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Induced Systemic Resistance in Two Genotypes of *Brassica napus* (AACC) and *Raphanus oleracea* (RRCC) by *Trichoderma* Isolates against *Sclerotinia sclerotiorum*

Jawadayn Talib Alkooranee[1,2,3], Tamarah Raad Aledan[1], Jun Xiang[4], Guangyuan Lu[5*], Maoteng Li[1,3*]

[1]College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, China
[2]Plant Protection Department, College of Agriculture, University of Basrah, Basrah, Iraq
[3]Hubei Key Laboratory of Economic Forest Germplasm Improvement and Resources, Huanggang, China
[4]Hubei Collaborative Innovation Center for the Characteristic Resources Exploitation of Dabie Mountains, Huanggang, China
[5]Oil Crops Research Institute, Chinese Academy of Agricultural Sciences, Wuhan, China
Email: [*]luwiz@163.com, [*]limaoteng426@mail.hust.edu.cn

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Abstract

Two different species, *Trichoderma viride* TV10 and *Trichoderma harzianum* TH12 from 30 *Trichoderma* isolates were selected out based on their high growth inhibition of the phytopathogen *Sclerotinia sclerotiorum* (Lib) de Bary, which reached 84.44% and 100%, respectively. Their untreated culture filtrates (CF) and culture filtrates treated with heat (CFH) also were tested for growth inhibition of the pathogen in potato dextrose agar (PDA). Morphological and molecular characterisation by internal transcribed spacer (ITS) PCR provided consistent identification of these isolates. The degree of infection and disease index (DI) of *S. sclerotiorum* were examined in *Brassica napus* (AACC) and *Raphanus alboglabra* (RR) and *Brassica alboglabra* (CC). The results revealed that *Raphanus alboglabra* showed higher disease resistance than that of *B. napus*. Biotic elecitors *T. harzianum* TH12 and *T. viride* TV10 and their CF and CFH demonstrated the ability to cause induced systemic resistance (ISR) in *B. napus* and *Raphanus alboglabra* against sclerotinia stem rot (SSR) disease. Furthermore, a high ability to reduce the degree of infection and DI in *B. napus* with the biotic elicitors *T. harzianum* TH12 and *T. viride* TV10 was observed, with numbers reaching 7.22% to 6.67% and 17.78% to 11.67%, respectively. When CF were used, reached 20.00% to 16.67% and 33.33% to 23.33%, respectively; with CFH, values reached 35.00% to 21.67% and 37.78% to 28.33%, respectively. While in *Raphanus alboglabra* the degree of infection and DI

[*]Corresponding authors.

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reached 0.00% and 0.00% with all biotic elicitors treatments. These results show that biotic elicitor treatments significantly ($P < 0.05$) can increase the fresh and dry weights of both roots and shoots as well as plant height compared with controls. The TH12 treatment in *B. napus* and *Raphanus alboglabra* ranked as most effective. This study showed for the first time the ability of genotype *Raphanus alboglabra* (RRCC) to demonstrate resistance against *S. sclerotiorum* with or without treatment by biotic elicitors and the ability of genotype *B. napus* (AACC) to demonstrate resistance to the pathogen after treatment with biotic elicitors.

Keywords

***Brassica napus*, *Raphanus alboglabra*, Induced Systemic Resistance, *Sclerotinia sclerotiorum*, *Trichoderma* spp.**

1. Introduction

The Brassicaceae family is composed of many of the economically important food crops. *Brassica napus* is one of the most important oilseed crops in the world after soybean and palm, as it provides oils for human consumption and is used as a forage for animal feed, biofuels, and soil conditioners [1] [2]. *B. napus* (AACC, 2n = 38) is an allopolyploid, resulting from the natural hybridisation between *B. rapa* (AA, 2n = 20) and *B. oleracea* (CC, 2n = 18) [3]. *Raphanus alboglabra* (2n = 34) was a new materials that obtained by hybridization between *Raphanus sativus* (RR, 2n = 16) and *B. alboglabra* (CC, 2n = 18) [4] [5].

Sclerotinia stem rot (SSR) disease is the most common diseases of canola plants caused by *Sclerotinia sclerotiorum* (Lib) de Bary, and its economical effects are associated with substantial losses in quality and quantity of seed yield [6] [7]. *S. sclerotiorum* is a necrotrophic pathogen that could cause disease in more than 400 host plants, and it can cause white mold disease if the conditions are suitable. Although some fungicides have been available to manage this disease, but, several negative effects on the environment have been observed. Excessive use and misuse of agrochemicals may lead to serious environmental consequences, which have reinforced the necessity to consider alternative strategies for the management of this plant disease [8] [9].

