Egyptian Journal of Biology, 2009, Vol. 11, pp 37-44
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Antagonistic effects of three species of *Trichoderma* sp. on *Sclerotinia sclerotiorum*, the causal agent of canola stem rot

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Abstract

Stem rot of canola (*Brassica napus*) caused by *Sclerotinia sclerotiorum* is one of the most serious of plant diseases. From 30 *Trichoderma* isolates, three different species *T. harzianum*-8, *T. atroviride* PTCC5220 and *T. longibrachiatum* PTCC5140, were selected on the basis of their high level of chitinase and/or glucanase activity, along with their rapid growth rate *in vitro*. These showed high growth inhibition of two phytopathogenic isolates of *Sclerotinia sclerotiorum* (S1 and S2), with *T. atroviride* the greatest effect, reducing growth by 85-93%. They showed coil formation and penetration structures against the hyphae of the pathogenic isolates. *T. atroviride* PTCC5220 can be used for assessment of field biocontrol against *S. sclerotiorum*.

Keywords: biocontrol, chitinase, β-1,3 glucanase, antifungal activity

Introduction

Sclerotinia sclerotiorum is worldwide in distribution and is pathogenic to more than 480 plant species including oil-seed crops (Boland 1990). Infection of oilseed plants can occur any time after seedling emergence. *Sclerotinia* diseases cause serious yield losses of oilseed crops including sunflower, soybean and canola (Gulhua 2003). Stem rot is one of the most significant *Sclerotinia* diseases in soybean and canola (Hind *et al*. 2003). Fungicides are frequently recommended for plant disease management, but may negatively impact the environment and non-target organisms (Brimner & Boland 2003). One of the strategies used to control pathogens is mycoparasitism whereby a species or strain of fungus directly attacks and feeds on other fungi (Harman 2000; Kendrick 1992).

Trichoderma spp. are active mycoparasites against a range of economically important aerial and soil-borne plant pathogens, and is successfully used as a biocide in greenhouse and field applications (Chet 1987; Papavizas 1985). The antagonistic mechanism of *Trichoderma* is a complex process involving chemotropism (Chet *et al*. 1981), lectin-mediated recognition (Inbar & Chet 1992, 1994, 1995), and formation of trapping and penetration structures (Elad *et al*. 1983a,b). This process is further supported by the secretion of extracellular enzymes such as chitinases (Carsolio *et al.* 1994; de la Cruz *et al*. 1992; Harman *et al*. 1993), β-glucanases (Haran *et al.* 1995; Lora et al 1995; Lorito et al 1994), and proteinases (Geremia *et al.* 1993). The effects of these compounds in phytopathogenic fungi include degradation of the cell walls, forming holes (Harman *et al.* 2004).

The objective of this study was to use *S. sclerotiorum* as a model microorganism to test the biocontrol activity of three speices of *Trichoderma* (*T. harzianum, T. atroviride* and *T. longibrachiatum*) via chitinase and glucanase production along with antifungal activity by using dual culture and slide culture methods.

Materials & Methods

Thirty isolates of the *Trichoderma* spp (Seyed Asli *et al.* 2004) were used for chitinase and β-1,3 glucanase enzyme production. The stock culture was stored on agar (1.5%) slants of MY medium (2% malt extract, 0.2% yeast extract, 1% maltose). Two isolates of *Sclerotinia sclerotiorum* (S1, S2), the causal agent of canola stem rot, were kindly provided by Prof. Ershad (Pest & Diseases Research Institute, Agricultural Research & Education Organization,

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Tehran). The fungus was propagated on potato dextrose agar (PDA) and sub-cultured into fresh medium as needed.

For chitinase production, *Trichoderma* isolates were grown in 200 ml of Czapeck-Dox medium (3 g $NaNo_3$, 0.5 g $MgSo_4.7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4.7H_2O$, 1 g KH_2PO_4 per litre), supplemented with 10% glucose, and contained in a 500-ml flask. The flask was inoculated with 2 ml conidial suspension (10^6 conidia/ml) of each fungus and incubated for 96 hours at 25ºC as stationary culture. Harvested mycelia were washed several times with 2% $MgCl_2$ and distilled water, and transferred to Czapeck-Dox medium supplemented with 1.5% colloidal chitin. The culture medium was incubated in an orbital shaker incubator at 25ºC at 100 rpm, harvested after 3 days, filtered, and centrifuged. The supernatant was used for the chitinase assay. Protein concentration was estimated spectrophotometrically at 280 nm (Bradford 1976) using bovine serum albumin as a standard protein.

