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ORIGINAL ARTICLE

Systemic protection against pearl millet downy mildew disease induced by cell wall glucan elicitors from *Trichoderma hamatum* UOM 13

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Abstract

The obligate oomycete *Sclerospora graminicola* (Sacc.) Schroet, is the incitant of downy mildew disease, which is the main constraint in pearl millet production worldwide. Different elicitors from *Trichoderma hamatum* UOM 13, e.g. mycelial extract and cell wall glucans, were assessed for their resistance elicitation efficiency and the possible underlying mechanisms. Both mycelial extract and cell wall glucans of *T. hamatum* UOM 13 positively influenced seed quality parameters of pearl millet, significantly enhanced seed germination and seedling vigor in comparison to the untreated control. Seed priming with cell wall glucan elicitors of *T. hamatum* UOM 13 suppressed downy mildew on susceptible pearl millet seedlings under greenhouse conditions by induction of systemic host resistance. Of the different elicitor delivery methods tested, transplant root dip was more effective than seed treatment and foliar spray. A combination of transplant root dip + seed treatment + foliar spray was significantly more effective than the single delivery methods. The induced resistance corresponded to up regulation of genes of important defense proteins upon pathogen inoculation. Transcripts of genes of defense enzymes glucanase, phenylalanine ammonia lyase, peroxidase and polyphenoloxidase were significantly increased due to the *T. hamatum* UOM elicitor effect. Expression of hydroxyproline-rich glycoprotein genes, known to play an important role in cell wall cross-linking, were also up regulated in response to *T. hamatum* UOM cell wall glucan treatment. This study emphasizes the role of *T. hamatum* UOM as a potential elicitor of downy mildew resistance in pearl millet and presents novel insights into the involvement of important defense proteins mediating such as resistance trigger.

Key words: cell wall glucans, defense proteins, induced resistance, pearl millet downy mildew, *Trichoderma hamatum*

Introduction

Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is one of the most important cereal crops in semiarid regions worldwide. In India it is cultivated on an area of 7 mln ha with an annual production of 9.25 mln t (Yadav 2015). Downy mildew caused by the obligate biotrophic oomycete *Sclerospora graminicola* (Sacc.) Schroet., is a major disease of pearl millet that significantly reduces production, and causes severe yield losses. Massive damage caused by downy mildew on pearl millet can reduce yields up to 60% and sometimes losses in individual fields can reach nearly 100%. The mean annual loss

across India is estimated to be 14% (Hash *et al*. 1999; Thakur *et al*. 2011).

Recent management strategies include breeding for resistance and application of fungicides, but these methods have serious limitations. *Sclerospora graminicola*, being highly variable, quickly breaks down the host resistance, rendering the hybrid susceptible. Metalaxyl (Apron), a popular and effective fungicide used for downy mildew control is not easily available to farmers. It is expensive for a crop like pearl millet and is known to be hazardous, having residual problems. Furthermore, with concerns regarding the use of fungicides due to their perilous effects on the environment and human health, newer plant protection strategies which rely less, or not at all, on chemicals are gaining worldwide importance and acceptance.

The potential of the free living filamentous fungi *Trichoderma* as elicitors of resistance has been well established in a variety of crop plants against a broad range of phytopathogens (Harman *et al*. 2004; Rao *et al*. 2015). The protective effect of *Trichoderma* treatment against plant diseases is mainly attributed to either biocontrol or the induction of resistance mechanisms (Harman 2006; Shafique *et al*. 2016). Induction of resistance, both local and systemic, by several strains of *Trichoderma* has been well demonstrated against various phytopathogens such as fungi, bacteria, virus and oomycetes (Nawrocka and Małolepsza 2013; Pieterse *et al*. 2014). Effective systemic resistance was induced by *Trichoderma* against the fungus *Rhizoctonia solani* in sunflower (Singh *et al*. 2013), against the bacteria *Pseudomonas syrnigae* in *Arabidopsis* (Segarra *et al*. 2009), against the *Cucumber mosaic virus* in *Arabidopsis* (Elsharkawy *et al*. 2013) and the oomycete *Plasmopara viticola* in grapes (Perazzolli *et al*. 2008).

Earlier studies from our laboratory showed that an endophytic *T. hamatum* UOM 13 isolated from pearl millet roots was efficient in suppressing downy mildew disease incidence both under greenhouse and field conditions (Nayaka *et al*. 2017). The present study was undertaken to elucidate the mechanism of induction of resistance by *T. hamatum* UOM 13 elicitors, like mycelial extract and cell wall glucans against pearl millet downy mildew disease. Furthermore, this study also aimed to determine the most effective methods of elicitor delivery as well as to study the pattern of accumulation of transcripts of some important defense enzymes that are said to have a role in the induction of resistance.

Materials and Methods

Host

Seeds of pearl millet cultivars 7042S and IP 18292, highly susceptible and highly resistant to *S. graminicola,* respectively, were obtained from the International Crop Research Institute for Semi Arid Tropics (ICRI -SAT), Hyderabad, India and the All India Coordinated Research Project on Pearl Millet (AICRP-PM), Mandor, Jodhpur, India.

Source of pathogen and inoculum preparation

Sclerospora graminicola was isolated from severely infected pearl millet cv. 7042S grown under field conditions (Safeeulla 1976). The pathogen was maintained under greenhouse conditions on its susceptible host prior to use. Leaves showing profuse sporulation of *S. graminicola* on the abaxial side were collected in the evening hours, and thoroughly washed under running tap water to remove sporangia. The leaves were then blotted dry, cut into small pieces, and maintained in a moist chamber to promote sporulation. The following morning, fresh sporangia were washed into distilled water. For use as inoculum, the resulting zoospore concentration was adjusted to 40,000 zoospores · ml^{-1} using a haemocytometer.

