array of plant defence response during plant-pathogen interaction [7]. These elicitors act as an indicator molecule at little concentrations and activate defence responses in the host plant [8,9]. Koike et al. [10] have reported that the induction of cucumber seedling hypocotyls lignification was due to culture filtrates of plant growth-promoting fungi (PGPF) such as *Trichoderma* sp., *Fusarium* sp., *Penicillium* sp., *Phoma* sp. and a sterile fungus following challenge inoculation with *Colletotrichum orbiculare*. amongst elicitors, trehalose, a non-reducing disaccharide distributed ubiquitously, acts as a biogenic cell wall elicitor [11]. Biochemically, trehalose has the ability to stabilize lipids and protein membrane [12]. The metabolism of trehalose is fundamental for few metabolic pathways in general, for example, biosynthesis, carbon assimilation, sugar status, and ruin of starch in plants [13–15]. In addition, disaccharide trehalose produced by microorganisms is an excellent metabolic osmoregulator that upregulates defence signalling during biotic and abiotic stresses [12,16,17]. Trehalose is produced in the plants during abiotic stresses such as drought, salinity and oxidative stress [18,19]. Subsequently, these plants can survive the extended dry conditions and when the favourable conditions return, the trehalose can partially or completely hydrolysed to reinitiate the plant growth. In other words, trehalose can act an osmoprotectant against salinity stress in wheat plants [20]. Thus, it stabilizes protein in its native state at high temperatures in living cells

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[21].

Trichoderma species are proposed chiefly as major PGPF in the natural environment with the ability to carry elicitors that could promote rapid plant growth, reproduction, high crop productivity, uptake of soil nutrients, greater yield, and resistance to abiotic and biotic stress [22–25]. In recent years, more and more research has been focussed on resistance against plant diseases exploited through induced systemic resistance (ISR) caused as a result of root symbiotic relationships of *Trichoderma* [26–28]. Along with the revelation of the diverse beneficial anti-fungal mechanism of *T. atroviride* and other *Trichoderma* species, the ability to promote plant growth and to increase other growth parameters such as the plant height, leaf area, and dry weight is evident from various researches [29–32].

Broccoli is a hardy biennial crop that belongs to the family Brassicaceae. It is grown as a cool-season annual plant and grows worldwide in a much cooler climate, forming tiny to large blue-green flower buds either in single or multiple flower heads [33]. The leaf spot fungal disease caused by *Alternaria brassicicola* is found to be the most damaging disease amongst Brassicaceae members at all phases of the plant growth from the nursery until the reproductive stage [34–37]. Therefore, disease management schemes using chemical and resistant varieties of plants against leaf spot disease of broccoli have constraints and inadequacies [38]. Thus, finding a suitable alternative method that complements resistance plant breeding with lesser chemical usage by innovative stratagems is inevitable. It can be achieved by inducing resistance in the host that enhances the plant defenses by PGPF [39,40]. However, there are minimal reports on the use of proposed PGPF elicitors for leaf spot disease management in broccoli. Hence, the present study aimed to (i) investigate the isolation and characterization of cell wall elicitor from PGPF, *Trichoderma atroviride*, (ii) evaluate the seed priming effect of elicitor for induction of resistance against leaf spot disease in broccoli, and (iii) ascertain the dual role of elicitor in promoting plant growth observed in elicited plants.

2. Materials and methodology

2.1. Microbial culture (Trichoderma atroviride) and growth conditions

2.1.1. Microbial culture

The isolate *Trichoderma atroviride (*TriAt_JSB2) having accession No. JQ665257*,* used in this study, was well characterized with multi-faceted beneficial characters including induce resistance and plant growth promoter [29]. This was available at plant healthcare and diagnostics centre, P.G. Department of Biotechnology and Microbiology, Karnatak University, Dharwad. The fungus was cultured on Potato Dextrose Agar (PDA) media at pH 7.0 and incubated for seven days at 27 °C. Emerging hyphae from the above culture plate were inoculated in 250 ml of Potato Dextrose Broth (PDB) and kept for seven days of incubation at 27 °C. Finally, the obtained dry fungal mat (5 gm) was used in the extraction of the fungal cell wall elicitor.

2.1.2. Extraction of cell wall elicitor from trichoderma atroviride (TaCWE)

Cell wall extract from *T. atroviride* isolate was formulated as per the method described by Jishaa et al. [41]. Briefly, mycelium from 7-day-old PDB culture was homogenized with liquid nitrogen and crushed using mortar and pestle. The homogenate was filtered using a muslin cloth, and then the obtained residues were washed with double distilled water, followed by chloroform and methanol (1:1). The suspension was washed later with acetone. Finally, the obtained suspension was air-dried, and the cell wall material was recovered. The extract from the mycelia cell wall was obtained by suspending 1 g of the cell wall in 100 ml of distilled water and autoclaved. Later, the suspension was centrifuged at 14,000 rpm for 10 min and filtered using a sterile syringe filter (0.45 μm Millex). The cell wall extract was smeared on a clean glass slide and fixed. Further, it was observed under a light microscope (Olympus, India) using lactophenol stain for morphological analysis.

2.1.3. Morphology of cell wall elicitor by atomic force microscopy (AFM)

The cell wall extract was suspended in a 10 mM KH_2PO_4 buffer; then, 2% glutaraldehyde (v/v) was used to fix onto the glass slide for 2 h at 25°C. Further, the cell wall morphology was scanned at different resolutions using the Flex-Bio Nanosurf system . This is used to understand the underlying structural membrane of the cell wall extract of *T. atroviride.*

2.1.4. Scanning electron microscopy (SEM) with energy dispersive x-ray spectroscopy (EDS)

The dried cell wall extract was coated with a 20–25 nm thick layer of carbon in an EC-32010CC using a graphite bar with a current intensity of 25A. Carbon is preferred to use in microanalysis due to its excellent electrical conductivity and transparent properties. The absorption of this layer of X-rays emitted by the element can influence the results obtained by the EDX JSM-IT500 [42].

2.2. Quantification of sugar present in the elicitor produced by Trichoderma atroviride

After their complete growth, by the enzymatic assay of trehalase, the trehalose concentration in the CWE from *T. atroviride* was quantified. The CWE was mixed with sterile distilled water (200 μL), followed by 30 μL of buffer solution containing NADP, glucose-6-phosphate dehydrogenase, hexokinase and ATP. The activity of this reaction mixture at $\lambda = 340$ nm was recorded. Later, 2 μL of trehalase enzyme was added, and this reaction mixture was left to stand for 8 mins at room temperature, and absorbence of all the samples was noted [43]. The test sample which showed the highest trehalose activity was used for further studies.

2.3. Identification and characterization of cell-wall elicitor from Trichoderma atroviride (TaCWE)

2.3.1. Functional groups analysis of elicitor by Fourier transform infrared spectroscopy (FTIR)

For the analysis of the sample by FTIR, a cell wall elicitor was finely ground and encapsulated in 200 mg of Potassium bromide (KBr) to characterize the molecular functional groups. The annotation of spectra at the Infrared (IR) region of 4000 to 400 cm^{-1} using Nicolet FT-IR 6700 (Thermo Fisher Scientific, India) and the outcome of the spectra was processed by OMNIC 7 software [44].