There are a variety of induced resistances that vary according to different signalling. Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are two forms of systemic resistance. In both SAR and ISR, plant defenses are released by previous infection thus the application of biotic and abiotic factors, with most agents urged to reduce disease in infected plants between 20% and 85%. SAR and ISR can be used as alternatives to fungicides [10]. ISR is mediated by the *NPR*1 gene, which is a key gene involved in disease resistance, and is phenotypically similar to SAR. However, the molecular pathways of each are different; SAR depends on the salicylic acid (SA) responsiveness [11], while ISR is dependent upon two pathways that respond to ethylene and jasmonic acid [12]. ISR can be defined by induction of defences in plants against many pathogens by application of plant growth-promoting microorganisms in the soil as well as by direct spreading on plants [13] [14]. Increased resistance in *B. rapa* and *Arabidopsis thaliana* leaves to the fungal pathogen *Colletotrichum higginsianum* and bacterial pathogen *Pseudomonas syringae* pv. *Maculicola* when treated with the plant activator Housaku Monogatari (HM) prepared from cell wall extracts of yeast appears to induce early activation of jasmonate/ethylene and late activation of SA pathways [15].

Trichoderma spp. are soil-borne and produce green spores, and they are among ascomycetes that are widespread throughout the world [16]. Fungi of the genus *Trichoderma*, which comprise a group plant growth-promoting fungi (PGPF), are able to colonise the intercellular parts of plant roots and stimulate systemic resistance in all parts of the plant, as demonstrated in cucumber [17]. The ability of *Trichoderma* spp. to induce systemic resistance in dicotyledonous and monocotyledonous plants has been demonstrated against different pathogens, including the fungus *S. sclerotiorum* in tomato and cauliflower [18], the bacteria *Ralstonia solanacearum* in tomato [19], the cucumber mosaic virus in *A. thaliana* [20], and the nematode *Meloidogyne incognita* in *Vigna radiate* [21]. The fungus *T. viride* can induce systemic resistance against *Fusarium oxysporum* and *Alternaria* spp. In addition, in black gram under greenhouse conditions, plants pre-treated with *T. viride* showed higher levels of antioxidant defensive enzymes that led to enhanced seed germination, increased growth, and decreased disease incidence compared with plants without treatment [22]. *T. harzianum* T12 spore suspension reduced the severity of disease of the bean rust fungus *Uromyces appendiculatus* even when autoclaved, and the T12 application on

CF had protective but no curative effects [23]. At present, the effects of *Trichoderma* isolates on *S. sclerotiorum* in *B. napus* have not been reported.

The objectives of this study were to determine the ability and effect of the biotic elicitors *T. harzianum* and *T. viride* as well as CF and CFH on the pathogen *S. sclerotiorum* on the following:

Inhibited pathogen growth in a Petridish, onather is ISR in genotypes *B. napus* and *Raphanus alboglabra* against *S. sclerotiorum*-caused SSR disease.

2. Materials and Methods

2.1. Experimental Layout and Design

One spring-type *B. napus* genotype and newly obtained *Raphanus alboglabra* were used as plant materials in this experiment and isolates of the fungus *S. sclerotiorum* were obtained from the College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, China. We chose these genotypes for comparison where *B. napus* is sensitive to sclerotinia stem rot (SSR) while we test *Raphanus alboglabra* for the first time against pathogens to know its ability to resist. Both genotypes of plants materials and *S. sclerotiorum* were used for greenhouse experiments during the entire period of investigation. The *S. sclerotiorum* fungus was maintained and cultured on PDA medium (200 g peeled potato, 20 g dextrose, 15 g agar, and 1 liter distilled water) in the dark at 22˚C ± 2˚C, and 5-mm-diameter mycelia agar plugs were punched fromthe growing margin after4 days. For rhizosphere inoculation, ascospores were harvested in sterile distilled water using a sterile brush and filtered through four layers of cheesecloth to remove the mycelia of the culture. The resulting spore suspension was adjusted to 1×10^6 spores/ml.

2.2. Biotic Elicitor Isolation

The study was conducted with 30 isolates of *T. harzianum* (TH1 to TH13) and *T. viride* (TV1 to T17) collected in 2014 from soil samples originating from rapeseed fields in Wuhan, Hubei province of China (114˚25'39.7"E; 30˚30'39.8"N). Samples were collected into polythene bags, labelled, and stored in the refrigerator until further processing. *Trichoderma* isolates were isolated in a specific selective medium containing the following (g/litre): 1.0 Ca $(NO_3)_2$, 0.26 KNO_3, 0.26 $MgSO_4 \cdot 7H_2O$, 0.12 KH (PO_4), 1.0 $CaCl_2$, 0.05 $C_6H_8O_7$ (citric acid); 2.0 sucrose, 20.0 agar, 0.05$C_{22}H_{23}ClN_2O_8$ (chlortetracycline), and 0.04 captan (50% wettable powder) [24].

2.3. Morphological Identification of *Trichoderma*

Trichoderma spp. isolates were tentatively identified on the basis of their morphological characteristics [25]. All measurements of morphological characters (size and shape of conidia, size of conidiophores, and presence or absence of sterile hairs) were taken from slide mounts prepared using the tape touch method in a drop of lacto fuchsin [26]. Plates were incubated at 28˚C ± 2˚C, and colonies were selected. *T. harzianum* TH12 and *T. viride* TV10 were obtained from 30 isolates from the pure fungi cultures in these experiments.