Elicitor preparation

Endophytic *T. hamatum* UOM 13 which had host resistance inducing potential was previously isolated and identified on the basis of microscopic and macroscopic characteristics, and further confirmed at the molecular level by amplifying and sequencing a portion of the internal transcribed spacer (ITS) region using primers ITS-1 (TCCGTAGGTGAACCTGCGG) and ITS-4 (TCCTCCGCTTATTGATATG) (White *et al*. 1990; Nayaka *et al*. 2017). A representative for each unique ITS sequence was BLAST queried to confirm species designation before being accessioned in Gen -Bank (KP 876050.1).

Trichoderma hamatum UOM 13 mycelia extract preparation

Trichoderma hamatum UOM 13 mycelial extract was prepared as described earlier by Ming *et al*. (2013). The *T. hamatum* UOM 13 was inoculated into 250 ml Erlenmeyer flasks, each containing 100 ml of liquid half-strength B5 medium. The biomass was removed by filtration after incubation on rotary shakers at 28°C and 180 rpm for 10 days. After washing three times in distilled water, mycelia were suspended in distilled water, autoclaved at 121°C for 40 min, and then filtered through three pieces of filter paper under vacuum. The filtrate obtained was the mycelia extract (ME) and this was stored at 4°C in a refrigerator and subsequently it was autoclaved at 121°C for 15 min.

Trichoderma hamatum UOM 13 cell wall glucan preparation

Cell wall glucan elicitors were extracted from *T. hamatum* UOM 13 according to the procedure described by Sriram *et al*. (2009). *Trichoderma hamatum* UOM 13 was cultured on potato dextrose broth for 7 days at 28°C. The mycelial biomass was collected by repeated washing with sterile distilled water. The mycelia were then resuspended in sterile water (1 g in 5 ml) and homogenized. The mycelial slurry was filtered through two layers of muslin cloth and the residue on the muslin cloth was collected. This residue was homogenized thrice in water, once in a mixture of chloroform and methanol (1 : 1) and once in acetone. This crude mycelial cell wall preparation was air dried. The cell wall glucans were extracted from the mycelial walls by suspending 1 g cell wall in 100 ml distilled water and autoclaved. The autoclaved suspension was filtered and clarified by centrifugation. The supernatant was used as cell wall glucan elicitor.

Effect of seed treatment with *Trichoderma hamatum* UOM 13 elicitor preparations on seed germination and seedling vigor

To determine the optimum concentrations of the elicitor preparations and the treatment duration that adversely affect pearl millet seed germination and seedling vigor, various concentrations of the elicitors were prepared and used to treat pearl millet seedlings for varying time durations.

The mycelia extract and cell wall glucan fractions were used at four concentrations viz., undiluted, 1 : 1, 1 : 2 and 1 : 3 v/v with sterile distilled water.

Trichoderma hamatum UOM 13 elicitors were applied as seed treatment to downy mildew susceptible pearl millet cultivar 7042S seeds. The seeds were surface-sterilized with 0.02% mercuric chloride for 5 min, and rinsed thoroughly in sterile distilled water. Seeds were coated with 1% gum arabic as an adhesive and as suspended elicitor preparations (5 ml/ 400 seeds). They were kept at 25±2°C in a rotary shaker for different time durations, 3, 6, 9, 12 and 24 h, to determine the optimum treatment time that would not affect pearl millet seed germination and seedling vigor. Seeds treated with distilled water served as non-treated controls.

Trichoderma hamatum UOM 13 elicitor treated seeds and controls were seeded onto paper towels. The brown germination paper was soaked in distilled water. Fifty seeds of pearl millet were placed equidistantly on the paper. Another presoaked paper towel was placed on the first one so that the seeds were held in position. The towels were then rolled and wrapped with polythene to prevent drying. After incubation for 7 days, the towels were unrolled and the numbers of germinated seeds were counted. Seedling vigor was analyzed at the end of 7 days of incubation by the method of Abdul Baki and Anderson (1973). The length of the roots and shoots of individual seedlings was measured to determine the vigor index. The vigor index was calculated using the formula: Vigor index = (mean root length + mean shoot length) (% germination). The experiment was carried out with four replicates of 100 seeds each and was repeated three times.

Evaluation of *Trichoderma hamatum* UOM 13 elicitor preparations on pearl millet downy mildew disease protection

Trichoderma hamatum UOM 13 elicitors, both as mycelia extract and cell wall glucan preparations when seed treated in undiluted form for 6 h recorded maximum germination and seedling vigor and therefore these concentrations were used as optimum seed treatment concentrations for further studies.

The effect of the following treatment methods on the incidence of downy mildew disease in pearl millet was investigated under greenhouse conditions.

1. Seed treatment

7042S seeds were surface-sterilized with 0.02% mercuric chloride for 5 min, and rinsed thoroughly in sterile distilled water. Seeds were coated with 1% gum arabic as an adhesive and as suspended elicitor preparations (5 ml/400 seeds) and kept at 25±2°C in a rotary shaker for 6 h. The treated seeds were sown in earthen pots filled with autoclaved soil, sand and manure at the ratio of 2 : 1 : 1.

2. Transplant root-dip

7042S seeds were plated on moist blotters and were incubated at 25±2°C in an incubator. Twenty-four hours later the roots of the seedlings were soaked in the *T. hamatum* UOM 13 elicitor preparations for 6 h and later transplanted into earthen pots filled with soil, sand and manure at the ratio of 2 : 1 : 1.

3. Foliar spray

7042S seeds were surface-sterilized with 0.02% mercuric chloride for 5 min, and rinsed thoroughly in sterile distilled water. Seeds were coated with 1% gum arabic as an adhesive and as suspended elicitor preparations (5 ml/400 seeds) and kept at 25±2°C in a rotary shaker for 6 h. The treated seeds were sown in earthen pots filled with autoclaved soil, sand and manure at the ratio of 2 : 1 : 1. The emerging seedlings were sprayed with the elicitor preparations till run-off using an atomizer.

4. Seed treatment + transplant root-dip

Seeds treated as described in (1) were plated on moist blotters and were incubated at 25±2°C in an incubator.