For β-1,3 glucanase measurement, *Trichoderma* isolates were cultured in Mandel's salt medium with optimum conditions (Ghoujeghi *et al* 2008). An 0.5-l Erlenmayer flask containing 200 ml of Mandel's medium and 20 g/l of dried *Rhizoctonia solani* cell wall at pH 5 was inoculated with 10^6 conidia (Zeilinger *et al* 1999). The culture was incubated in an orbital shaker incubator at 28°C at 150 rpm. After 48 hours of incubation, culture media were collected, concentrated by 1:1 (v/v) cold acetone, and centrifuged at 12000 rpm (4°C) for 20 min. The pellet was resuspended in 200 µl of 50 mM acetate buffer (pH=5) and used for the β-1,3 glucanase assay.

Chitinase activity was assayed with 200 µl of colloidal chitin (5 mg/ml), and 200 µl of enzyme solution. The mixture was incubated for 60 min at 40ºC, and the reaction stopped by adding 1 ml of NaCl (1%) and centrifuging at 6000g for 5 min. The supernatant was boiled with 100 µl of potassium tetraborate buffer for 3 min. 3 ml of DMAB reagent [10 g of dimethyl amino benzaldehyde in 100 ml of glacial acetic acid (12.5%) and 10 M chloridric acid (87.5%)] was added to the reaction and incubated at 40ºC for 20 min, and the amount of N-acetylglucosamine (GLcNAc) produced in the supernatant determined by the method described by Zeilinger et al (1999) using GLcNAc as a standard. One unit of enzyme activity was defined as the amount of enzyme that catalyses the release of 1 µmol GLcNAc in 60 min at 40ºC.

β-1,3 glucanase activity was assayed with 250 µl of 5% (w/v) laminarin in 50 mM acetate buffer (pH=5) with 250 µl enzyme solution at 50°C for 60 min, followed by determination of the reducing sugars with the Nelson-Somogyi method (Somogyi 1952; Nelson 1957). The amount of reducing sugars released was calculated from the standard curve for glucose. One unit of enzyme activity was defined as the amount of enzyme that catalyses the release of 1µM glucose in 1 min at 50°C.

For dual cultures, a mycelial plug of each *Trichoderma* species isolate (5 mm diameter) was incubated on potato dextrose agar, about 1 cm from the edge of each petri dish. A mycelial plug of *S. sclerotiorum* removed from the colony margin of a 3-day-old culture grown on potato dextrose agar was placed 6 cm away from the plug of the *Trichoderma* isolate in the same petri dish. Petri dishes similarly inoculated with *Trichoderma* or *S. sclerotiorum* isolates alone were used as controls. Plates were incubated at 22ºC for 4 days, and were examined after 24, 48, 72 and 96 hr for the formation of inhibition zones between *Trichoderma* and *S. sclerotiorum* isolates. At end of the incubation period, radial growth was measured. Radial growth reduction was calculated in relation to growth of the control as follows:

$$\% \text{ Inhibition of radial mycelial growth} = [(C-T)/C] \times 100$$

Where C is the radial growth measurement of the pathogen in control plates, and T is the radial growth of the pathogen in presence of *Trichoderma*.

For slide cultures, a clean slide was placed on an L-shaped glass rod in a 9cm diameter petri dish and autoclaved. Then a small amount of molten water agar was poured and evenly spread over the slide to make a thin agar film. One end of the slide was kept free of the

medium to facilitate handling. Inocula from each *Trichoderma* or *Sclerotinia* isolate were placed separately on the slide 1 cm apart from each other. A few ml of sterile water was added to the petri dish to prevent drying, and the slide incubated at 22ºC for 3-5 days. At the end of incubation period, regions where the hyphae of *Trichoderma* met the hyphae of the pathogen were observed under a light microscope for the presence of coil formation and penetration structures, or wall disintegration.

Results

Chitinase was produced with colloidal chitin as the carbon source after incubation of the *Trichoderma* isolates previously grown in glucose – supplemented medium. Out of 30 *Trichoderma* isolates, *Trichoderma* sp-T1, *Trichoderma* sp-T9, *T. longibrachiatum* PTCC5140 and *T. harzianum*-8 showed greater chitinase-specific activity (Table 1).