2.3.2. Liquid chromatography and mass spectroscopy (LCMS) of elicitor

The cell wall extract was mixed thoroughly with ethyl acetate and acetone in the ratio of 1:1 (v/v) and vortexed, then 50 ml of extract was dried at 50 °C for complete extraction. Later, 5 ml of methanol was used to dissolve the residue obtained, and 0.5 ml of the residue sample was used for LCMS analysis. The LC detection was followed by mass spectrometry (MS) confirmation using an AB Sciex API 200 machine. The AB Sciex API 200 used for LC was equipped with Phenomenexgemini 3 μm 50 mm into a 2 mm column. The methanol: formic acid (80:20, v/v) solvent was used in a reverse-phase in the isocratic mode for 3 min, and 10 μL was introduced at precise room temperature. The MS/MS coupled to the LC with a triple Quadrupole mass analyser and mass spectrometer equipped was run in a positive mode with an API source in which the vacuum gauge was maintained at 10e-5 Torr with a source temperature of 400 °C. The mass spectrometer was performed in a full scan (0–400 mass range) multiple reaction monitor (MRM) mode.

2.4. Seed material

Broccoli plant seeds (*Brassica oleracea* (L) var *italica*) cultivar Shogun that were highly susceptible to leaf spot disease were collected from the authentic suppliers, Brian Bell Pvt. Ltd, Goroka, and these seeds were used throughout the experiment.

2.5. Pathogen

The fungus *Alternaria brassicicola* (accession no. MN700129), which was isolated previously from our group [37], was grown in potato dextrose agar (PDA) for seven days at 23 °C and was used as a pathogenic source in this study.

2.6. Seed priming of TaCWE as an inducer

At first, the susceptible broccoli seeds (cv. Shogun) were surface sterilized with 0.2% sodium hypochlorite for 5 min and cleansed carefully using sterile distilled water 2–3 times. The sterilized seeds were then treated with isolated TaCWE at 5.0, 10.0, and 25.0 mg ml^{-1} for12 h, respectively, by keeping them in an incubatory rotary shaker at 25 ± 2 °C. After incubation, the treated seeds were air-dried aseptically at room temperature and used for further studies. Treated seeds with only distilled water were used as control. Both seeds, treated and untreated, were planted in February and observed through April during a wet season under greenhouse conditions. The treatments included i) susceptible plants treated with sterile distilled water (SDW) used as control, ii) susceptible plants inoculated with *A. brassicicola* pathogen, iii) susceptible plants treated with *T. atroviride* elicitor (CWE), and iv) susceptible plants treated with *T. atroviride* elicitor (CWE) and pathogen inoculated.

2.7. Effect of TaCWE on seed germination and seedling vigour

Seeds treated with TaCWE and control seeds (SDW) were plated in petriplates on moistened three-layered blotter discs at equal distance from each other, and the germination was evaluated in percentage [45]; additionally, a paper method was used on a set of treated seeds to note down the seedling vigour [46]. After incubating for ten days, percentage of germination, root length, shoot length, and vigour index were calculated. The experiment involved four replicates of 100 seeds each, and it was repeated thrice.

Vigour index = Seed germination (%) * [Mean Root Length + Mean Shoot Length]

2.8. Demonstration of elicitor as resistance inducer against broccoli leaf spot disease under greenhouse conditions

To assess the efficacy of elicitor for induction of systemic resistance (ISR), for each treatment, sixteen randomly selected broccoli plants (two-week-old) were challenge inoculated with the pathogen (*A. brassicicola*) suspension of 9×10^5 conidia ml^{-1} (7-day-old culture) in sterile distilled water. In contrast, plants without inoculation were maintained as control. The inoculated plants were roofed with plastic bags for three days and incubated at 20 °C under greenhouse conditions with 85% relative humidity. Regular observations were made on the plants for the typical leaf spot symptoms, such as pale to dark spots with concentric rings and brown necrotic lesions on the leaves. The development of symptoms was finally scored after 4-weeks of pathogen inoculation.

2.9. Assessment of elicitor priming on plant growth promotion under greenhouse conditions

The susceptible plants (one-month-old) to leaf spot disease were raised from seeds of (*Brassica oleracea* (L) var *italica*) under greenhouse conditions in earthen pots filled with peat moss, sand, and FYM (1:2:1). The potted plants were observed from day one to check on the expected growth of the plant for three months until it got matured and withered. The treated and untreated plants were monitored for growth parameters such as plant height, leaf size, stem girth, early flowering, and maturity.

2.10. Statistical analysis

Each experiment was performed using four different replicates. The obtained data were analysed using SPSS Inc.18.0 for analysis of variance (ANOVA). The magnitude of the F value ($P \leq 0.05$) determines the significant effects of treatments used. Treatment means were separated by Tukey's Honestly Significa nce Difference (HSD) test.

3. Results

3.1. Microbial culture and growth conditions

After seven days of incubation on Potato Dextrose Agar (PDA), *T. atroviride* isolate (TriAt_JSB2) showed consistent growth in all the Petri plates (Fig. 1 **a**) and a thick fungal mat appeared in the 500 mL Erlenmeyer's flask (Fig. 1 **b**).

3.2. Observation of cell wall elicitors from Trichoderma atroviride (TaCWE) under bright field compound microscope

The extracted cell wall elicitors were light yellow, and it was stained with lactophenol cotton blue and observed under 40x zoom in a bright field compound microscope. The cell wall extracts from the tested fungal isolate (TriAt_JSB2) showed the ghost cell appearance, indicating the secretion of putative cell wall elicitors in the cell-free suspension (Fig. 2**a**). On the other hand, the normal cells of *T. atroviride* showed an intact cell wall (Fig. 2**b**).

3.3. Morphological analysis of TaCWE by atomic force microscopy (AFM) and scanning electron microscopy (SEM) with energy dispersive spectroscopy (EDS)

Morphological and elemental analysis of the isolated cell wall elicitor was carried out to understand the surface morphology of the elicitor. SEM analysis of the TaCWE displayed reproducible surface architecture in all the fields of study. The extraction procedure proved to be non-invasive as the surface morphology appeared undamaged without significant morphological changes, as shown in Fig 3. The elicitor was found to be 10.16 μm in length and 2.148 μm in width, as evident in the AFM analysis (Fig. 3**a**). The scanning electron microscope with EDS detected the presence of elicitors on the surface area of the fungal cell wall and the accumulation of carbon, nitrogen, oxygen, and hydrogen elements. (Fig. 3**b**).