Twenty-four hours later the roots of the seedlings were soaked in the *T. hamatum* UOM 13 elicitor preparations for 6 h and later transplanted into earthen pots filled with soil, sand and manure at the ratio of 2 : 1 : 1.

5. Seed treatment + foliar spray

Seeds treated and sown in pots as described in (1) were sprayed with the elicitor preparations till run-off using an atomizer.

6. Transplant root-dip + foliar spray

Seeds treated and transplanted into pots as described in (2) were sprayed with the elicitor preparations till run-off using an atomizer.

7. Seed treatment + transplant root-dip + foliar spray

Seeds treated and transplanted as described in (4) were sprayed with the elicitor preparations till run-off using an atomizer.

7042S seeds treated with sterile distilled water served as the control. 7042S seeds treated with the systemic fungicide, metalaxyl (Apron 35 SD at 6 g · kg^{-1} seeds) served as a fungicide check and pearl millet seeds of cultivar IP 18292 served as a resistant check.

Each treatment consisted of 4 replications, 10 pots per replication, and 10 seedlings per pot. Treatments were arranged in a randomized complete block design. Three-day-old seedlings were challenge-inoculated by the whorl inoculation method (Singh and Gopinath 1985) with a zoospore suspension of *S. graminicola* at a concentration of 4×10^4 zoospores · ml^{-1} prepared as described previously. In the whorl inoculation method, droplets of *S. graminicola* zoospores were dropped onto the leaf whorl formed by the emerging seedlings and allowed to flow down to the base. These pathogen-inoculated plants were maintained under greenhouse conditions (90–95% RH, 20–25°C), and observed for disease development. The plants were rated for disease when they showed any of the typical downy mildew symptoms such as sporulation on the abaxial leaf surface, chlorosis, stunted growth, or malformation of the earheads. Downy mildew disease incidence was recorded at 30 DAS (days after sowing) and final counts were made at 60 DAS. The experiment consisted of 4 replicates of 100 seedlings each and was repeated twice.

Optimum time interval for maximum resistance after treatment with *Trichoderma hamatum* UOM

This included two sets of experiments. In the first set, seeds treated with *T. hamatum* UOM 13 cell wall glucan elicitor as described above were sown in earthen pots filled with autoclaved soil, sand and manure at the ratio of 2 : 1 : 1. The emerging seedlings were challenge-inoculated with the zoospore suspension of *S. graminicola* by adding 4–5 drops (0.5 ml) to the leaf whorl of each plant at intervals of 1, 2, 3, 4, 5 and 6 days between seedling emergence and pathogen inoculation in different sets of plants. In the second set pearl millet seeds were plated on moist blotters and were incubated at 25±2°C in an incubator. Thirty-six hours later the roots of the seedlings were soaked in the *T. hamatum* UOM 13 cell wall glucan elicitor for 6 h and later transplanted into earthen pots filled with soil, sand and manure at the ratio of 2 : 1 : 1. The seedlings were then challenge-inoculated with zoospore suspension of *S. graminicola* (40,000 zoospores · ml^{-1}) following the whorl inoculation procedure with a time gap of 1, 2, 3, 4, 5 and 6 days in different sets of plants. Both of the above sets of plants were maintained under greenhouse conditions, and observed for the downy mildew disease reaction. Downy mildew disease data were recorded as described earlier.

Biochemical analysis of *Trichoderma hamatum* UOM 13 cell wall glucan elicitor seed treatment on pearl millet defense against downy mildew

Plating of treated seeds

7042S seeds were plated on moist blotters and were incubated at 25±2°C in an incubator. Thirty-six hours later the roots of the seedlings were soaked in the *T. hamatum* UOM 13 cell wall glucan elicitor for 6 h after which the seedlings were transferred to another plate. 7042S and IP 18292 seeds treated with distilled water served as susceptible untreated controls and resistant checks, respectively.

Harvesting of seedlings

Two-day-old seedlings were root-dip inoculated with a zoospore suspension of 4×10^4 zoospores · ml^{-1}, and incubated in the dark at 25±2°C. Seedlings were harvested 0, 3, 6, 9, 12, 24, 48 and 72 h post inoculation (hpi) and immediately wrapped in aluminum foil and stored at –80°C until further use.

Quantitative real-time PCR analysis (qPCR) for defense enzymes and HRGPs

The relative quantitation of phenylalanine ammonia lyase (PAL) (NM_001174615.1), peroxidase (POX) (EU492461), glucanase (GLU) (EU725041.1), polyphenol oxidase (PPO) (AY881993.1), and hydroxyproline-rich glycoprotein (HRGP) (GQ223398), mRNAs in pearl millet seedlings was done by using gene-specific primers, designed with Primer Express version 3.0

Table 1. Primer sequences used for qRT-PCR amplification

Target gene	Forward primer sequence [5′ to 3′]	Reverse primer sequence [5′ to 3′]
GLU	AGCATTCGCAGCCATTCCTA	TGCATGCACGGATTATGGGT
PAL	ATGGAGTGCGAGAACGGCC	CTGCGCGATGCTGAGGCT
POX	CCCCAGAAGCACATTTGTGA	CATGGCTGCGGGCGGAG
PPO	AGTCGAGGTTTGGCCACCAT	CCACCTGATGCGCTCGATG
HRGP	GCCTAAGCCGAAGCCACCAA	GCGTGTAGGTCGGAGGAGTT
PP2A	TGAGAGCAGACAAATCACTCAA	AAGAGCTGTGAGAGGCAAATAA

GLU – glucanase, PAL – phenylalanine ammonia lyase, POX – peroxidase, PPO – polyphenol oxidase, HRGP – hydroxyproline-rich glycoprotein, PP2A – protein phosphatase 2A

software (Applied Biosystems) (Nayaka *et al*. 2017) (Table 1). Protein phosphatase 2A (PP2A) served as the endogenous reference gene. Primer specificities were confirmed by agarose gel electrophoresis of the RT-PCR products. Each qPCR reaction (20 µl) consisted of 1× SYBR Green PCR master mix (SYBR Green mix, Applied Biosystems), 3 pmol of each primer and 20 ng each of cDNA and StepOnePlus™ Real-Time PCR Systems (Applied Biosystems) was used. qPCR steps were: denaturation at 95°C for 10 min, 40 cycles of 15 s at 95°C, 60 s at 60°C. At the end of each reaction, a melting curve was created using a single cycle consisting of 15 s at 95°C and 60 s at 60°C. This was followed by a slow temperature increase to 95°C at the rate of $0.3°C \cdot s^{-1}$. The quantification of target mRNAs used a comparative Ct method (Livak and Schmittgen 2001).