Table 1: Specific activity of chitinase and β-1,3 glucanase from different isolates of *Trichoderma* species.
Results are averages of three replicates; ± standard error; PTCC= Persian Type Culture Collection; Guilan, Hamadan, and Uremia are different provinces of I.R. of Iran

			Specific activity (U mg^{-1})	
No	**Isolates**	**Location**	**chitinase**	**β-1,3 glucanase**
1	*T. longibrachiatum*-5	Guilan	5 ± 0.2	13.5 ± 2.7
2	*T. viridae*-1	Guilan	6 ± 0.8	4.7 ± 0.2
3	*T. viridae*-2	Guilan	9 ± 0.7	18.9 ± 2.7
4	*T. harzianum*-7	Guilan	7 ± 0.7	14.9 ± 1.7
5	*T. harzianum*-8	Guilan	35 ± 2.1	15 ± 2.1
6	*T. hamatum*-12	Guilan	9 ± 0.6	15.5 ± 3.6
7	*T. virens*-9	Guilan	7 ± 0.8	10.1 ± 1.8
8	*T. virens*-10	Hamadan	9 ± 0.8	14.2 ± 2.8
9	*T. koningii*-11	Uremia	11 ± 1.5	14 ± 1.5
10	*T. parceramosum*-4	Guilan	16 ± 2.1	18 ± 2.1
11	*T. parceramosum*-3	Guilan	19 ± 2.2	16.7 ± 3.2
12	*T. longibrachiatum*-6	Guilan	15 ± 2.5	13.5 ± 2.5
13	*T. viridae*	PTCC5157	14 ± 1.2	18.2 ± 2.1
14	*T. atroviride*	PTCC5220	11 ± 1.8	20 ± 3.1
15	*Trichoderma* sp.	PTCC5138	10 ± 1.5	18.2 ± 2.5
16	*Trichoderma* sp-T1	Kerman	32 ± 3.5	19.3 ± 3.5
17	*T. harzianum*-T2	Kerman	20 ± 2.9	19.5 ± 2.9
18	*Trichoderma* sp-T3	Kerman	22 ± 3.1	16.7 ± 3.1
19	*Trichoderma* sp-T6	Kerman	12 ± 2.4	14 ± 2.4
20	*T. harzianum* -T7	Kerman	16 ± 2.3	10.2 ± 2.4
21	*Trichoderma* sp-T9	Kerman	28 ± 3.4	17 ± 1.9
22	*T. harzianum*-T10	Kerman	15 ± 1.9	16.8 ± 1.9
23	*Trichoderma* sp-T11	Kerman	10 ± 1.2	21.4 ± 3.2
24	*T. harzianum* -T12	Kerman	7 ± 1.1	10.9 ± 1.7
25	*Trichoderma* sp-T13	Kerman	6 ± 1.4	13.2 ± 1.4
26	*T. atroviride* -T24	Kerman	12 ± 2.3	19.4 ± 2.3
27	*Trichoderma* sp-T26	Kerman	5 ± 0.9	15.4 ± 3.4
28	*T. koningi*	PTCC5139	6 ± 0.7	14.9 ± 2.7
29	*T. longibrachiatum*	PTCC5140	23 ± 2.4	12.9 ± 2.4
30	*T. reesei*	PTCC5142	6 ± 0.8	22 ± 2.8

For β-1,3 glucanase enzyme production, three isolates (*T.atroviride* PTCC 5220, *T.reesei* PTCC5142, and *T.atroviride*-T24) showed high β-1,3-glucanase activity (Table 1).

Of the seven selected isolates, three (*T.harzianum*-8, *T.atroviride* PTCC5220, and *T. longibrachiatum* PTCC5140) were shown to have a high mycelial growth rate (data not shown) which is associated with the ability to control plant pathogens. These three isolates were assessed for their antagonistic effects on the two isolates of *S. sclerotiorum*.

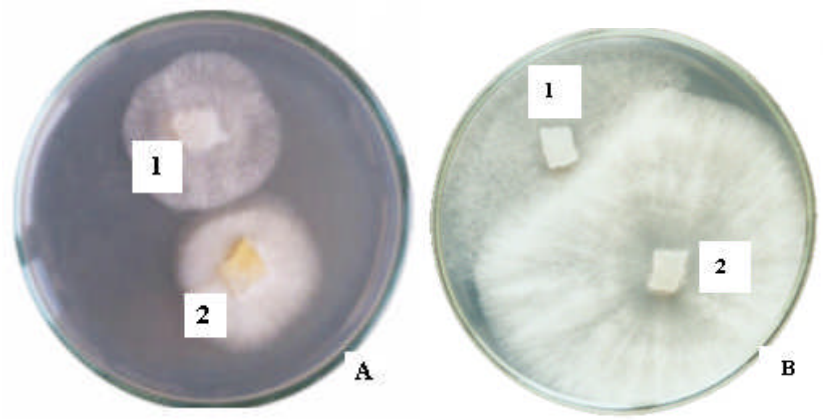

Figure 1: Antifungal activity (dual culture method) of *Trichoderma atroviride* PTCC5220 (2) on *Sclerotinia sclerotiorum* S1 (1). The dual culture was incubated on PDA for 48 h (A) and 72 h (B) at 22°C.