3.4. Fourier transform infrared spectroscopy (FTIR) analysis of elicitor

FTIR spectroscopy analysis was carried out to identify the functional groups of the isolated elicitor trehalose. Thespectra were recorded in absorption mode with the Perkin Elmer spectrometer. Successively,
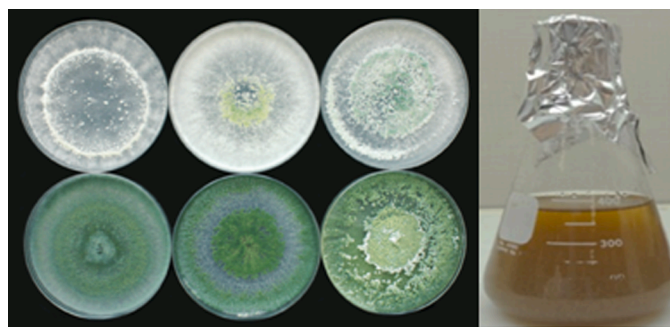

Fig. 1. (a) Seven-day-old *T. atroviride* (TriAt_JSB2) on Potato Dextrose Agar (PDA) Petri plates producing luxury mycelia growth. (b) Pure fungal mat obtained on Potato Dextrose Broth (PDB) after 12-days of incubation.
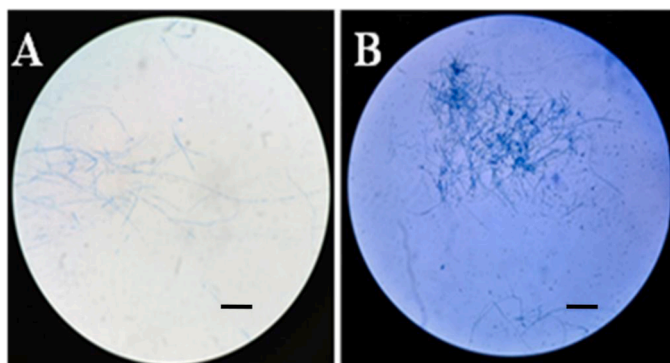
Fig. 2. (A). Ghost cell appearance (release of active fraction in the suspension) observed in the cell wall extract of *Trichoderma atroviride* isolate (TriAt_JSB2). B). Typical intact cell wall visualized in unextracted *Trichoderma atroviride* culture. Bar scale: 20 μM.

identifying the functional groups present in the obtained spectra with different wavelengths was evaluated against the standard values. FTIR spectra showed absorption bands from 3010.62 cm^{-1} to 2853.56 cm^{-1}, corresponding to O–H, - CH, -CH$_2$ stretching forms in **Fig. 4**. In the IR spectra, a broad peak disclosed at 3350 cm-1 represents the O—H stretching form, at 1746 cm-1 and 1655 cm-1 absorption peaks represent *cis* double bond compounds with C = O bonds in aldehyde and amide compounds. The obtained stretching pattern explicitly confirms

asymmetric fatty acids and accumulation of methyl, and methylene group compounds, as illustrated in **Table 1**.

3.5. Activity of trehalase in the elicitor extract

Trehalase activity is a significant marker for analysing responses of hosts towards mitigation of biotic stress. The highest trehalase activity of 1.92 μmol glucose equivalent ml^{-1} was observed in the *T. atroviride* CWE (TaCWE). Therefore, trehalase activity in *T. atroviride* implies that it is a potent candidate for the identification of biotic elicitors to induce resistance against biotic and abiotic stresses.

3.6. Detection of trehalose sugar by liquid chromatography-mass spectroscopy (LC-MS)

Trehalose sugars are important mitigators of abiotic stresses due to their osmolytic activity. LC-MS analysis was carried out with a fraction of the elicitor in *T. atroviride* to identify whether trehalose was present in the cell wall. Standard trehalose was eluted at 3.79 min in the LC-MS, and the TaCWE sample had a single peak eluted at a retention time of 3.78 min. Both indicated the presence of trehalose in the cell wall fraction of the fungi tested (**Fig. 5**).

3.7. Seed priming effect of elicitor on seed germination and seedling vigour under laboratory conditions

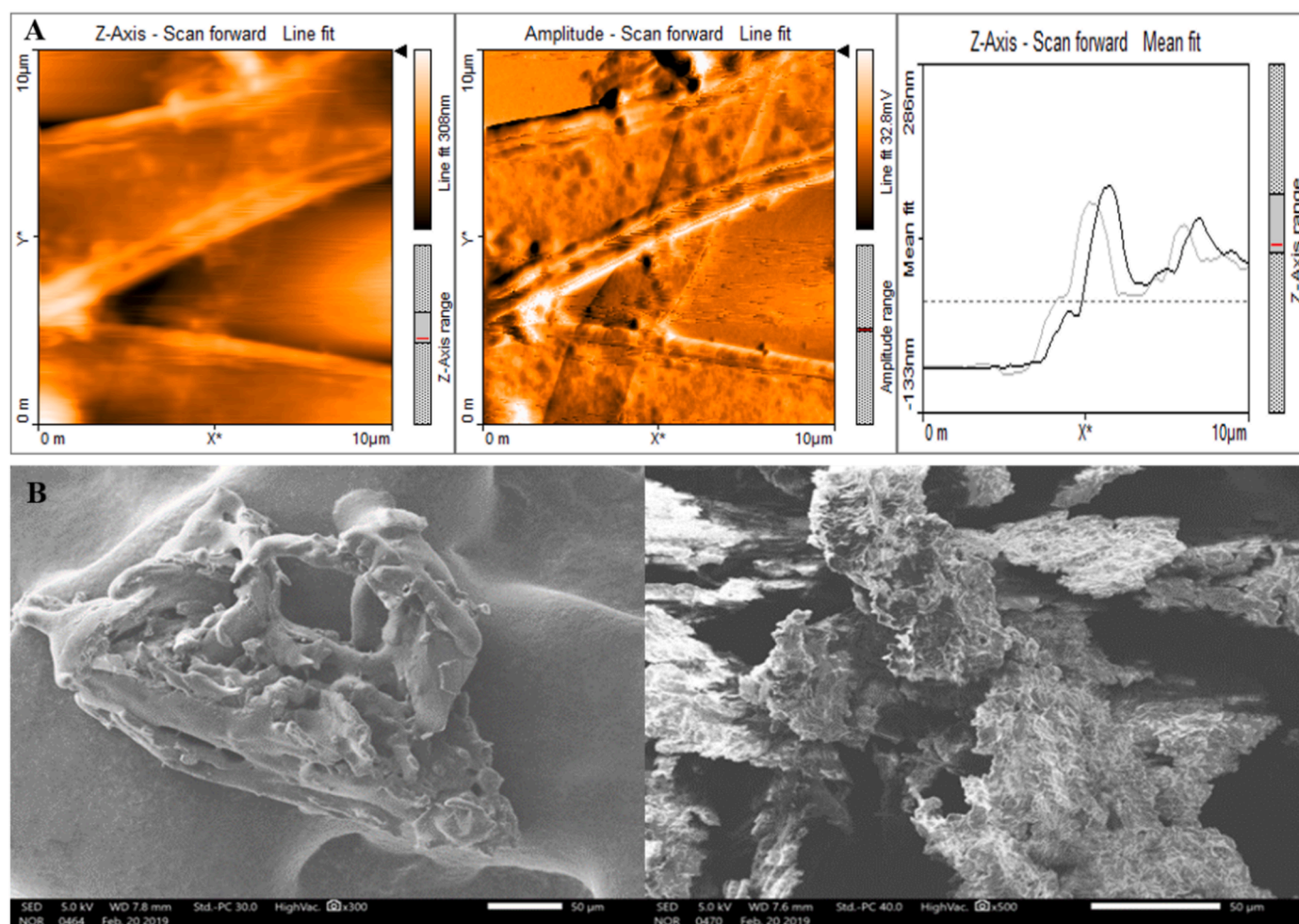Amongst the tested concentrations, the seed priming with 10.0 mg