2.4. PCR Amplification of ITS Regions of *Trichoderma* Isolates

Total DNA was extracted according to the method described by Chakraborty *et al*. [27], and the DNA samples were stored at −20˚C in small aliquots. The ITS regions were amplified using the universal primers ITS1 (5'-TCTGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') according to White *et al*. [28]. PCR was performed in a total volume of 100 µl, which contained 78 µl deionised water, 10 µl 10× Taq pol buffer, 1 µl 1-U Taq polymerase enzyme, 6 µl 2-mM dNTPs, 1.5 µl 100-mM reverse and forward primers, and 1 µl 50-ng template DNA. PCR was programmed with an initial denaturing at 94˚C for 5 min followed by 30 cycles of denaturation at 94˚C for 30 sec, annealing at 59˚C for 30 sec, extension at 70˚C for 2 min, and a final extension at 72˚C for 7 min. The PCR product (20 µl) was mixed with loading buffer (8 µ1) containing 0.25% bromophenol blue and 40% (w/v) sucrose in water and then loaded in 2% agarose gel with 0.1% ethidium bromide for examination via horizontal electrophoresis.

2.5. Production of Spore Suspensions, CF, and CFH

Fifteen mycelial 1-cm square disks of actively growing TH12 and TV10 were inoculated separately with 300 ml

of PDB and incubated with a rotary shaker (85 rpm) at 25˚C ± 2˚C. After 20 days, the mycelial mat was harvested and grinded to form a spore suspension. The cell concentration was then adjusted to 1.5×10^7 CFU/ml (TH12) and 1×10^7 CFU/ml (TV10). Pure cultures of biotic elicitors were maintained on respective agar slants and stored at 4˚C for further use. Cell-free CF from 20-day-old *T. harzianum* TH12 and *T. viride* TV10 grown on PDB were prepared by centrifugation (12,000 × g for 15 min) followed by filter sterilisation with a 0.4-µm filter unit; the supernatants were collected and used as enzyme solutions. Some CF for biotic elicitors were heated to 100˚C for 15 min (*i.e.*, CFH) to collect non-volatile metabolites produced by biotic elicitors.

2.6. Assay of Mycoparasitic and CF Effects

To determine the effects of isolates on mycelia growth of the targeted pathogen *S. sclerotirum* in dual-culture techniques, *Trichoderma* isolate suspensions and their cell free (CF) and CFH biotic elicitors were added to molten PDA media (40˚C ± 3˚C) to obtain a final concentration of 25% (v/v) each, which were mixed properly prior to plating. The media was poured in Petri dishes at 20 ml per plate. Plates were inoculated separately with 5-mm mycelia plugs of the pathogen *S. sclerotiorum* placed in the centres of the plates. The inoculated plates were incubated at 25˚C ± 1˚C for 5 days. Percent mycelial growth inhibition of each pathogen was calculated using the formula:

$$\text{Inhibition } \% = \text{C} - \text{T}/\text{C} \times 100$$

where C = control (radial growth of the pathogen) and T = treatment (radial growth of the pathogen after inhibition by the antagonist).

2.7. Plant Cultivation under Greenhouse Conditions

Both genotypes *B. napus* and *Raphanus alboglabra* were planted in 25-cm-diamter pots containinga 3-kg mixture of sand and peat moss at a ratio of 1:1 that had been autoclaved twice for 30 min within a 24-h interval. Five seeds of each genotype were sown into each pot. Both genotypes were grown under greenhouse conditions at 18/14 (±1) ˚C (day/night) temperature and a light intensity of 150 µE/m^2/s^1 for 12-h light/dark cycles for 35 to 40 days. Irrigation was applied by drenching twice a week.

2.8. Screening of Fungal Strains Based on ISR-Eliciting Potential and Effects on Plant Growth

To screen the fungal biotic elicitors capable of eliciting ISR, 15-day-old both *B. napus* and *Raphanus alboglabra* plants were treated with water (control) or inoculated with suspensions 100 ml (1.5×10^7 CFU/ml) of *T. harzianum* TH12 and (1×10^7 CFU/ml) *T. viride* TV10 as well as their CF and CFH by spraying the leaves and stems, as opposed to soil drenching. The *S. sclerotiorum* tested was grown in PDB media for 4 weeks incubated with a rotary shaker (85 rpm) at 22˚C ± 2˚C. The pathogen propagules of each flask containing 30 ml media was blended and then mixed with the upper soil surface of each pot 1 day after application of the last biotic elicitor. Each treatment consisted of three replicates. Disease incidence and intensity were recorded at 15 days after treatment using a rating from 0 (no stem discolouration) to 5 (discolouration, sclerotia, premature ripening of the whole plant, and low seed production). Numerical values of 0, 1.25, 2.5, 3.75, and 5, respectively, were assigned to the 1 to 5 rating, and the disease index (DI) was calculated following the formula of Dueck *et al.* [29]:

$$\text{DI} = \frac{(1.25 \times \text{Y2}) + (2.5 \times \text{Y3}) + (3.75 \times \text{Y4}) + (5 \times \text{Y5})}{\text{Total plant}} \times \frac{1}{0.05}$$

where DI = disease index and Y2, Y3, Y4, and Y5 = numbers of plants with scores of 2 to 5, respectively.

