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Trichoderma oligosaccharides priming mediates resistance responses in pearl millet against downy mildew pathogen

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ABSTRACT

Fungal cell wall oligosaccharides are being focused on the biological management of crop diseases by elicitation of defense responses. In the present study, an approach was taken to enhance the pearl millet disease resistance using biotic elicitors for eco-friendly management against downy mildew pathogen through seed priming approach. Crude oligosaccharides extracted from four different *Trichoderma* spp. enhances the disease protection ability in pearl millet. Seed priming with *T. asperellum* along with the osmopriming agent, mannitol had shown better protection with improved seedling vigor compared to controls. Modulation of defensive enzymes such as peroxidase and lipoxygenase also confirms the elicitation of resistance responses in the host with increased enzyme activity at different time interval patterns.

1. INTRODUCTION

Pearl millet [*Pennisetum glaucum* (L.) R.Br.] (PM) crop production is severely hampered by several biotic stresses. Downy mildew (DM) disease caused by the oomycete obligate pathogen, *Sclerospora graminicola* (Sacc.) Schroet. is one of the major biotic constraints. Downy mildew (DM) disease accounts for a yield loss of PM up to 20–40% annually [1]. The spread of DM disease is favored by high relative humidity (85–90%) with moderate temperature (20– 30ºC) [2]. Studies on plant-oomycete interactions are fundamental for research inventions as it gives a way ahead to develop economically with improved disease resistance crop against the pathogen [3, 4]. The biological control mechanisms, such as antibiosis, antagonism, mycoparasitism and induction of plant defense responses have all been accredited in *Trichoderma* spp. [5]. *Trichoderma* and its direct interaction with plant pathogen involve cell-wall degrading substances including antibiotics attributes as important factors for mycoparasitism and antibiosis [6, 5, 7, 8]. The production of the extracellular cell-wall degrading enzymes such as chitinase, cellulase, protease and β-(1,3) glucanase by *Trichoderma* spp. have a vital role in the inhibition of the fungal pathogens and induced resistance of host plant system [9, 10]. Earlier reports reveal the efficiency of *Trichoderma* spp. as biofertilizers/ biocontrol agents for crop production in the field or greenhouse agriculture farming systems [5, 11] as an alternative choice to the chemical fungicides [12]. Root colonization by the antagonist *Trichoderma* has been studied using conventional microbiological techniques [13]. The inhibitory activities of *T. harzianum* and *T. viride* culture filtrate against *Fusarium moniliforme* was due to the production of volatile compounds and release of extracellular enzymes, such as those with amylolytic, pectinolytic, proteolytic and cellulolytic activities [14]. Oligomers of chitin and glucan are fungal elicitors generated from fungal cell walls and are measured as primary signals responsible for the initiation of plant resistance reactions [18]. Chitosan (poly-(1,4)-β-D-glucosamine) is known to induce systemic resistance in PM and defends through the establishment of defense responses [15,16]. *Trichoderma*-derived cell wall degrading enzymes and fungal metabolites from *T. asperellum* CCTCC-RW0014 have a synergistic inhibitory effect to control fungal pathogen *Fusarium oxysporum* f. sp. *cucumerinum* [17]. It is well-known that several oligosaccharides from fungal cell wall components stimulate phytoalexin secretion and lignin or callose formation in plants [19, 20]. The present work was aimed to study the possible effects of crude oligosaccharides from *Trichoderma* spp. on PM seed quality parameters and its ability to induce resistance in PM against DM pathogen.

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2. MATERIALS AND METHODS

2.1 Host and Pathogen

Highly susceptible PM seeds to DM pathogen i.e., cv. 7042S were obtained from the International Crop Research Institute for Semi-Arid Tropics, Patencheru, India, under a material transfer agreement and were used throughout the study. Pearl millet (PM) seeds of cultivar 7042S, were surface sterilized with 0.2% sodium hypochlorite for 2 min and rinsed in distilled water for 2-3 times.