Fig. 3. Cell wall morphology analysis of *T. atroviride* cell wall elicitor displaying the reproducible surface architecture by AFM analysis. (A). SEM shows the putative elicitor on the surface of the cell wall of *T. atroviride* and (B). The cell surface after removal of the elicitor at 50 μm. .
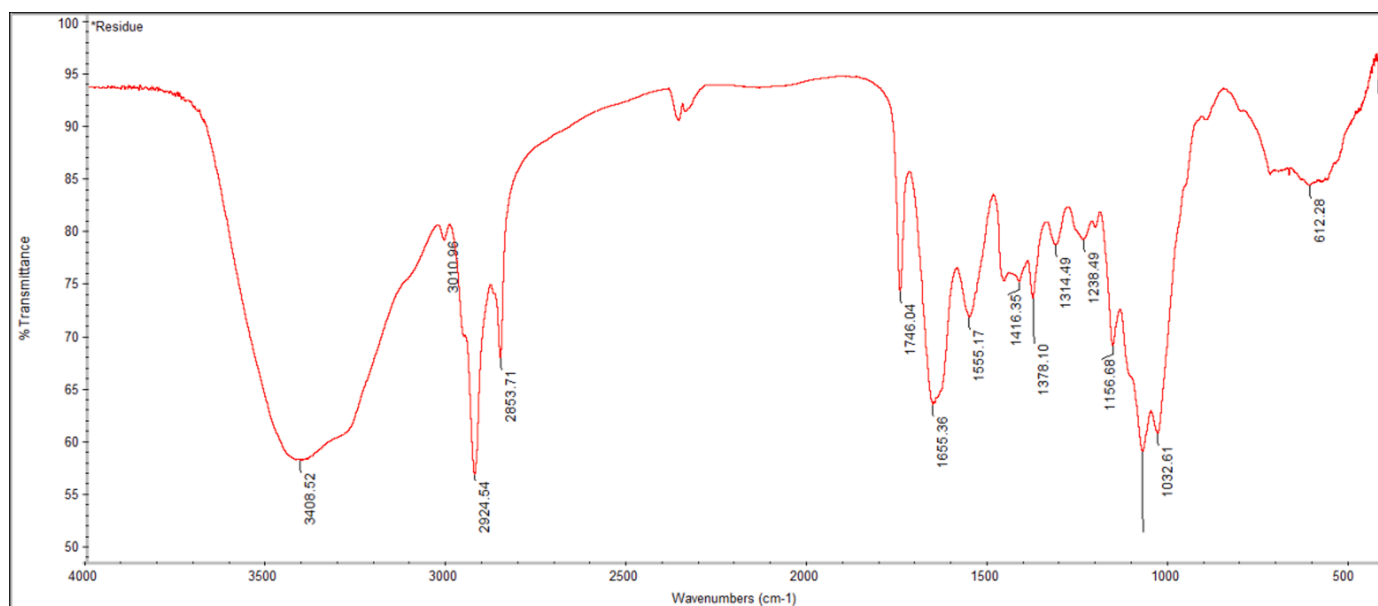
Fig. 4. Chemical analysis of cell wall elicitor expressing the spectra of key molecular functional groups in the Infrared (IR) region from 4000 to 500 cm^{-1} by Fourier transform infrared spectroscopy.

Table 1

FTIR spectral annotation of cell wall elicitor showing identification of functional groups.

IR regions	Functional groups
3408	-OH stretching
3010, 2924, 2853	-CH, -CH$_2$, -CH$_3$ stretching
1746	-C = O (Aldehyde)
1655	-C = O of amide (Proteins and peptides)
1555	-NH Amide bond (Proteins and peptides)
1416	-CH$_2$ bending
1378	- C-O-H bending
1238	-C-N, C-OH, not predictable
612	C-H bending & ring puckering

ml^{-1} of elicitor (TaCWE) uncovered significantly ($P < 0.05$) enhanced early emergence (3rd-day post-priming), germination (94%), and seedling vigour (2601). It was followed by seeds primed with 25.0 mg ml^{-1}, which recorded early emergence at the 4th-day post-priming, germination (89%), and seedling vigour (1977). Whereas 5.0 mg ml^{-1} primed seeds showed fair enhancement of emergence, rate of germination, and seedling vigour. In the untreated control, the emergence recorded at 6-day post-treatment with germination of 77% and seedling vigour of 1155, respectively, were noticed (Fig. 6).

3.7. Elicitation of resistance by seed priming with elicitor (TaCWE)

Plants pre-treated with TaCWE elicitor with 10 mg ml^{-1}and
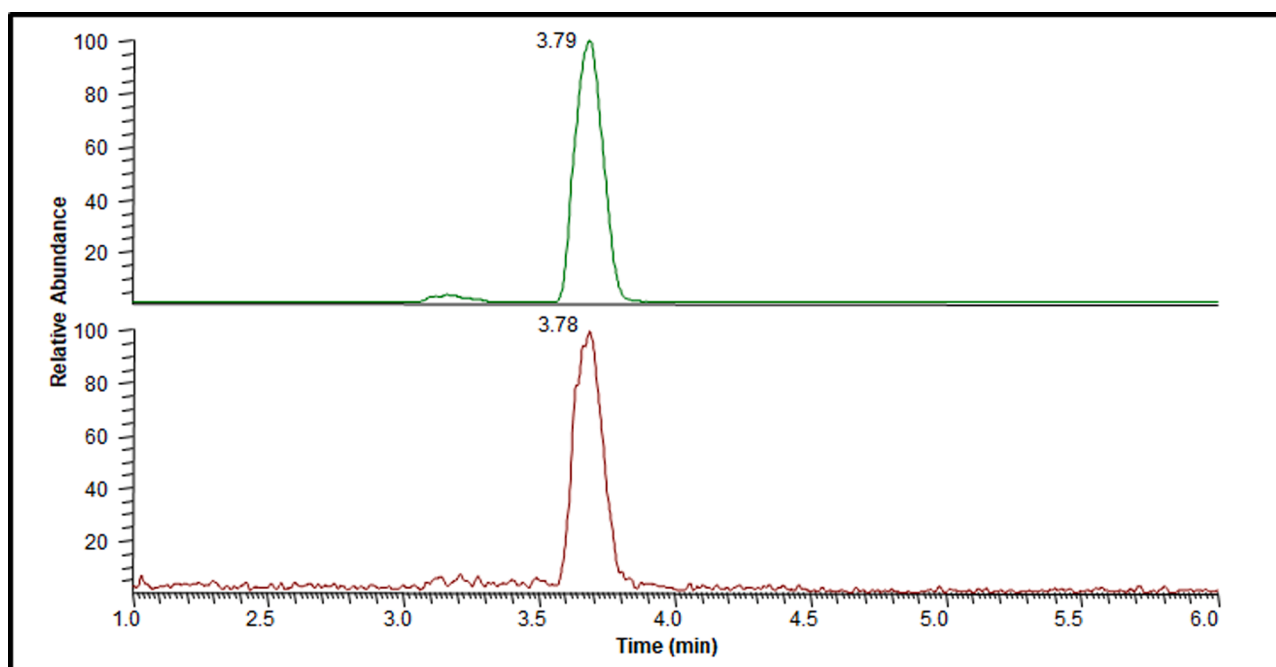