Data analysis

Data from greenhouse and field experiments were analyzed separately for each experiment and were subjected to arcsine transformation and analysis of variance (JMP Software; SAS Institute Inc., Cary, NC). Significance effects of PGPR treatments were determined by the magnitude of the F-value ($p = 0.05$). Treatment means were separated by Tukey's HSD test.

Results

Effect of seed treatment with *Trichoderma hamatum* UOM 13 elicitor preparations on seed germination and seedling vigor

Trichoderma hamatum UOM 13 elicitor preparations, both as mycelial extract and cell wall glucans, significantly enhanced seed germination and seedling vigor in comparison to the untreated control. However, the rate of enhancement varied considerably with the concentration, duration and type of elicitor treatment. *Trichoderma hamatum* UOM mycelial extract treatment showed the best performance when used as an undiluted concentrate when compared to the other dilutions. Undiluted *T. hamatum* UOM mycelial extract, when treated for 6 h, recorded the highest seed germination (97%) and seedling vigor (1997) in comparison to all other concentrations and time durations. Similarly, *T. hamatum* UOM 13 cell wall glucans when treated with an undiluted concentration for 6 h showed the highest seed germination (95%) and seedling vigor (1983) in comparison to all other concentrations and time durations. The untreated control had a maximum germination of 92% and seedling vigor of 1,851 (Table 2).

Evaluation of *Trichoderma hamatum* UOM 13 elicitor preparations on pearl millet downy mildew disease protection

In general, all the tested treatments protected pearl millet against downy mildew disease, but the degree of protection varied considerably. *Trichoderma hamatum* UOM 13 elicitor preparations, both as mycelial extract and cell wall glucans, significantly reduced the incidence of downy mildew disease compared to the untreated control. Furthermore, the reduction of downy mildew varied with the method of elicitor treatment (Table 3).

Among the elicitors, the *T. hamatum* UOM 13 cell wall glucans treatment was more efficient in downy mildew reduction than the *T. hamatum* UOM 13 mycelial extract. It was observed that for both elicitors, the combination treatment method of seed treatment + transplant root-dip + foliar spray recorded maximum downy mildew disease reduction. Among individual treatment methods, with both of the elicitors, the transplant root-dip method showed the highest downy mildew reduction potential. With mycelial extract there was no significant difference between transplant root-dip and the combination of seed treatment + transplant root-dip + foliar spray treatment. Additionally, none of the elicitors were in par with the resistant and fungicide checks in

Table 2. Effect of seed treatment with different concentrations of mycelia extract and cell wall glucans of *Trichoderma hamatum* UOM 13 on pearl millet seed germination and seedling vigor

Treatment	Concentration	Duration of treatment [h]	Seed germination [%]	Seedling vigor
Trichoderma hamatum UOM 13 mycelia extract (ME)	undiluted	3	90 b	1935 b
		6	97 c	1997 d
		9	92 bc	1988 cd
		12	86 ab	1971 c
	1 : 1	3	89 b	1901 ab
		6	94 bc	1963 bc
		9	91 bc	1957 bc
		12	86 ab	1942 b
	1 : 2	3	92 bc	1928 b
		6	91 bc	1945 b
		9	85 ab	1911 ab
		12	84 a	1903 ab
	1 : 3	3	87 ab	1932 b
		6	89 b	1938 b
		9	90 b	1916 ab
		12	90 b	1911 ab
Trichoderma hamatum UOM 13 cell wall glucans (CWG)	undiluted	3	92 bc	1917 ab
		6	95 c	1983 cd
		9	89 b	1955 bc
		12	82 a	1924 b
	1 : 1	3	90 b	1978 cd
		6	91 bc	1981 cd
		9	87 ab	1977 c
		12	84 a	1974 c
	1 : 2	3	85 ab	1966 bc
		6	85 ab	1975 c
		9	89 b	1971 c
		12	86 ab	1962 bc
	1 : 3	3	87 ab	1889 a
		6	87 ab	1876 a
		9	85 ab	1871 a
		12	85 ab	1862 a
Control (distilled water)	–	3	91 bc	1816 a
	–	6	89 b	1834 a
	–	9	92 bc	1848 a
	–	12	90 b	1851 a

Percentages of seed germination and vigor index are the mean from three repeated experiments. Vigor index was calculated from percentage germination and mean root and shoot lengths of the seedlings. The values are the mean from three experiments. Means designated with the same letter in a column were not significantly different according to Tukey's HSD test at p = 0.05

downy mildew reduction which showed 4.7 and 8.3% downy mildew, respectively. The untreated control showed a 97.2% incidence of downy mildew disease.