Downy mildew (DM) pathogen sick plot was maintained at the Department of Biotechnology, University of Mysore, Mysuru (N 24º18', E 79º 26', 903 m altitude) since last three decades under the ICAR program, provided the source of *S. graminicola*. Infected leaves were collected in evenings, cleaned under running tap water, blot-dried and placed in a moist chamber at 20°C and > 95% relative humidity (RH). Fresh sporangia formed on the leaves were collected in distilled water and the spore load was attuned to 4×10^4 zoospores ml^{-1} and further used as inoculum in various experiments.

2.2 Extraction of oligosaccharides from *Trichoderma* spp.

Trichoderma spp., namely *T. asperellum, T. atroviride, T. longibrachiatum and T. brevicompactum* were obtained from the department stock culture, which are basically isolated from the root rhizosphere soil sample of the monocot plants. The *Trichoderma* spp. was mass-multiplied on potato dextrose broth for 12-14 days at 28 ± 2°C. At the end of the incubation phase, mycelia were collected and dried at 60°C for 48 h. Mycelium (100 g) was extorted overnight with acetone (250 ml at 20°C) and the powder was subjected to alkaline treatment consisting of 100 ml of 0.1M NaOH at 60°C for 2 h. The supernatant was neutralized to pH 7 with 50% acetic acid and stored overnight at 4°C. The resultant sample was centrifuged (16,500g, 20 min at 20°C) and the supernatant was collected and lyophilized [21]. The presence of oligosaccharides in the samples was confirmed by Molisch test [22] and reducing sugars were estimated by phenol-sulfuric acid method [23].

2.3 Effect of seed priming with *Trichoderma* oligosaccharides on PM seed quality parameters

Pearl millet seeds cv. 7042S were treated with crude oligosaccharide extracts alone and also along with 1% mannitol as a priming agent in the same concentration of oligosaccharides in 0.5, 1, 2, 4, 6, and 8 mg/ml for 12 h at room temperature on a shaker at 150 rpm. Distilled water and 1% mannitol treatment served as controls [24, 25]. Germination test was performed by the paper towel method according to the standard measures of International Seed Testing Association [26]. Seedling vigor was evaluated by following the method of Abdul Baki and Anderson [27]. Four samples of 100 seeds for each treatment were used and the experiment was replicated thrice. The vigor index (VI) was calculated using the formula:

VI = (mean root length + mean shoot length) × percentage of germination

2.4 Effect of seed priming with *Trichoderma* oligosaccharides on PM-DM disease response under greenhouse conditions

In the greenhouse study, primed seeds were sown in pots containing sterilized soil: decomposed cow dung manure (3:1 v/v). Seed priming was performed as described earlier. Seeds treated with the metalaxyl at 6 g/ kg dose served as a positive control treatment. A randomized complete block design was laid out for the experiment. Zoospore suspension of *S. graminicola* was whorlly challenge-inoculated for two-day-old seedlings at a concentration of 4×10^4 zoospores ml^{-1} [28]. Under greenhouse conditions, the challenge-inoculated plants were maintained (90-95% RH, 20-25°C temperature). Each treatment consists of eight replications of five pots with eight seedlings each and repeated thrice.

Disease incidence was observed by recording the number of plants that showed typical DM symptoms like, sporulation on the abaxial leaf surface, stunted growth, chlorosis, or malformation of the panicles. The experiment was concluded 60 days after sowing.

2.5 Defense-related enzyme analysis

Seedlings were grown on wet blotter discs in petriplates (25 seeds/plate). Three-day-old seedlings were inoculated with a zoospore suspension of 4×10^4 ml^{-1} by root-dip method and incubated in the dark at 25 (±1) °C [29]. Seedlings (1 g fresh weight) were collected in the different time interval at 0, 6, 12, 18, 24, 30, 36, 42, 48, 60, 72, and 96 h after challenge pathogen inoculation and then grind to a fine powder in liquid nitrogen to extract enzymes and used for assays. The protein content was examined using the dye-binding method of Bradford [30] with bovine serum albumin (Sigma, USA) as standard.