Fig. 5. Liquid chromatography elution profile of standard trehalose at 3.79 (A) mins followed by detecting the trehalose sugar in TaCWE at 3.78 mins (B).
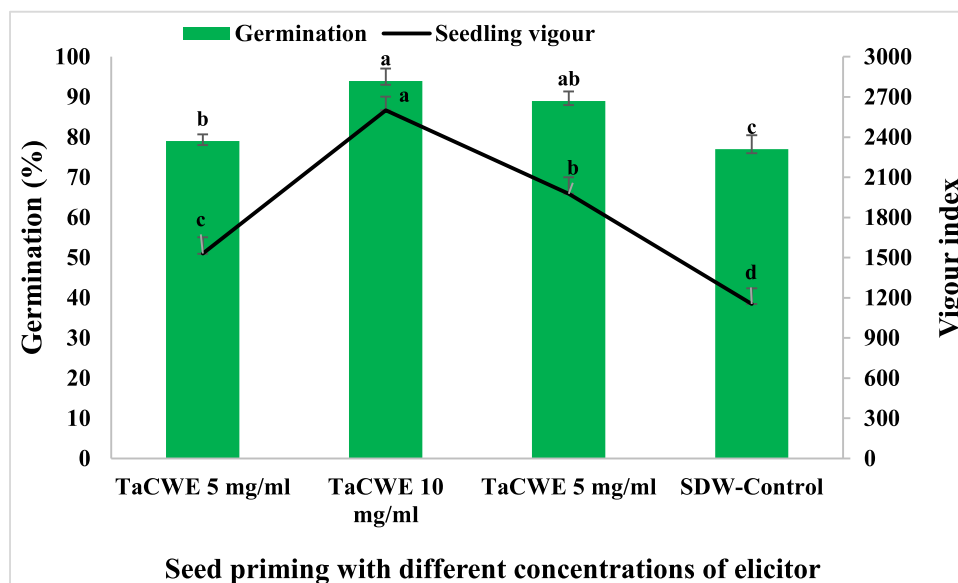
Fig. 6. Effect of seed priming with different concentrations of elicitor (TaCWE) on seed germination and seedling vigour. Values are the mean of four replicates ($n = 4$). Bars represent standard errors. Letters on bars indicate significant difference between control and treated samples based on Tukey's honestly significant differences (HSD) test ($P \leq 0.05$). .

inoculated with the pathogen were able to induce a remarkable 71.7% protection against leaf spot of broccoli caused by *A. brassicicola* after 34 days of sowing (4th day after inoculation). This protection or resistance was slightly reduced to 70% at 35-day sowing; the plants treated with elicitor alone with 10 mg ml^{-1} were also protected (57.3 and 57.9% respectively) at 3rd and 4th day after inoculation against leaf spot disease (**Fig. 7**A). Plants treated with elicitor (5 and 25 mg ml^{-1}) and challenged with the pathogen also offered significant ($P < 0.05$) disease suppression after 34 days of sowing by recording disease protection of 49.7% and 57%, respectively. The plants that received the elicitor treatment of 5 and 25 mg ml^{-1} alone recorded adequate disease protection but were still significant compared to the control plants. Conversely, the pathogen inoculated plants recorded a maximum disease incidence of 95% by exhibiting typical pale to darkened spots with sunken brown lesions on the leaves (**Fig. 7**B). In contrast, the distilled water-treated control plants showed stunted growth and chlorosis on the leaves. The pots showing the expression of disease symptoms on elicitor-treated and untreated broccoli plants under greenhouse conditions are represented in **Fig. 7**B.

3.8. Plant growth promotion after elicitor priming

The plants raised with elicitor treatment with all the concentrations showed more profuse growth than the control or pathogen inoculated plants. Primed broccoli plants with elicitor after five weeks recorded a positive growth effect in plant height and early flowering compared to the control (**Fig. 8**). A significant plant height of 227 cm (56.7%) and 199 cm (49.1%) was attained in the plants raised with elicitor (10 and 25 mg ml^{-1}), respectively. It was observed that the plants primed with 10 mg ml^{-1} produced early flowering 12-days prior to the control plants (85-days). Likewise, treated plants (10 mg ml^{-1}) recorded significant leaf size and height enhancement when assessed with control and pathogen inoculated plants and in other aspects, including disease resistance (**Fig. 8**).

Further, the induced resistance and growth promotion experiments showed a significant constructive correlation between the elicitor-primed plants in compared with the control plants irrespective of the time gap with or without pathogen inoculation (**Fig. 9**). A spacio-temporal elicitor effect was clearly evidenced in this study wherein plants that received treatment with pathogen showed induced

protection from 3rd and 4th day against pathogen attack. The same treatment without pathogen attack demonstrated a plant immune induced response positively to the plant growth promotion compared to non-induced plants or control plants.

4. Discussion

Plants, being sessile, are in continual acquaintances with various biotic and abiotic stresses [28]. However, plants keep adapting to such variations by constantly evolving and rapidly gaining a greater degree of systemic resistance [23,47]. Many studies on plant growth-promoting fungi (PGPF) *Trichoderma* spp. have shown profound beneficial effects in agriculture to enhance plant growth and induction of disease resistance [24,48,49]. Various elicitors have been documented, and the most central amongst them are the cell wall elicitors [50]. The elicitors either directly or indirectly induce resistance in plants against pathogen infection through Microbe Associated Molecular Pattern (MAMPS) or Pathogenesis-related (PR) resistance [51–53]. However, little information is known about the plant responses to cell wall elicitors extracted from PGPF, and the resistance mechanism confers on the host.