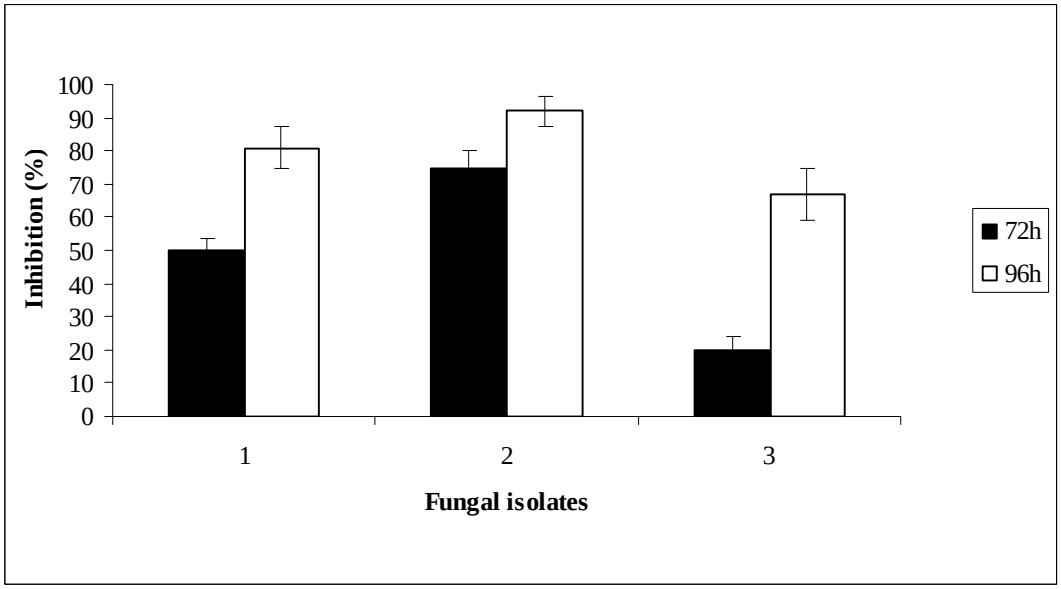

Figure 2: Antifungal activity (% inhibition) on *S.sclerotiorum* S1 by *T.harzianum*-8 (1), *T.atroviride* PTCC5220 (2) and *T.lingibrachiatum* PTCC5140 (3) using the dual culture method. The cultures were incubated on PDA for 72 and 96 h at 22°C. Each bar represents the average of three independent measurements.

In single cultures, the fungus grew rapidly and covered the entire agar surface of plates after 5 days. In co-inoculated cultures of *Trichoderma* and *S. sclerotiorum* , the colony margin of *S. sclerotiorum* zones opposite the colonies of *Trichoderma* gradually became flattened and bent, with clear inhibition after incubation for 3 days (Figure 1). Thus these *Trichoderma* isloates are antagonistic to *Sclerotinia*. When *S. sclerotiorum* S1 was cultivated in the same plate with different isolates of *Trichoderma*, inhibition of its radial growth was observed after 72 and 96 hours (Figure 2). Over shorter periods of 24 and 48 hours the growing fungi did not contact each other on the plate; the first time of mycelial contact was observed after 72 hours

of incubation. The results showed that *T.harzianum, T.atroviride,* and *T.longibrachiatum* inhibited the mycelial growth of *S.sclerotiorum* S1 after 72 h incubation, the effect increasing after 96 h (Figure 2). The same pattern of inhibition was observed for *S. sclerotiorum* S2 after 72 and 96 hours (Figure 3), with the greatest growth reduction caused by *T.atroviride.*