Based on the percent disease incidence and intensity, the mean degree of infection was calculated following the formula of Krüger [30]:

$$\%\,\text{Q} = \frac{(n \times 0) + (n \times 10) + (n \times 20) + \cdots (n \times 100)}{n}$$

where Q = degree of infection and n = numbers of assessed plants whose percentages were 0 to 100.

Fresh and dry (70˚C for 48 h) weights of shoots and roots as well as plant height were determined. Five plants

were chosen from each pot for these determinations at 45 days after sowing.

2.9. Statistical Analysis

Antagonistic effects of biotic elicitor isolates and their cell-free CF and CFH against *S. sclerotiorum* and all greenhouse experiments were analysed as completely randomised designs with three replications using GenStat software, and means were compared using least-significant difference tests [31].

3. Results and Discussion

3.1. Morphological Identification of *Trichoderma* spp.

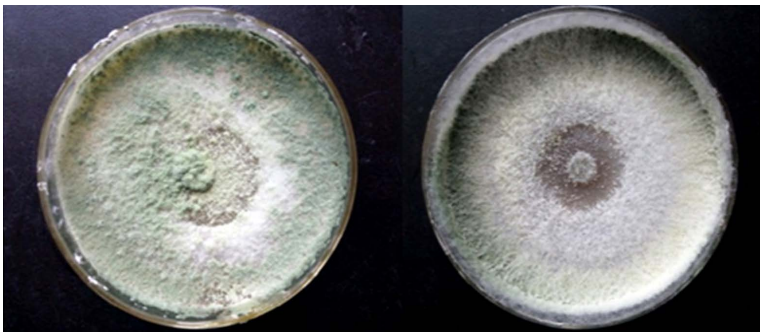
Thirteen isolates of *T. harzianum* and 17 of *T. viride* were isolated from rhizospheres of healthy rapeseed plants. Morphological characteristics have been used to characterise and discriminate *Trichoderma* species since 1969 [32]. *Trichoderma* isolated in PDA culture media after 7 days of incubation at 28˚C ± 2˚C showed differences in mycelial culture growth and patterns of sporulation (**Figure 1**). The characters of mycelia colour patterns varied noticeably between the *Trichoderma* isolates, from colourless to yellow and white; their shapes consisted of concentric rings, and hyphae were septated, smooth-walled, and hyaline. The conidia were green to yellow-green or dark green colour and their shapes were rough and subglobose. Conidia production was more intense in the centre but declined toward the margins. Morphological studies can provide information to identify *Trichoderma* species, but it is insufficient because *Trichoderma* species have relatively few morphological characters and limited disparity, which may cause interference and misidentification [33]. Furthermore, cultural conditions also can affect morphological characteristics [34].

3.2. Molecular Characterisation of TH12 and TV10 Isolates

The *Trichoderma* genus is important from a practical perspective due to the ability of strains, belonging to various species, to inhibit and reduce effects of fungal plant pathogens. To use certain strains for plant protection as useful bio products, it is necessary to identify and characterise the species. The effects of environmental conditions on physiological and morphological characteristics have made accurate identification extremely difficult [35]. For better identification, molecular techniques are very useful. The ITS regions of rDNA of *T. harzianum* TH12 and *T. viride* TV10 were amplified using primers ITS1 and ITS4. ITS 5.8SDNA fragments approximately 625base pairs in length (according to estimates via agarose gel electrophoresis) were successfully amplified from *T. harzianum* TH12 and *T. viride* TV10 (**Figure 2**). Amplified PCR products of 5.8S rDNA genes from *T. harzianum* TH12 and *T. viride* TV10 isolates were sequenced; BLAST on National Centre for Biotechnology Information (NCBI) was then used to confirm the species identities of *T. harzianum* TH12 and *T. viride* TV10, which were morphologically identified earlier.

3.3. Detection Ability of Antagonists of *Trichoderma* Isolates *in Vitro*

The isolates were tested for their ability to reduce mycelia growth of *S. sclerotiorum* fungi using dual-culture



(a) (b)

Figure 1. Two different isolates of (a) *T. harzianum* TH12 and (b) *T. viride* TV10 on potato dextrose agar after 7 days of incubation.