2.6 Peroxidase (POX) assay (EC 1.11.1.7)

Seedlings (1g) extracted with 10 mM potassium phosphate buffer (pH 6.9) and the supernatant was collected at 4°C was used as enzyme source. Enzyme assay was performed as explained by Hammerschmidt et al. [31]. The reaction mixture (3 ml) includes 0.25% (v/v) guaiacol in 10 mM potassium phosphate buffer (pH 6.9) containing 10 mM hydrogen peroxide. Crude enzyme extract addition initiates the reaction and measured spectrophotometrically at 470 nm absorbance (Hitachi U-3900, Japan).

The variation in absorbance (ΔA_{470}) was divided by the tetraguaiacol molar extinction coefficient (26.6 mM^{-1} cm^{-1}) and the enzyme activity expressed as µmol of H_2O_2 min^{-1} mg^{-1} of protein [32]. The experiment was done thrice and average enzyme activity was recorded.

2.7 Lipoxygenase (LOX) assay (EC 1.13.11.12)

Lipoxygenase activity was examined by following the method of Borthakur et al. [33]. Enzyme source was obtained by grinding the 0.5 g seedlings extract with 5 ml of 0.2 M sodium phosphate buffer (pH 6.5) and a supernatant was collected as an enzyme source. The activity was measured spectrophotometrically

by observing the occurrence of the conjugated diene hydroperoxide at 234 nm. LOX assay substrate was prepared by following the procedure described by Axelrod et al. [34]. Linoleic acid (28 mg) and an equal weight of Tween-20 plus 2ml of distilled water were added. An Appropriate amount (50 µl) of 2N NaOH was added to attain a clear solution. The solution was made up to 10 ml with distilled water. Fresh substrate was prepared for each time and used for the enzyme assay. The reaction mixture consists 2.7 ml of sodium phosphate buffer (0.2 M, pH 6.5) and 0.3 ml of substrate. The reaction was started by the addition of enzyme extract and the absorbance at 234 nm was noted for 3 min using Hitachi U-3900 spectrophotometer.

The difference in absorbance (ΔA_{234}) was divided by the molar extinction coefficient (23,000 $M^{-1}cm^{-1}$) of hydroperoxide formed and the enzyme activity expressed as µmol min^{-1} mg^{-1} protein. The experiment was done thrice and average enzyme activity was recorded.

2.8 Statistical analysis

Data from different treatments were evaluated for each experiment and subjected to arcsine transformation and analysis of variance using SPSS Inc. 17.0. Significant results of treatments were determined by F values ($P \leq 0.05$). Average of the treatment was separated by Tukey's honestly significant differences (HSD) test.

3. RESULTS

3.1 Effect of seed priming with *Trichoderma*-mediated oligosaccharides on seed germination parameters of PM

Seed priming with crude oligosaccharides in different concentrations has not shown any inhibition parameters of seed germination and vigor. Though, there was no significant ($P \leq 0.05$) variation observed in treated seedlings germination percentage, seedling vigor was enhanced significantly ($P \leq 0.05$) in treated seedlings compared to the control treatments, in which, *T. asperellum* at 4mg/ml with 1% mannitol had shown maximum germination percentage of 93% with seedling vigor of 1757. Metalaxyl seed treatment shows 89% seed germination and 1591 seedling vigor and it not significantly ($P \leq 0.05$) different from the distilled water control. Amongst the crude oligosaccharides treatment of *Trichoderma* spp., from *T. longibrachiatum* shows minimum efficacy in improving the seed germination and its vigor, which is not significantly different from the control treatments (Figure 1).