In the present study, we analysed the cell wall elicitor's surface morphology and chemical nature. Based on the characterisation studies, the purified cell wall elicitor fraction has been demonstrated to possess trehalose as its essential source. In a recent survey by Djanaguiraman et al. [54], the FTIR analysis of synthetic elicitor nanoceria showed the absorption bands at 3341, 1634, 1327, and 582 cm^{-1}. The results showed the presence of high crystallinity and purity in the synthesized nanoceria. AFM measured the size and morphology of nanoceria. The results of AFM indicated that synthesized nanoceria had an average diameter of 15 ± 5 nm. The current research exhibited amides, carboxylic acid, and aldehydes in the isolated elicitor with unique FTIR frequencies.

The LC-MS analysis revealed that the pure form of trehalose was detected at an elution rate of 3.78 min. Further, when analysed by SEM, the cell wall elicitor displayed undamaged surface architecture, and the elicitor was found to be 2.16 μm in length and 2.148 μm in width. Collaborating our results, Hayner et al. [55] also reported that the trehalose produced by *Escherichia coli* was able to elute at the same retention time and found that LC-MS is the most suitable method for the detection of trehalose in any biological samples.
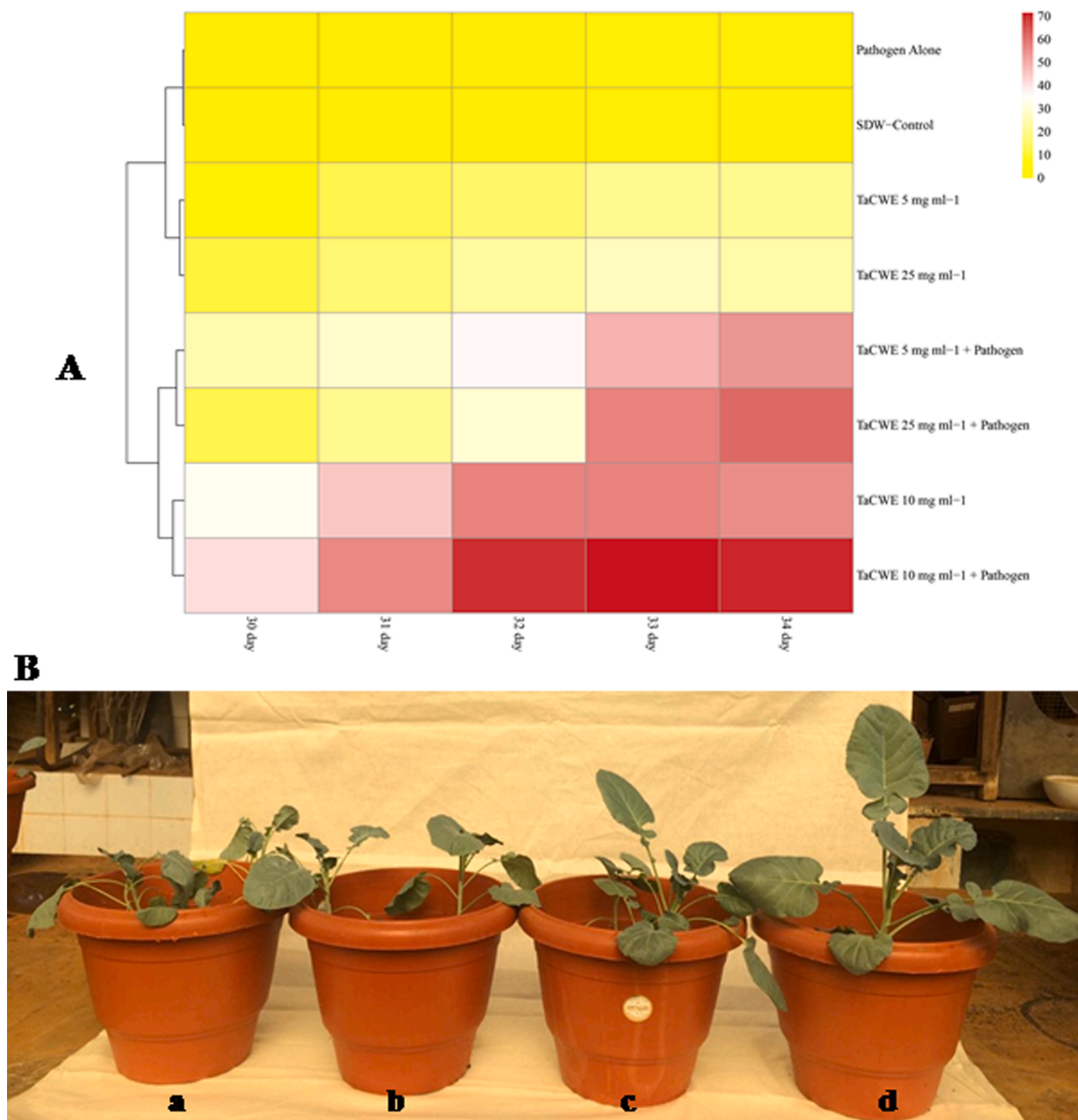
Fig. 7. (A) Nature of induced resistance against leaf spot disease observed in broccoli plants (cv shogun) raised from TaCWE elicitor-treatment with or without pathogen inoculation as expressed in terms of time-gap between (1 to 5 days, 30, 31, 32, 33 and 34-days-old broccoli plants post-inoculation) under greenhouse conditions. The significance of induced resistance is based on the intensity of the colour scale generated by the heatmap software. (B) The photos above show that the control plants (Fig 7a) and pathogen inoculated (Fig 7b) had some disease symptoms and the treated plants and challenge inoculated with the pathogen had no disease symptoms (Fig 7c). The plants sprayed with elicitor (10 mg ml^{-1}) of *T. atroviride* (Fig 7d) showed overwhelming growth without any disease symptoms on the broccoli plant. .

Interestingly, a high amount of trehalase activity (1.92 μmol glucose equivalent ml^{-1}) in the *T. atroviride* cell wall extracts was also recorded. Our results agree with Elbein et al. [11], who recorded a high concentration of trehalose from yeast culture. Previous studies also demonstrated increased activity of trehalose in the mycorrhizae fungi *Rhizophagus irregularis*, and it was reported that the production of trehalose helps the host plant with improved nutrition and protection against biotic and abiotic stresses [56,57].