Trichoderma hamatum UOM 13 mycelial extract treatment showed the least downy mildew incidence of 18.6 when applied as seed treatment + transplant root-dip + foliar spray followed by 19.7% downy mildew when treated as transplant root-dip. Similarly, *T. hamatum* UOM 13 cell wall glucans treatment showed the least downy mildew incidence of 11.3%

Table 3. Effect of mycelia extract and cell wall glucans of *Trichoderma hamatum* UOM 13 applied by different delivery methods on the suppression of pearl millet downy mildew disease

Mode of treatment	Downy mildew disease incidence	
	T. hamatum UOM 13 ME	*T. hamatum* UOM 13 CWG
Seed treatment	22.7 d	19.3 f
Transplant root-dip	19.7 cd	16.1 e
Foliar spray	24.6 e	21.7 h
Seed treatment + transplant root-dip	19.3 c	14.2 d
Seed treatment + foliar spray	24.2 e	22.4 i
Transplant root-dip + foliar spray	23.7 de	20.6 g
Seed treatment + transplant root-dip + foliar spray	18.6 bc	11.3 c
Resistant check	4.7 b	4.7 b
Fungicide check	8.3 a	8.3 a
Untreated control	97.2 f	97.2 j

Percentages of downy mildew incidence are the mean from two repeated experiments. The values are the mean from two experiments. Means designated with the same letter in a column are not significantly different according to Tukey's HSD test at p = 0.05

when applied as seed treatment + transplant root-dip + foliar spray followed by 16.1% downy mildew when treated as transplant root-dip (Table 3).

Optimum time interval for maximum resistance after treatment with *Trichoderma hamatum* UOM 13

Since *T. hamatum* UOM 13 cell wall glucans treatment was more efficient in downy mildew disease reduction compared to mycelial extract treatment, it was further studied for the nature of resistance induced, by following the spatial and temporal separation method. The results showed that resistance offered by *T. hamatum* UOM 13 cell wall glucans was systemic. Initially the *T. hamatum* UOM 13 cell wall glucans treatment showed downy mildew disease incidence of 18.9% when the time gap was 1 day. The downy mildew incidence was further reduced to 18.1% on the second day and 14.7% on the third day. This resistance was consistently maintained throughout the experimental period thus indicating that a minimum of 3 days was required for the total resistance build up. The trend was similar in the second set of experiments where the inducer treatment was given as root dip inoculation. Initially, on the first day gap the downy mildew incidence was 18.6%. This was further reduced to 17.6% and 15.1% on the second and third day gaps, respectively. This protection percentage was maintained throughout the experimental period (Table 4).

Quantitative real-time PCR analysis (qPCR) for defense enzymes and HRGPs

The effect of the *T. hamatum* UOM 13 cell wall glucan treatment on the accumulation and expression of various defense genes after pathogen inoculation was studied by real-time PCR analysis. Important defense genes like: glucanase, phenylalanine ammonia lyase, peroxidase, polyphenol oxidase and hydroxyproline-rich

Table 4. Demonstration of the systemic nature of downy mildew resistance induction in pearl millet by cell wall glucans of *Trichoderma hamatum* UOM 13 following spatial and temporal separation of inducer and the challenger

Time gap after pathogen inoculation (days)	Downy mildew disease incidence	
	whorl inoculation	root-dip inoculation
1	18.6 d	18.9 d
2	17.6 bc	18.1 c
3	15.1 a	14.7 a
4	16.3 b	15.9 b
5	17.9 c	18.1 c
6	19.6 e	19.9 e

Percentages of downy mildew incidence are the mean from two repeated experiments. The values are the mean from two experiments. Means designated with the same letter in a column are not significantly different according to Tukey's HSD test at p = 0.05

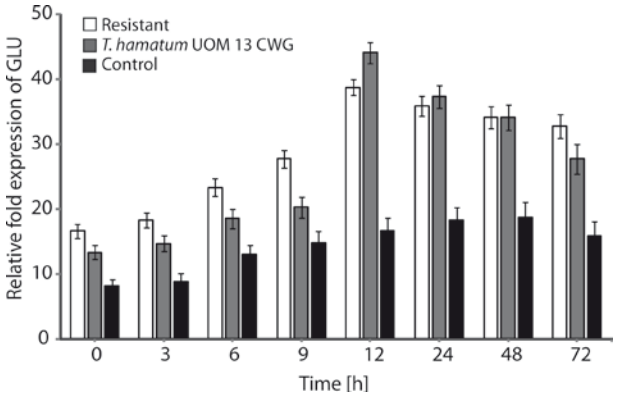

Fig. 1. Relative expression levels of β-1,3 glucanase (GLU) genes in *Trichoderma hamatum* UOM 13 cell wall glucans treated 7042S in comparison to untreated 7042S (control) and IP 18292 (resistant check) pearl millet varieties upon inoculation with *Sclerospora graminicola*. Expression levels were measured by PCR and normalized to the constitutive PP2A gene. Values are mean of a single experiment carried out in triplicate. The bars indicate ±SE

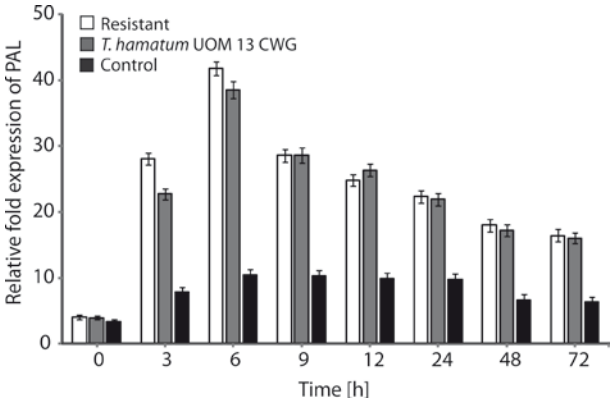

Fig. 2. Relative expression levels of phenylalanine ammonia lyase (PAL) genes in *Trichoderma hamatum* UOM 13 cell wall glucans treated 7042S in comparison to untreated 7042S (control) and IP 18292 (resistant check) pearl millet varieties upon inoculation with *Sclerospora graminicola*. Expression levels were measured by qPCR and normalized to the constitutive PP2A gene. Values are means of a single experiment carried out in triplicate. The bars indicate ±SE