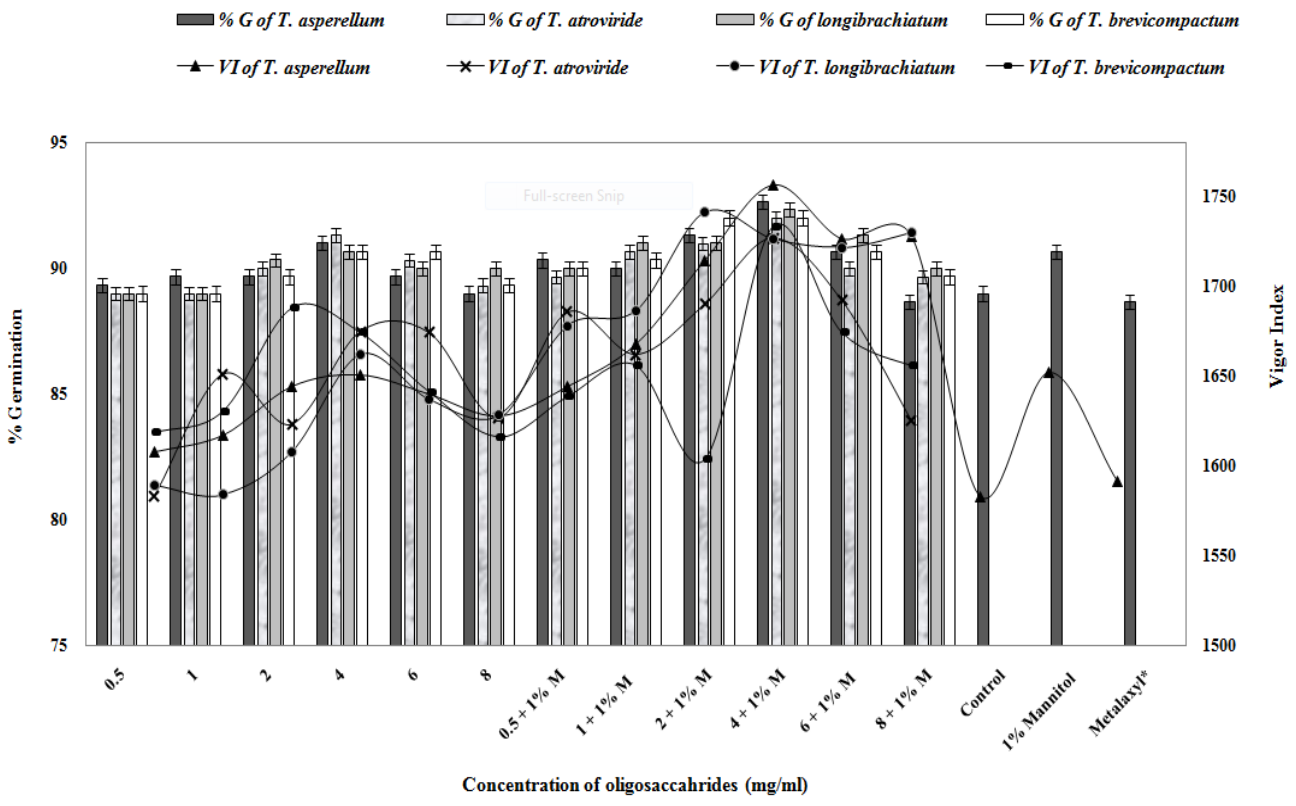

Fig. 1: Effect of seed priming with *Trichoderma* spp. mediated oligosaccharide extracts on seed germination and seedling vigor of pearl millet. Values are means of three independent replicates. % G – percent germination; SV – seedling vigor. *Metalaxyl was used as seed dressing at of 6 g/kg seed.

Table 1: Greenhouse experiments showing DM disease protection upon seed priming with *Trichoderma*-mediated oligosaccharides.