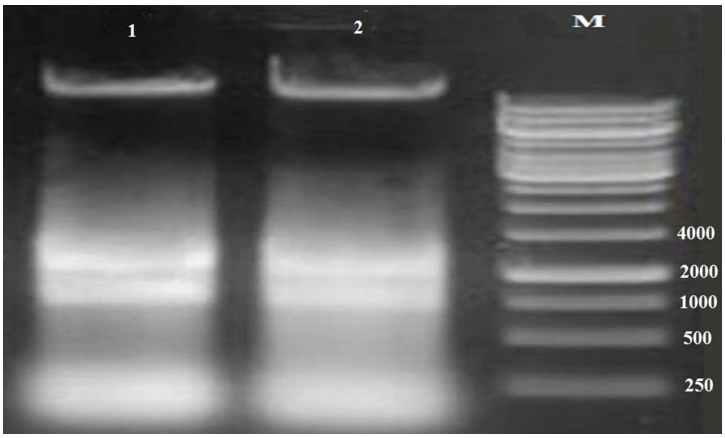


Figure 2. Polymerase chain reaction (PCR) amplification products obtained from total DNA extracted from *Trichoderma* spp. using internal transcribed spacer (ITS) 1 and ITS4 primers. Lane 1 = *T. viride* TV10. Lane 2 = *T. harzianum* TH12. M = DNA marker.

techniques. The results revealed that the degree of inhibition varied greatly, ranging from 1.67% to 100% (**Table 1**), which facilitated of the biotic elecitors. On the basis of percent inhibition of the radial growth of the test pathogens, two specific isolates, *T. harzianum* TH12 and *T. viride* TV10, exhibited higher antagonistic potential were selected. These isolates (TH12 and TV10) reduced the growth of the pathogen *S. sclerotiorum*, with inhibition zones reaching 100 and 84.44%, respectively. Their cell-free CF caused the inhibition zones to reach 100 and 61.48%, respectively, and the inhibition zones of their CFH reached 50.00% and 35.18%, respectively (**Figure 3**). These results indicate that among the different strains of the same fungal genus isolated from the same ecological niche, a significant difference exists in terms of their antagonistic behaviour against a pathogen. *Tricoderma* is a genus of saprophytic fungi. These *Trichoderma* fungi are present in various soils and rhizosphere microflora, and they are well known as biological control agents against various plant pathogens. It should be noted that percent inhibition of radial *S. sclerotiorum* growth by different isolates of fungi varied widely in the present study ($P < 0.05$). These isolates overgrew the pathogen and completely suppressed it within 7 days of inoculation. These results indicate that *T. harzianum* and *T. viride* strongly can prevent mycelial growth of *S. sclerotiorum* in plates [36]-[38]. Generally, considerable variation in the inhibitory properties of resistant biotic elicitor isolates exists and is discernible. This could be due to differences in the quantity and quality of the material produced. Some strains inhibit the growth of fungal pathogens through production of volatile and non-volatile antibiotics and production of low-molecular-weight diffusible compounds or antibiotics [39].

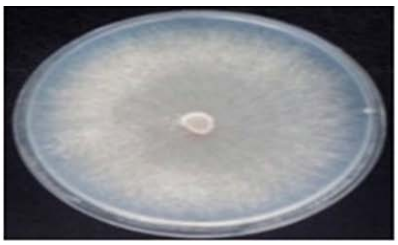
Trichoderma isolates are capable of producing many powerful plant-degrading enzymes and more than 200 types of antibiotics that are highly toxic to any macro- and microorganism [40]. Different mechanisms have been proposed for their mycoparasitic secretion of lytic enzymes, such as cellulase (β-1, 4-glucanase), proteases, and chitinases, which degrade cell walls [41]. Production of the antibiotic is likely located in the tips of growing hyphae, with components responsible for the antagonism secreted at the points of contact with the pathogen [42]. *T. harzianum* and *T. viride* CF to inhibit growth of *Fusarium moniliforme* pathogens might be due to the production of volatile compounds and liberation of extracellular enzymes, such as those with amylolytic, pectinolytic, proteolytic, and cellulolytic activities [43].

3.4. ISR of Biotic Elicitors to *S. sclerotiorum* in *B. napus* and *Raphanus alboglabra*

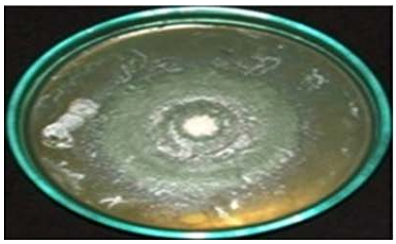
The results showed that the pathogenic fungus *S. sclerotiorum* effect is significant in *B. napus* and *Raphanus alboglabra* plants compared to non-infected plants with the fungus pathogen (**Figure 4**). The results in **Table 2** show that *B. napus* and *Raphanus alboglabra* plants grown in soils amended with TH12, TV10, CF, and CFH resulted in reductions of disease symptoms compared to control plants. The treatments were sprayed on rapeseed leaves and stems 1 day prior to inoculation with *S. sclerotiorum* in the greenhouse. The disease was evaluated by determining rates of disease intensity in plants after 15 days of challenge by examining the growth of the pathogen. Treatment with the biotic elicitors CF and CFH significantly ISR in treatment plants compared to control

Table 1. *In-vitro* screening of *Trichoderma* isolates against *S. sclerotiorum*.