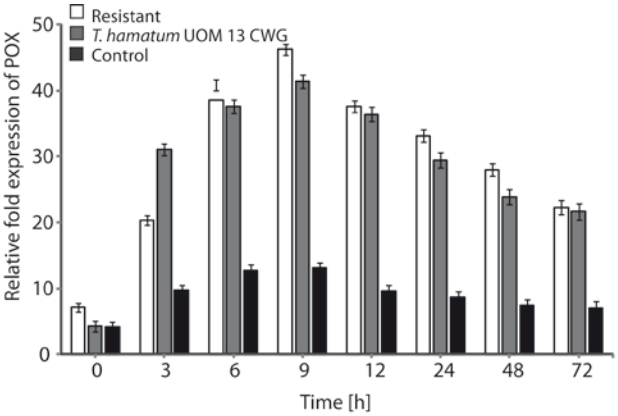

Fig. 3. Relative expression levels of peroxidase (POX) genes in *Trichoderma hamatum* UOM 13 cell wall glucans treated 7042S in comparison to untreated 7042S (control) and IP 18292 (resistant check) pearl millet varieties upon inoculation with *Sclerospora graminicola*. Expression levels were measured by qPCR and normalized to the constitutive PP2A gene. Values are means of a single experiment carried out in triplicate. The bars indicate ±SE

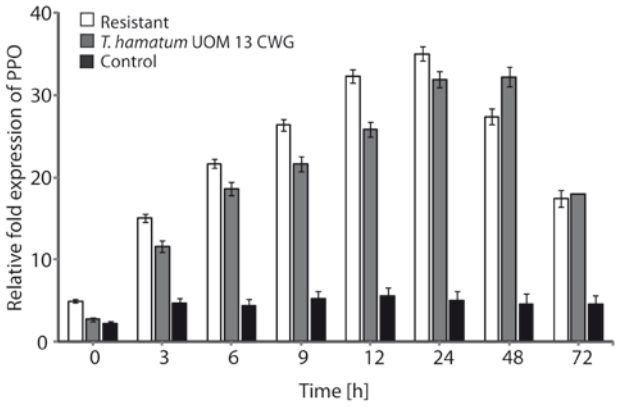

Fig. 4. Relative expression levels of polyphenoloxidase (PPO) genes in *Trichoderma hamatum* UOM 13 cell wall glucans treated 7042S in comparison to untreated 7042S (control) and IP 18292 (resistant check) pearl millet varieties upon inoculation with *Sclerospora graminicola*. Expression levels were measured by qPCR and normalized to the constitutive PP2A gene. Values are means of a single experiment carried out in triplicate. The bars indicate ±SE

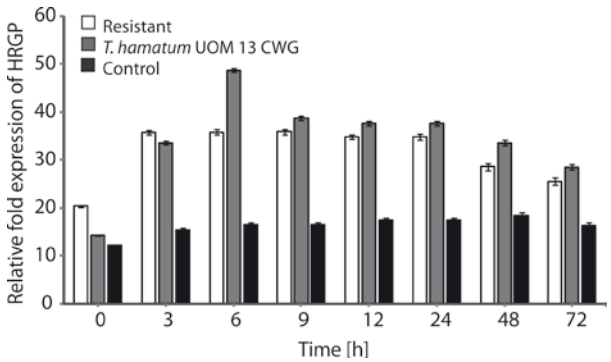

Fig. 5. Relative expression levels of hydroxyproline-rich glycoprotein (HRGP) genes in *Trichoderma hamatum* UOM 13 cell wall glucans 7042S in comparison to untreated 7042S (control) and IP 18292 (resistant check) pearl millet varieties upon inoculation with *Sclerospora graminicola*. Expression levels were measured by qPCR and normalized to the constitutive PP2A gene. Values are means of a single experiment carried out in triplicate. The bars indicate ±SE

glycoproteins were analyzed in elicitor treated seedlings in comparison to the resistant check and untreated control seedlings following pathogen inoculation.

An overall, constitutive level of gene expression was observed for all the genes studied which gradually increased after pathogen inoculation. Furthermore, for all the genes studied the expression level was significantly higher in *T. hamatum* UOM 13 cell wall glucan treated seedlings and resistant check seedlings than in the control seedlings at all time intervals after pathogen inoculation.

In *T. hamatum* UOM 13 cell wall glucan treated seedlings GLU gene expression gradually increased from 3 hpi and peaked at 12 hpi. It was observed that at 12 hpi GLU gene expression in *T. hamatum* UOM 13 cell wall glucan treated seedlings was higher than the resistant check seedlings (Fig. 1). At 12 hpi GLU gene expression in *T. hamatum* UOM 13 cell wall glucan treated and resistant check seedlings were 1.13 and 2.6 fold higher than in the control seedlings. It was observed that at 12 and 24 hpi GLU expression in *T. hamatum* UOM 13 cell wall glucan treated was higher than resistant seedlings. In control seedlings maximum GLU expression was observed at 48 hpi.

In *T. hamatum* UOM 13 cell wall glucan treated seedlings PAL gene expression gradually increased and peaked at 6 hpi and then decreased. In resistant check seedlings PAL peaked at 6 hpi and decreased thereafter. At 9 and 12 hpi PAL expression was equal to and slightly higher than resistant seedlings. At 6 hpi PAL expression in *T. hamatum* UOM 13 cell wall glucan treated seedlings was 3.7 fold higher than control seedlings (Fig. 2).

In *T. hamatum* UOM 13 cell wall glucan treated seedlings after inoculation there was a sudden spike in POX expression from 3 hpi, which peaked at 9 hpi. At 3 hpi POX gene expression was 1.52 fold higher than resistant seedlings. At 9 hpi POX was 3.15 fold higher than the control seedlings. At all time points POX expression was significantly lower than *T. hamatum* UOM 13 cell wall glucan treated and resistant check seedlings (Fig. 3).

In *T. hamatum* UOM 13 cell wall glucan treated seedlings PPO gradually increased and peaked at 48 hpi. At this point PPO expression was higher than resistant check and control seedlings by 1.18 and 7.0 fold, respectively. Up to 24 hpi PPO expression was higher in resistant seedlings than in *T. hamatum* UOM 13 cell wall glucan treated seedlings (Fig. 4).