Treatment	Conc. mg/ml	% Downy mildew disease protection			
		T. asperellum	***T. atroviride***	***T. longibrachiatum***	***T. brevicompactum***
Crude oligosaccharides	0.5	29.9 ± 0.54^{opq}	23.4 ± 0.67^{pq}	24.0 ± 0.64^{pq}	26.6 ± 1.07^{opq}
	1	35.8 ± 1.33^{lmno}	37.1 ± 0.13^{mno}	29.8 ± 1.44^{opq}	33.1 ± 1.25^{nop}
	2	47.0 ± 1.17^{fgh}	46.5 ± 0.44^{fgh}	31.7 ± 0.81^{lopq}	33.4 ± 1.86^{nop}
	4	57.7 ± 1.06^{bc}	48.7 ± 0.26^{cdef}	38.7 ± 0.78^{klm}	38.8 ± 1.18^{klm}
	6	45.1 ± 1.21^{ghi}	39.7 ± 1.93^{klm}	36.7 ± 1.86^{mno}	27.9 ± 1.36^{opq}
	8	33.1 ± 1.25^{nop}	46.7 ± 1.29^{fgh}	32.6 ± 1.57^{nop}	30.5 ± 0.79^{opq}
Crude oligosaccharides + 1%mannitol	0.5	34.9 ± 1.75^{mno}	31.9 ± 0.84^{opq}	33.9 ± 1.72^{nop}	$31.8 \pm 1.74l^{opq}$
	1	47.8 ± 1.80^{efg}	43.6 ± 1.51^{hij}	38.3 ± 0.93^{lmn}	39.6 ± 1.07^{klm}
	2	54.3 ± 1.04^{bcd}	48.5 ± 0.68^{def}	36.5 ± 0.46^{mno}	41.7 ± 1.65^{jkl}
	4	61.7 ± 0.80^{b}	54.9 ± 1.86^{bcd}	34.0 ± 1.56^{nop}	42.0 ± 2.2^{jkl}
	6	50.4 ± 1.82^{cde}	44.9 ± 1.86^{ghi}	42.6 ± 1.57^{ijk}	37.7 ± 1.11^{lmn}
	8	47.0 ± 1.12^{fgh}	48.2 ± 0.52^{efg}	34.6 ± 2.33^{mno}	35.0 ± 2.58^{mno}
Control	-	-			
1% Mannitol	-	21.4 ± 0.59^{q}			
Metalaxyl*	-	90.5 ± 0.71^{a}			