Trichoderma isolates	Inhibition ability test	
	Mean radial growth (mm)	% Inhibition (seventh day of inoculation)
T. harzianum		
TH1	29.3	67.44
TH2	38.7	57
TH3	58	35.56
TH4	23.5	37.89
TH5	35	61.11
TH6	72.6	19.33
TH7	68.3	24.11
TH8	42.5	52.78
TH9	50.6	43.78
TH10	29.3	67.44
TH11	77.8	13.56
TH12	0	100
TH13	42.7	52.56
LSD	**4.628**	**4.125**
T. viride		
TV1	68.8	23.56
TV2	88	2.22
TV3	84.6	6
TV4	25.3	71.89
TV5	70	22.2
TV6	53.6	40.44
TV7	88.5	1.67
TV8	60.2	33.11
TV9	34.8	61.33
TV10	14	84.44
TV11	80.3	10.78
TV12	77	14.44
TV13	38.5	57.22
TV14	47.8	46.89
TV15	65	27.78
TV16	70.5	21.67
TV17	65.6	37.11
LSD	**6.385**	**5.776**

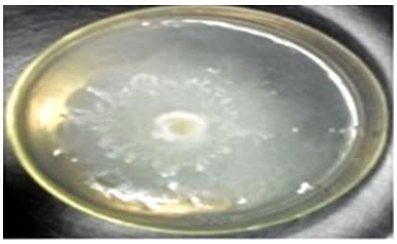


S. sclerotiorum (Control)



TH12 + *S. sclerotiorum*

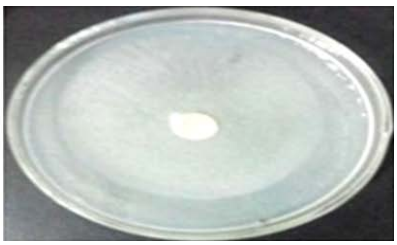
Cell-free CF TH12 + *S. sclerotiorum*



Cell-free CF with heat TH12 + *S. sclerotiorum*

TV10 + *S. sclerotiorum*

Cell-free CF TV10 + *S. sclerotiorum*



Cell-free CF with heat TV10 + *S. sclerotiorum*

Figure 3. Antagonistic activities of *T. harzianum* TH12 and *T. viride* TV10 as well as their cell-free culture filtrates (CF) and culture filtrates treated with heat (CFH) against *S. sclerotiorum*. LSD = least significant difference.

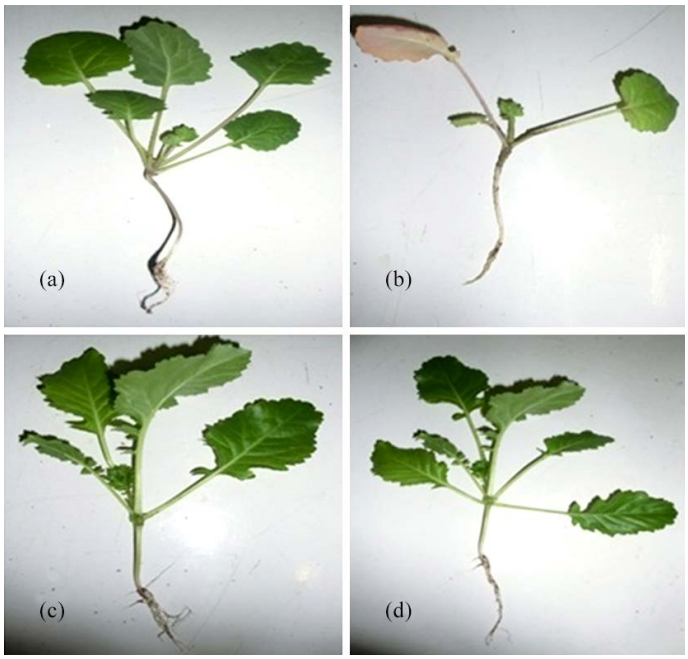


Figure 4. Effects *S. sclerotiorum* on *B. napus* (AACC) and *Raphanus oleracea* (RRCC). (a) = non-pathogen (AACC). (b) = AACC infected. (c) = non-pathogen (RRCC). (d) = RRCC infected.

Table 2. Effect of *T. harzianum* TH12 and *T. viride* TV10 and their CF and CFH on *B. napus* and *Raphanus alboglabra* infected by *S. sclerotiorum* in greenhouse conditions.