In *T. hamatum* UOM 13 cell wall glucan treated seedlings HRGPs accumulation gradually increased and peaked at 6 hpi. HRGPs expression in *T. hamatum* UOM 13 cell wall glucan treated seedlings was 1.3 and 2.9 fold higher than resistant and control seedlings, respectively. It was observed that at all time points HRGPs gene expression was higher in *T. hamatum* UOM 13 cell wall glucan treated seedlings than in the resistant check and control seedlings (Fig. 5).

Discussion

Induced resistance is the increased expression of natural defense mechanisms against different plant pathogens, triggered either by biotic or abiotic stimuli. Among the biotic inducers of resistance, *Trichoderma* sp. including *T. hamatum* have been well studied for their role in plant growth promotion, biocontrol and induction of systemic resistance against a wide variety of diseases in many crop plants (Chet *et al*. 1981; Elad 2000; Shoresh and Harman 2008; Błaszczyk *et al*. 2014; Pieterse *et al*. 2014).

The results of the present study indicated that the endophytic *T. hamatum* UOM 13 isolate is highly efficient in improving pearl millet seed quality parameters and also in protecting pearl millet plants against downy mildew disease. Application of *T. hamatum* UOM 13 elicitor preparations, both as mycelia extract and cell wall glucans, significantly increased pearl millet seed germination and seedling vigor and also induced systemic resistance against pearl millet disease and significantly reduced downy mildew incidence by eliciting systemic host resistance.

Analysis of seed quality parameters consequent to *T. hamatum* UOM 13 elicitor treatments revealed that both mycelia extract and cell wall glucans had positive effects on pearl millet seed germination and seedling vigor with cell wall glucans being the superior of the two elicitors. Mycelial extract, when used as an undiluted concentrate for a treatment time of 6 h, was very effective in promoting germination and seedling vigor in comparison to the diluted concentrations. Similarly, the cell wall glucans at an undiluted concentration for a treatment time of 6 h was very effective in promoting pearl millet seed germination and seedling vigor when compared to the other tested dilutions.

Our findings are in line with earlier studies where *Trichoderma* and their elicitor treatments have enhanced seed quality parameters in various crop plants. *Trichoderma hamatum* isolate DIS 219b, isolated as an endophyte from *Theobroma cacao* (cacao) pods, promoted root growth of cacao seedlings (Bae *et al*. 2009). *Trichoderma* isolates showed significant, positive effects on germination, root length and vigour index in soybean (Tancic *et al*. 2013). Seedling growth, germination rate, vigour index and speed of germination of rice seeds were significantly enhanced by treatment with *Trichoderma* sp., SL2 (Doni *et al*. 2014).

Enhancement of seed quality parameters, including seed germination, seedling vigor, and seedling

emergence by *Trichoderma* sp., is attributed to the production of a wide array of secondary metabolites and phytohormones such as auxins and gibberellins and their analogs, as well as proteins such as pyrone and peptaibols, vitamins, and phenolic compounds (Clear and Valic 2005; Vinale *et al*. 2008, 2009; Lorito *et al*. 2010). Production of auxins and gibberellins by *Trichoderma* spp. were the key factors for enhancing rice seedling length (Martínez-Medina *et al*. 2011; Chowdappa *et al*. 2013). Harzianolide, a secondary metabolite produced by *Trichoderma,* was particularly responsible for the enhancement of root length in tomato (Cai *et al*. 2013).

The series of greenhouse experiments conducted to evaluate the efficacy of *T. hamatum* UOM 13 elicitor preparations in controlling pearl millet downy mildew disease showed that both of the elicitor preparations significantly reduced downy mildew disease incidence compared to the untreated control and checks. *Trichoderma hamatum* UOM 13 cell wall glucan elicitor treatment was found to be more effective than mycelia extract in suppressing downy mildew disease. The protection offered by *T. hamatum* UOM 13 was significantly higher than that of the control and was comparable to the resistant and fungicide checks used.

Our results corroborate earlier studies which have shown that the plant protecting ability of *Trichoderma* is by induced resistance and a wide range of *Trichoderma* based formulations are commercially available which are known to induce resistance against a broad spectrum of plant pathogens in several crops (Woo *et al*. 2006; Regliński *et al*. 2012; Mahdizadehnaraghi *et al*. 2015). Particularly, *T. hamatum* have been known to induce effective resistance against several plant pathogens. *Trichoderma hamatum* 382 (T382) significantly suppressed *Phytophthora* root and crown rot of cucumber caused by *P. capsici* by induction of host resistance (Khan *et al*. 2004). Spatio-temporal separation of cell wall glucan treatment and *S. graminicola* inoculation and subsequent evaluation for the downy mildew disease incidence established that the protection offered against downy mildew disease was through induction of resistance which was both systemic and durable. Spatial separation of *T. hamatum* and *P. capsici* established the systemic nature of resistance induction in cucumber (Khan *et al*. 2004). *Trichoderma hamatum* efficiently controlled vascular wilt disease of lentils caused by *Fusarium oxysporum* (El-Hassan *et al*. 2013).

Elicitation of defense responses is effected by a large array of signaling molecules produced by microorganisms. Important elicitors include crude fungal cell wall fragments or defined molecules such as purified proteins and avirulence gene products (Nimchuk *et al*. 2003). Furthermore, it has been suggested that plant immunity stimulation does not necessarily require living organisms, but metabolites from *Trichoderma* may also act as potent elicitors of plant resistance (Dana *et al*. 2001). This hypothesis further emphasizes our findings that *T. hamatum* UOM 13 possess elicitor molecules in its mycelial extract and cell wall glucan fractions which were responsible for eliciting systemic resistance against pearl millet downy mildew disease. *Trichoderma viride*, *T. virens* and *T. harzianum* mycelia extract treatment elicited resistance against *Rhizoctonia bataticola* (dry root rot of chickpea) and *Fusarium oxysporum* f. sp. *ciceris* (wilt of chickpea) (Dubey *et al*. 2011). *Trichoderma harzianum* cell wall glucan elicitor treatment induced resistance against *P. capsici* in red pepper plants (Sriram *et al*. 2009).