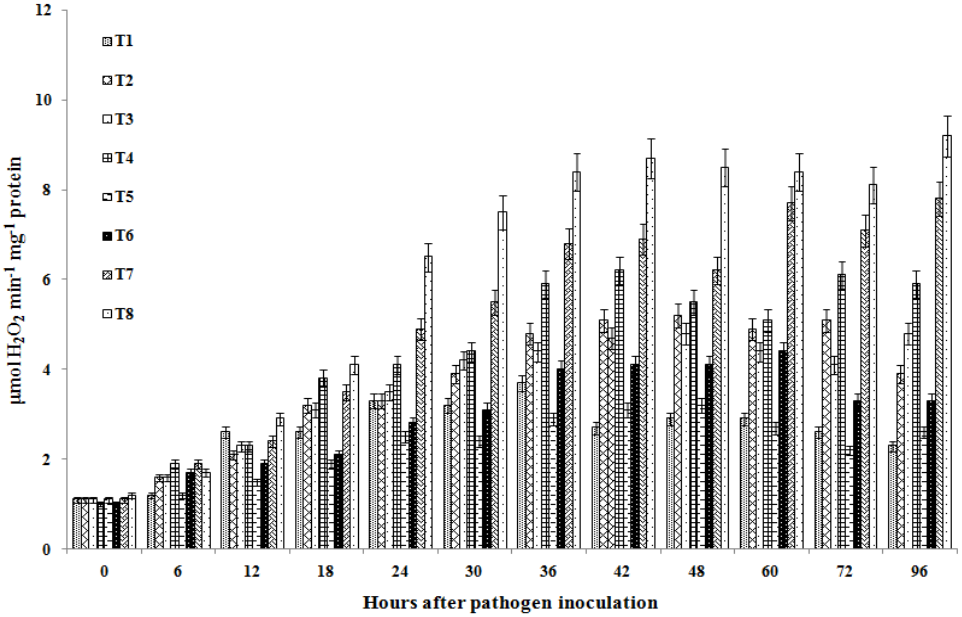

Fig. 2: Temporal profile of accumulation of peroxidase (POX) enzyme in pearl millet (PM) seedlings upon seed priming with oligosaccharide from *T. asperellum*. Lines on the bars indicate the standard error. T1 - control; T2 – 1% mannitol; T3 - Seed priming with oligosaccharides of *T. asperellum*; T4 – Seed priming with oligosaccharides of *T. asperellum* + 1% mannitol; T5 - control + pathogen; T6 - 1% mannitol + pathogen; T7 - Seed priming with oligosaccharides of *T. asperellum* + pathogen; T8 - Seed priming with oligosaccharides of *T. asperellum* + 1% mannitol + pathogen.

3.2 Oligosaccharides stimulates resistance responses in PM against DM under greenhouse conditions

Under greenhouse conditions, *T. asperellum* with mannitol (1%) shows significant ($P \leq 0.05$) protection compared to the other treatments. It was observed that mannitol (1%) treatment alone had not shown any protection against DM pathogen. Further, when it is used in combination with crude oligosaccharide elicitors, it acts as a priming agent for seed treatment with elicitor, which will enhance protection ability of the treatment. *T. asperellum* (4 mg/ml) with 1% mannitol offers maximum protection with least disease incidence of 37.2 % with 61.7 % disease protection. However, positive control treatment, metalaxyl offers least disease incidence of 10.7 % compared all other treatments (Table 1).

3.3 Modulation in defense enzyme activities

The temporal modulation changes in the defense enzyme activity of POX and LOX in treated and control seedlings with or without pathogen inoculation was illustrated in Figures 2 and 3. Besides, a constitutive level of POX and LOX enzyme activities was recorded in all the tested seedlings. In the case of *T. asperellum* treatment with 1% mannitol, POX and LOX enzyme activities were apparent at 0 h, which steadily increased and attained highest at 42 and 96 h for POX and 48 h for LOX, at which the activity has been increased two folds higher than in control-inoculated seedlings (Figures 2 and 3). Distilled water treated control and 1 % mannitol treatments showed least enzyme activities at all time intervals confirming the susceptibility of the selected cultivar to DM pathogen.