The current study witnessed that seeds primed with trehalose at 10 mg ml^{-1} showed enhanced emergence, germination (94%), and seedling vigour (2601). Again, the current study results were supported by the outcomes of Gong et al. [58], where seeds primed with elicitors isolated from the endophytic fungi provided a remarkable increase by 30% in the seeds germination rate of *C. goeringii*, and also reported that the mixed
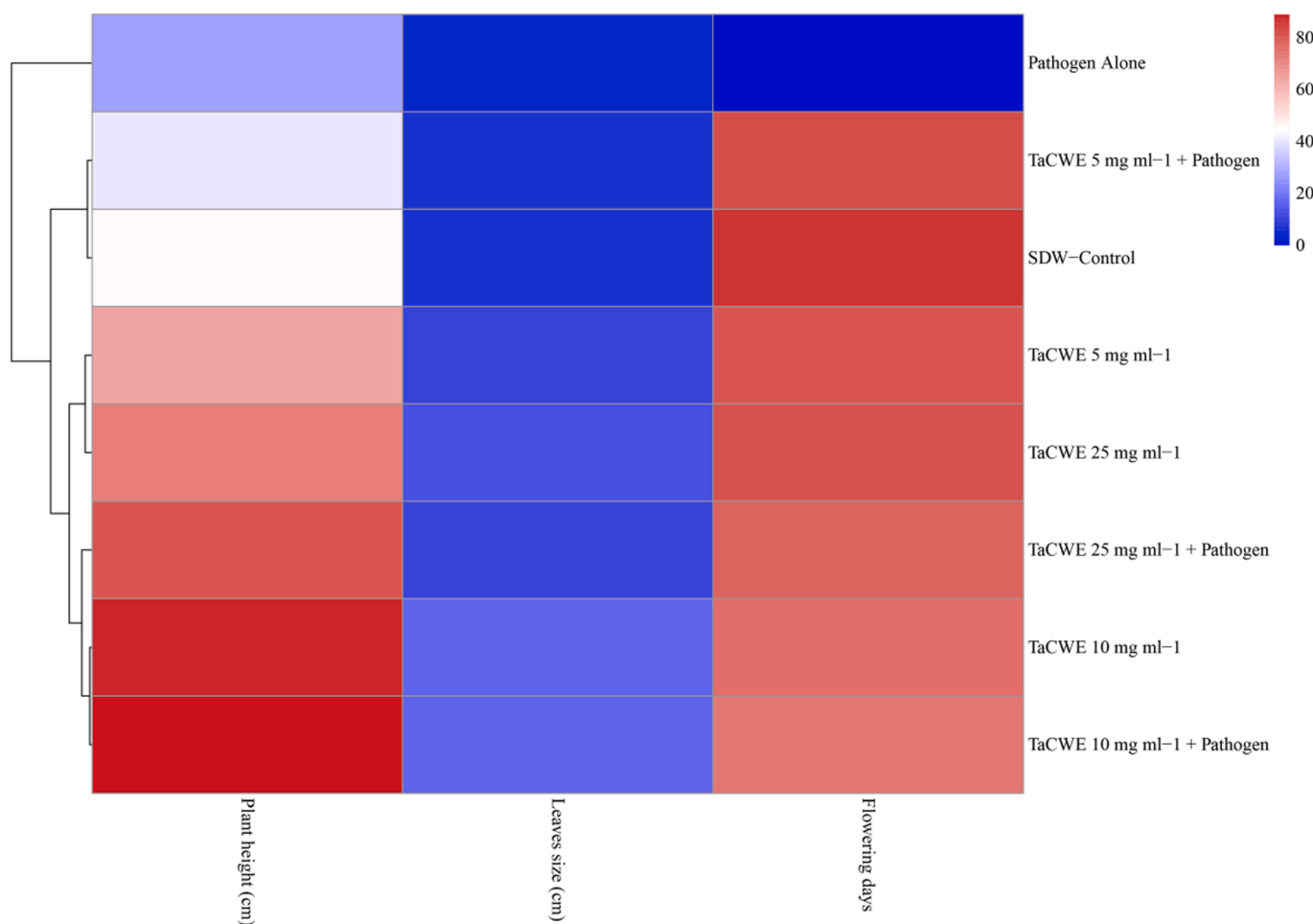
Fig. 8. Efficacy of plant growth parameters witnessed on broccoli plants (cv shogun) raised from TaCWE elicitor-treatment with or without pathogen inoculation. The intensity of the colour scale indicates the significance of the data developed by heatmap clustering.

fungal elicitors promoted the growth of tissue culture seedlings. Similar results were also revealed by Bhagobaty and Joshi [59]. The authors revealed that the treatment of culture broth of *P. verruculosum* exhibited the ability to promote seed germination in mung beans and chickpea under in vitro conditions. Another study by Anupama et al. [60] on seed treatment of tomato with oligosaccharides extracted from *A. solani* indicated a significant increase in seedling germination, growth, and vigour.

Moreover, seed treatment with the combination of fungal oligosaccharides and different strains of PGPR [61] revealed a significant surge in the germination rate of 93.33% and 2733 seedling vigour when compared to the control and the individual treatment of oligosaccharide elicitor, which showed 75% germination and 887 seedling vigour. Previously, our group demonstrated that exogenous pearl millet seed priming with trehalose stimulates early and enhanced seed germination and vigour [12]. These results substantiate the application of trehalose on stimulating germination and seedling vigour.

The collective and innovative evidence specifies that trehalose and its derivatives as signal molecules to stimulate plant resistance against diverse biotic factors [15,62]. More importantly, the present study showed that seed priming with trehalose at low concentration could potentially protect broccoli plants against leaf spot disease, as evidenced by their induced resistance and subsequent plant growth under *Alternaria brassicicola* stress. Several investigators have reported the positive application of trehalose that confers resistance against *Blumeria graminis* in wheat [63,64], downy mildew disease in pearl millet [12], and tobacco mosaic disease [65]. Further, augmentation of resistance against

green peach aphid in *Arabidopsis* using exogenous trehalose application with trehalose phosphate synthase11 (tps11) knockout in mutant plants was documented [66]. There is an immense impact of trehalose on the plant defence and metabolism responses; in contrast, the trehalose biosynthetic pathway plays a vital role in the pathogen body during infection. It was found out that instead of regular T6P synthesis taking place when a pathogen attacks the plant body, an alternative pathway such as the oxidative pentose phosphate pathway occurred in the case of the rice blast fungus *Magnaporthe grisea*. Thus, the TPS1 gene needed to colonize the plant tissue [67] was disengaged from pathogenicity [68]; by passing the production of T6P resulted in the expression of the virulence-associated gene. Therefore, the results from previous research studies and the current results authenticate the importance of exogenous trehalose treatments against phytopathogens. In particular, seed treatment with *T. atroviride* activated the production of trehalose sugar, which is an effective and efficient way of controlling leaf spot disease in broccoli. In countries like Papua New Guinea, broccoli is mainly cultivated by low-income farmers who cannot afford the repeated use of expensive chemical fungicide [47]. Subsequently, this method would be more suitable for large-scale production with less use of chemical fungicides.