Biotic elicitors and *S. sclerotiorum*		% Degree of infection	% Disease index	Root weight (g)		Shoot weight (g)		Shoot length (cm)
				Fresh	Dry	Fresh	Dry	
B. napus AACC								
Non pathogen		0.00	0.00	2.545	0.072	6.120	0.947	20.33
Control		81.40	83.50	1.600	0.016	3.846	0.143	14.40
T. harzianum	TH12	7.22	6.67	2.390	0.065	5.745	0.880	17.32
	*CF TH12	20.00	16.67	2.317	0.047	5.579	0.733	21.67
	*CFH TH12	35.00	21.67	2.253	0.041	5.272	0.634	18.93
T. viride	TV10	17.78	11.67	2.350	0.058	5.614	0.755	21.11
	CF TV10	33.33	23.33	2.324	0.050	4.437	0.677	20.33
	CFH TV10	37.78	28.33	2.226	0.038	4.228	0.539	18.20
	L.S.D	6.218	13.99	0.180	0.017	0.291	0.0507	1.223
Raphanus alboglabra								
Non pathogen		0.00	0.00	2.75	0.075	5.55	1.14	18.33
Control		0.00	0.00	2.70	0.071	5.40	0.95	18.00
T. harzianum	TH12	0.00	0.00	3.00	0.093	5.95	1.30	21.33
	CF TH12	0.00	0.00	2.88	0.090	5.88	1.18	20.67
	CFH TH12	0.00	0.00	2.75	0.078	5.59	1.25	18.60
T. viride	TV10	0.00	0.00	2.98	0.090	5. 90	1.28	21.32
	CF TV10	0.00	0.00	2.90	0.086	5.95	1.25	20.33
	CFH TV10	0.00	0.00	2.76	0.075	5.63	1.20	18.33
	L.S.D	0.00	0.00	0.736	0.120	0.526	0.138	1.67

Tables with the some treatments are not significantly different ($P = 0.05$). *CF = culture filter of resistant biotic elecitor. *CFH = culture filter of resistant biotic elecitor treated with heat. LSD = least significant difference.

plants (**Table 2**). There was no significant difference in inhibitory efficacy between the cell suspensions for the biotic elicitors CF and CFH. Suppression of SSR on plant leaves by cell suspensions was observed when biotic elicitor cells were applied to plant leaves before plants were inoculated with *S. sclerotiorum*. This indicates antifungal substances produced by biotic elicitor cells can penetrate well inside plant tissues to exhibit therapeutic effects and exert preventive effects against SSR disease. Therefore, once *S. sclerotiorum* infects plant tissue, it may be affected not at all or only slightly by antifungal substances produced *in vitro* by biotic elicitors [44] [45].

Biotic elicitors in plant roots play a role in improving plant health. ISR and plant growth promotion are the important mechanisms by which selected mycorrhizal fungi and plant growth-promoting rhizobacteria in the rhizosphere prime the entire plant to promote defence against a broad range of pathogens [46]. A wide range of organisms associated root of plant, such as *Trichoderma* spp., prime plant immune systems for enhanced defence, indirectly activating the defensive system [22] [23]. Atagonistic activities of resistant biotic elicitors and their CF have been reported against several fungal plant pathogens. The present results show that the effects of the pathogenic fungus *S. sclerotiorum* are significant in rapeseed compared to untreated plants. The degree of infection and disease index in treatments were 81.40% and 83.50%, respectively, in genotype *B. napus*, and both were 0.00 in genotype and *Raphanus alboglabra*. The present results also indicate that the pathogenic fungus causes reduced root and shoot fresh and dry weights and reduced shoot height (**Table 2**).

The absence of resistance source in *B. napus*, AACC has dramatically restrained resistance breeding against phytopathogen. Newly, many of wild *Brassica oleracea* (*B. incana*) accessions (CC) identified with high level of resistance against *S. sclerotiorum* pathogen which causes severe crop losses in oilseed production all around the world [47] [48].

A high level of resistance to *S. sclerotium* was reported in parental species of rapeseed such as *B. oleracea* cytodeme and was evaluated for resistance over 2 years. Relative to RS lines exhibited stronger resistance against *S. sclerotiorum* than "Zhongyou 821", a cultivar of *B. napus* has partial resistant; RS lines are one of the most important sources of resistance in current rapeseed against Sclerotinia stem rot [49]. Significant differences were showed across the two growing seasons of *B. incana* and *B. oleracea* var. *alboglabra* between parents and the progeny for measures of both stem and leaf sclerotina resistance [50].

The biotic elicitors significantly reduced ($P \leq 0.5$) the SSR disease incidence in the rapeseed infected with *S. sclerotiorum*. The highest reduction in SSR degree of infection and DI were 7.22% and 6.67%, respectively, treated with TH12. The degree of infection and DI of the CF reached 20.00% and 16.67%, respectively (**Figure 4**), and the CFH treatment reached 35.00% and 21.67%, respectively, in *B. napus*. On the other hand, the degree of infection and DI reached 17.78% to 11.67% of that treated with TV10. Its CF reached 33.33% and 23.33% and CFH reached 37.78% and 28.33%, respectively, in *B. napus*. In and *Raphanus alboglabra*, values of 0.00% for both in all treatments were observed.