Efficient management of plant diseases is dependent on the selection and application to the appropriate site. The application methods vary according to the nature of the elicitor, host plant and the pathogen in question. However, *Trichoderma* elicitors are mainly applied as seed treatment, seed biopriming, seedling dip, soil application and wound dressing (Kumar *et al*. 2014). Of the different methods of elicitor treatment used in this study, it was found that *T. hamatum* elicitors when applied as transplant root-dip were more effective than seed treatment or foliar spray. Most plant pathogens reach the host through vulnerable sites, e.g. the spermosphere, rhizosphere and phyllosphere where they establish themselves. Therefore, protection of such initial entry points by seed treatment or soil drench is more effective than foliar spray. In the pearl millet-downy mildew interaction, the main entry point for the downy mildew pathogen is through the root or coleoptile tissues which are very soft, allowing zoospores to get into them very easily. Since the two--day-old seedling stage is the most susceptible stage and the inoculation by root dip favors easy pathogen entry, seed treatment by elicitor has to be carried out as early as possible. With foliar spray the time is delayed and therefore the effect of the elicitor is reduced due to the relatively higher rate of pathogen establishment.

Treating the seeds is the most convenient method of fungicide application as it provides protection of the plants at their most vulnerable stage of development (Worrall *et al*. 2012). Many studies have shown that root drench treatment works better than foliar spray (Srivastava *et al*. 2011). Soil drench of *T. harzianum* and *T. viride* was more effective than seed treatment in suppressing root rot of vegetables (Abdel-Kader *et al*. 2012). Delivering *Trichoderma* elicitors through a combined application of different delivery systems will synergize the priming effect and thereby suppress the pathogenic propagules.

Our results further showed that a combination of treatment methods is more effective than individual treatments. *Trichoderma hamatum* UOM 13 cell wall glucan treatment as a combination of seed treat-

ment + transplant root dip + foliar spray was more effective in downy mildew suppression than individual treatment methods. Several studies have shown that combinations of different methods of elicitor application could be more effective in disease management than a single method of application (Vidhyasekaran *et al*. 1997; Vidhyasekaran and Muthamilan 1999). A combined application of seed and foliar spray treatments of mixed formulations of *T. hamatum* and *T. viride* were found superior in the management of *Sclerotinia* rot of mustard than individual treatments (Gaur *et al*. 2010). *Trichoderma viride,* when applied as a combination of seed treatment and soil application, was more effective against powdery mildew and leaf blight diseases in sesame than individual treatments (Jeyalakshmi *et al*. 2013). Seed and foliar application of *T. viride* reduced linseed *Alternaria* blight better than individual treatment (Singh *et al*. 2013).

Trichoderma spp. prime the host plants which results in rapid and elevated defense responses following a pathogenic infection (Tucci *et al*. 2011). Triggering of host resistance by *Trichoderma* corresponds with enhanced synthesis of phytoalexins, PR proteins, defense enzymes and other compounds (Dana *et al*. 2001). In our present study, we also evaluated the possible mechanism of *T. hamatum* UOM 13 induced resistance by analyzing the change in expression of various defense genes in the treated and untreated pearl millet seedlings after inoculation with the downy mildew pathogen. The results indicated that *T. hamatum* UOM 13 induced resistance in pearl millet positively correlated with the up regulation of genes of defense enzymes such as glucanase, phenylalanine ammonia lyase, peroxidase, polyphenoloxidase and the cell wall protein hydroxyproline rich glycoproteins. All these defense proteins were expressed early and on significantly higher levels in treated seedlings than in the untreated control seedlings and were comparable in their expression with that of the resistant control. *Trichoderma harzianum* cell wall glucans induced resistance against damping off, collar rot and fruit rot in pepper and corresponded with elevated levels of phenols and glucanase (Sriram *et al*. 2009). *Trichoderma* sp. induced resistance against dry root rot and wilt of chickpea was associated with enhanced activities of glucanase and chitinases (Dubey *et al*. 2011). *Trichoderma hamatum* T382 induced systemic resistance against *Botrytis cinerea* in *Arabidopsis thaliana* which involved the triggering of defense enzymes, including glucanases, chitinases and other pathogenesis related proteins of the SA and Et-pathways (Mathys *et al*. 2012). The ability of *T. hamatum* strain GD12 to elicit systemic resistance against Magnaporthe oryzae in rice, and *S. sclerotiorum* in lettuce was traced to its unique genomic regions with the potential to encode novel bioactive metabolites such as defense enzymes and proteins (Studholme *et al*. 2013). Mayo *et al*. (2015) recorded up regulation of seven defense related genes including glucanase during *Trichoderma* induced resistance against *Rhizoctonia solani* in beans. Two isolates of *T. harzianum*, T7 and T14 exhibited elicitor activities and suppressed *Macrophomina phaseolina* caused charcoal rot of soybean by augmenting activities of peroxidase and phenolics (Khaledi and Taheri 2016).

In summary, our study demonstrated that the application of *T. hamatum* UOM 13 cell wall glucans as elicitors is highly efficient in inducing systemic downy mildew resistance in pearl millet. Our results also suggested that combined methods of application of the elicitor agent work more efficiently than individual applications. Furthermore, analysis of this induced resistance at the molecular level was found to be mediated by the up regulation of some of the important defense enzymes such as glucanase, phenylalanine ammonia lyase, peroxidase, polyphenoloxidase and the cell wall crosslinking protein hydroxyproline rich glycoproteins. These up regulated defensive proteins could serve as important markers of resistance to screen resistant pearl millet cultivars and also to screen potent *Trichoderma* cultures with elicitor activities.

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