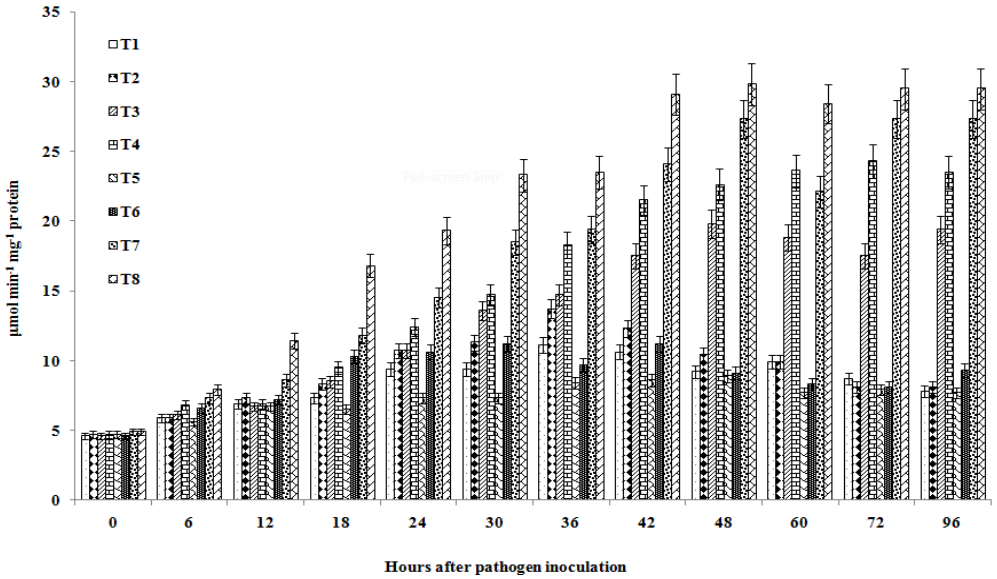

Fig. 3: Temporal profile of accumulation of lipoxygenase (LOX) enzyme in pearl millet (PM) seedlings upon seed priming with oligosaccharide from *T. asperellum*. Lines on the bars indicate the standard error. T1 - control; T2 – 1% mannitol; T3 - Seed priming with oligosaccharides of *T. asperellum*; T4 – Seed priming with oligosaccharides of *T. asperellum* + 1% mannitol; T5 - control + pathogen; T6 - 1% mannitol + pathogen; T7 - Seed priming with oligosaccharides of *T. asperellum* + pathogen; T8 - Seed priming with oligosaccharides of *T. asperellum* + 1% mannitol + pathogen.

4. DISCUSSION

Plant-pathogen interactions are most essential aspect for minimizing the economic deficits caused by pathogens in crops [35]. In the present work, we examined the efficacy of the *Trichoderma* spp. crude oligosaccharides on the PM seed quality parameters and in inducing the DM disease protection ability. Furthermore, correlation pattern was observed in defense enzyme activities with disease protection studies under laboratory and green house conditions. Our earlier report illustrates the efficacy of *Trichoderma* spp. crude oligosaccharides in elicitation of defense responses in PM-DM interaction [25]. In comparison to earlier work, in which, *T. virens* along with 1 % mannitol had shown higher defense enzyme activities compared other treatments along with significant disease protection ($P \leq 0.05$). Mannitol acts as an osmopriming agent in combination with crude elicitors. Roopa et al. [24] illustrate the similar observation by osmopriming with mannitol enhancing the seed quality parameters and planting value in PM. As a sustained part of the our earlier work, in the present exertion, *T. asperellum* with 1% mannitol shows significant ($P \leq 0.05$) protection with minimum disease incidence along with elevated defense enzyme accumulation till the time interval of 96 hours after pathogen inoculation. This study demonstrates the role a close relationship of *Trichoderma* spp. isolated from the monocot root rhizosphere samples is capable of protecting PM host by induction of DM disease resistance.

With mounting ecological attentiveness, the hub of controlling plant diseases has been changed in the direction of feasible and sustainable alternative approaches [36]. Trichoshield, a talc formulation consisting of spores of *T. harzianum*, *T. lignorum*, *Gliocladium virens* and *Bacillus subtilis* seed treatment has improved seed germination factors, vegetative and reproductive growth parameters and provide better protection against DM pathogen under field conditions compared to the individual isolates of *T. harzianum*, *T. lignorum*, *G. virens* and *B. subtilis* [37]. Biocontrol strain *T. harzianum* Th10 mediated cell wall glucan elicitor shows better glucanase activity and phenol accumulation in treated seedlings contrast to control seedlings [38]. Several reports on DM–PM interaction with significance to priming with elicitors have been done. Our present findings are also in concurrence with the findings enhanced levels of defense-related enzymes observed in crude oligosaccharide treated PM seedlings and further increased after *S. graminicola* infection specifies that seed treatment of PM with oligosaccharides makes an incompatible atmosphere for infection, production and sporulation by *S. graminicola* which directs to the disease inhibition [39, 40, 41]. Oligosaccharides extracted from the cell wall of *T. asperellum* shows significantly ($P \leq 0.05$) enhanced defense activity in PM plant and control the DM infection. Hence, this study puts effort to formulate different species of *Trichoderma* oligosaccharides isolated from monocot rhizospheric zones and amalgamate into a biological treatment.

5. CONCLUSION

The present work throws an insight into the efficacy of *Trichoderma* spp. oligosaccharides extracted from the mycelium in improving the PM growth as well as in controlling the DM disease. Further, it confirms the osmopriming activity of the mannitol along with oligosaccharide combinations in improving the seedling vigor and enhancing the disease protection and

thereby developing an efficient biological disease management approach to control the oomycete pathogen.

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