Furthermore, we have also noticed the susceptible broccoli cultivar's increased potential after trehalose treatment towards induced resistance and promoted plant growth under biotic stress conditions compared to the control. Similarly, Baldi et al. [69] reported that one of the most assuring strategies for crops yield enhancement is the elicitation technique. Biotic and abiotic elicitors, those derived exclusively from fungi,
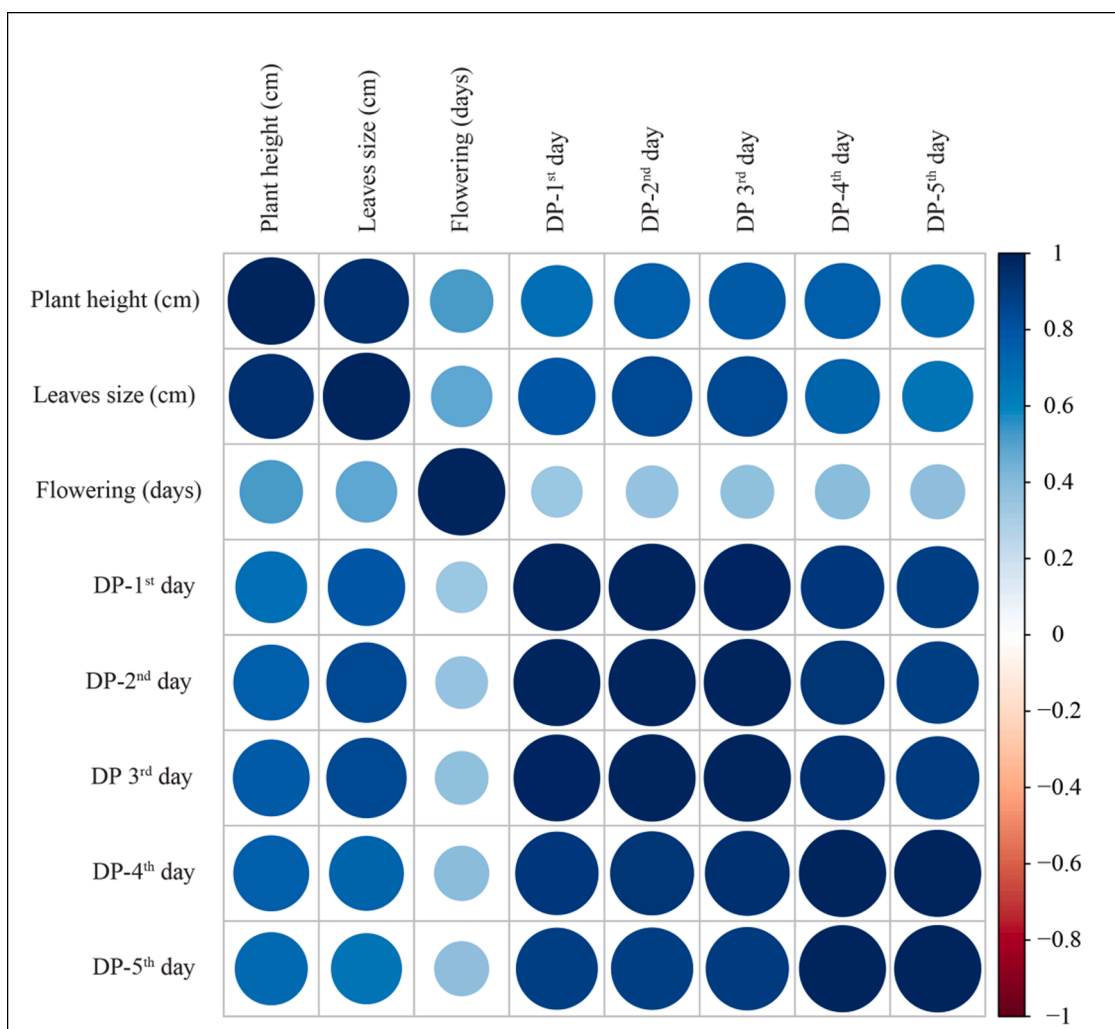
Fig. 9. Dipartite constructive correlation of induced resistance and plant growth in response to elicitor treatment to broccoli plants with or without *Alternaria brassicicola* infection was developed using "corrplot" in R 4.3.2 version.

enhance the secondary metabolites in plant cell suspension cultures. Such secondary metabolites could be used for economically feasible elicitation methods like seed treatment. A comprehensive study by Akram et al. [70] confirmed significant growth promotion in radish (*Raphanus sativus* L.) with 25 mM trehalose seed treatment concerning fresh and dry biomasses and chlorophyll *a* and total soluble sugar contents in the plants. In addition to various fungi isolated from the rhizosphere currently used for plant growth promotion, several symbiotic arbuscular mycorrhizae, like fungus, are also tested for their elicitor activity. *Piriformospora indica* and its elicitors have been used against biotic and abiotic stresses. Overall, in the present study, the pattern of inducing resistance or protection witnessed a positive correlation with that of plant growth promotion, and were consistently reproducible amongst the tested biological replicates, which demonstrate the spacio-temporal elicitation of induced resistance in broccoli plants priming with TaCWE.

It has been well documented that in line with this trehalose sugar, other sugars, viz. sucrose, fructose, and glucose, are required in the metabolic pathways and various signalling mechanisms in plants [71, 72]. Baenas et al. [73] illustrated that after spraying with sucrose (146 mM) for 5-days, there was an elicitation in the biomass weight of 5 different *Brassicaceae* sprouts. Similarly, an elicitor protein of rice blast fungus M. oryzae, namely MoHrip2 (Magnaporthe oryzae hypersensitive protein 2), has demonstrated enhanced plant growth and resistance against rice blast after treating with the elicitor [74].

The most remarkable finding of the current work has opened up a new perspective into the interactions of pathogen and elicitor with the evidence provided from FTIR and LC-MS analysis about the trehalose production in both plants treated with elicitor alone and elicitor treated together with pathogen inoculated plants. It should have incited the immune response of broccoli plants before and after challenge inoculation with *A. brassicicola* pathogen by actuating a defence gene that reveals ISR. There is less or no severity of disease incidence observed in the treated broccoli plants, and hence it is directly proportional to the increase in the yield. In other words, the application of *T. atroviride* as an eco-friendly biological control agent appears to be working well. It can be considered one of the most promising cell wall elicitors that plants could produce when pathogens contact them.

5. Conclusion

The current investigation has established decisively a strong correlation between the seed priming with *Trichoderma atroviride* at a specified amount and the trehalose production in broccoli, which enhanced plant growth and induced systemic resistance against leaf spot disease. Overall, the study has portrayed the trehalose sugar as a bio-stimulant for growth promotion and involved in the induction of resistance against leaf spot diseases of broccoli plants caused by *A. brassicicola* using the *T. atroviride* isolates as inducers for seed priming. Such a trehalose elicitor with evidence of its disease resistance is considered the

most favourable, eco-friendly, non-toxic, and biogenic product that provides natural growth promotion and a bio-control agent to the growing agricultural sector alongside the existing conventional chemical fertilizers in the market. It can be recommended to develop newer formulations to be used for integrated plant disease management in the cultivation of various cruciferous crop plants, especially broccoli. Overall, the study has portrayed the trehalose sugar as a bio-stimulant for growth promotion and involved in the induction of resistance against leaf spot diseases of broccoli plants caused by *A. brassicicola* using the *T. atroviride* isolates as inducers for seed priming.

Declaration of Competing Interest

Authors have declared that no competing interests exist.

Author contributions

Experiment design and monitor: SJ. Experiments performed: SDB. Data compilation: SJ, SMJ. Supply of reagents/consumables: SJ. Manuscript writing: SDB, SMJ and SJ. Preparation of Figures/Graphs: SJ. All the authors read and approved the revised manuscript.

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