These results were consistent with those of Yoshioka *et al.* [51], who described the ability of a cell-free CF of *Trichoderma asperellum* STK-1 to induce systemic resistance in *A. thaliana*, decrease lesion development and growth of the pathogen *P. syringae* pv. Tomato DC3000. *Trichoderma harzianum* T39, when applied before 48 to 72 h of inoculation, reduced downy mildew disease approximately 63% on susceptible grapevine cultivars caused by *Plasmopara viticola*in greenhouse conditions [52]. The PGPF *Penicillium* spp. GP16-2 and its cell-free CF were used to stimulate systemic resistance in *A. thaliana* against the bacterial leaf speck pathogen *P. syringae* [13]. They act against plant pathogens in several ways, either by lytic enzymes, antibiotic-mediated suppression, mycoparasitism and other by product production, induction of host resistance, or competition for nutrients [9]. SA accumulation and SA signalling have been demonstrated to facilitate resistance to the pathogens *Colletotrichum higginsianum* and *P. syringae* pv. *maculicola* in *B. rapa* and *A. thaliana* after treatment with plant activator HM [15]. Tested isolates of *Trichoderma* spp. can produce organic acids, such as gluconic and fumaric acids that permit the solubilisation of phosphates and decrease soil pH, micronutrients, and mineral cations, such as Mg, Mn, and Fe which are useful for plant metabolism [53] [54]. Jian *et al.* [55] also demonstrated that the same biogenic factors can provide protection in pea plants by causing increases in H_2O_2 (hydrogen peroxide) production, ascorbic acid, antioxidant enzymes, and lipid peroxidation against the pathogen *S. sclerotiorum*. The resistant biotic elicitor modes of action were not measured experimentally in this study. Amendment with biotic elicitors increased root and shoot fresh and dry weights and height of rapeseed plants and differed significantly compared to control plants infected by the pathogen (**Table 2**). The TH12 treatment ranked as most effective on *B. napus* with 2.390, 0.065, 5.745, and 0.880 g (root fresh weight, root dry weight, shoot fresh weight, and shoot dry weight, respectively) and 20.33 cm (height). This was followed by TV10, which reached 2.350, 0.058, 5.614, and 0.755 g and 21.11 cm, respectively, followed by CFH of TV10, which reached 2.324, 0.050, 4.437, and 0.677 g and 20.33 cm, respectively. The CFH of TV10 provided the lowest protection against the disease (2.226, 0.038, 4.228, and 0.539 g and 18.20 cm, respectively). The TH12 treatment ranked as most effective on *Raphanus alboglabra*, reaching3.00, 0.093, 5.95, and 1.30 g and 21.33 cm, respectively.

This was followed by TV10, which reached 2.98, 0.090, 5.90, and 1.28 g and 21.32 cm, respectively. The TH12 CF treatment reached 2.88, 0.090, 5.88, and 1.18 g and 20.67 cm, respectively, and the TH12 CFH treatment reached 2.75, 0.078, 5.59, and 1.25 g and 18.67 cm, respectively. Autoclaved *T. harzianum* T12 spores were not significantly different from living T12 spores at reducing uredial pustule percentage and reducing activity of bean rust disease caused by *Uromyces appendiculatus*, but autoclaving *T. harzianum* spores eliminated their ability to reduce rust spore infections [23]. *Trichoderma* species have the ability to suppress pathogens belonging to many other genera. This ability is due to the production of non-volatile and volatile organic compounds, extracellular lytic enzymes, high proliferation rates, and high competitive saprophytic abilities [56]. Their abilities to protect plantscan be attributed to the upregulation of a variety pathogenesis-related proteins and defence-related proteins (e.g., peroxidases, chitinases, glucanases, and specific phytoalexins), and enzymatic activities usually increase during a pathogenic attack [17] [57]. Protection of plants by *Trichoderma* spp. using

ISR is considered of fundamental importance today compared with the "classical" direct, antagonistic, and competition methods that occur with a pathogen in host plants [2] [53].

Finally, some plants do not have adequate protection to most potential pathogens that continually attack plants, such as fungi, bacteria, and viruses. This feature is referred to as "non-host resistance", such as *B. napus* in this study. This genotype of plants needs biotic or abiotic elicitors to induce resistance against various phytopathogens. The *T. harzianum* and *T. viride* and their CF and CFH can be used as elicitors of resistance in *B. napus*. Moreover, some genotypes have the ability to resist various phytopathogens. This feature is referred to as "host resistance", such as genotype *Raphanus alboglabra*; this study is the first to reveal the ability of *Raphanus alboglabra* to demonstrate resistance to SSR disease caused by *S. sclerotiorum*.

4. Conclusion

The application of useful resistant biotic elicitors for protection and increased rapeseed crop production is now practised in agriculture. The resistant biotic elicitors may better adapt to environmental changes, protect against a broader range of pathogens of plant varieties, increase the genetic diversity of biocontrol systems that continue longer in the rhizosphere without negatively impacting the environment, and utilise a wider array of ISR mechanisms.

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