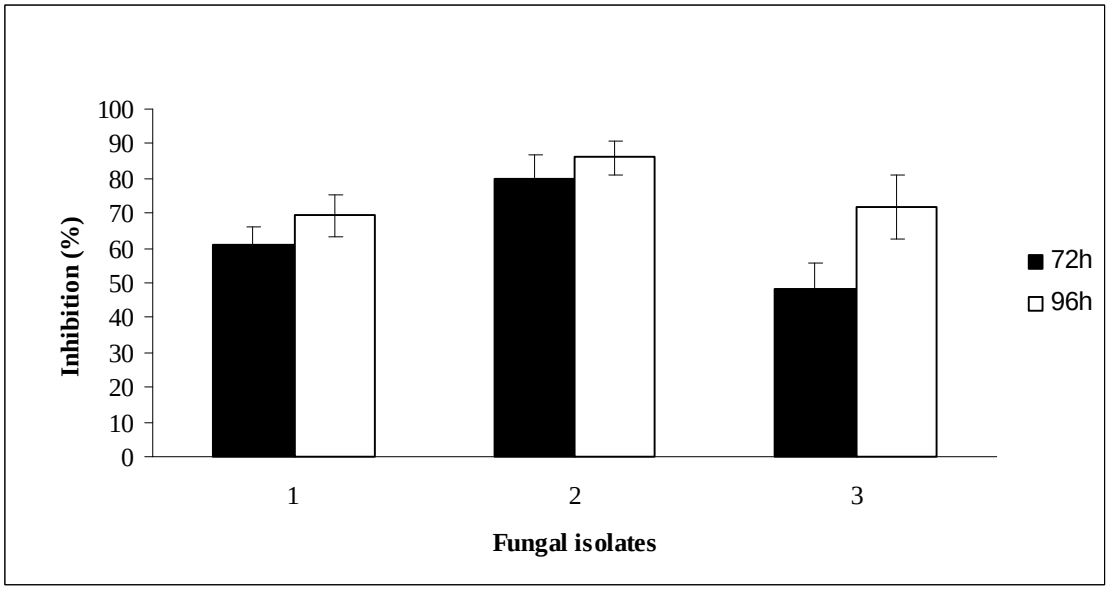

Figure 3: Antifungal activity (% inhibition) on *S.sclerotiorum* S2 by *T.harzianum*-8 (1), *T.atroviride* PTCC5220 (2) and *T.lingibrachiatum* PTCC5140 (3) using the dual culture method. The cultures were incubated on PDA for 72 and 96 h at 22°C. Each bar represents the average of three independent measurements.

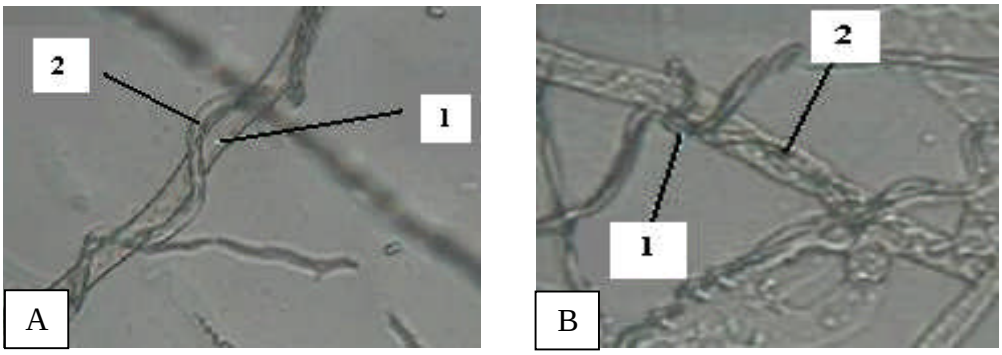

Figure 4: Antifungal activity (using the slide culture method) of *T.atroviride* PTCC5220 (2) on *S.sclerotiorum* S1 (1). The slide culture of the fungi was incubated on a thin layer of water agar for 3-5 days at 22°C. The coil structure formation (A) and hook-like structure (B) are observed.

In the slide-culture assay, once the fungal hyphae came into contact, the *Trichoderma* species attached to the pathogenic fungus, and were able to coil around it and form hook-like structures that aided in penetrating the host cell wall (Figure 4).

Discussion

Stem rot of canola (*Brassica napus*) caused by *S. sclerotiorum* is one of the serious and most destructive of plant diseases (Hind *et al*. 2003; Molloy *et al.* 2004). The disease has been found in all canola fields in Iran, causing up to 54% annual losses (Barary 2001). Canola cultivars with substantial and durable resistance to this pathogen are not easy to obtain by traditional breeding methods due to the limitation of germplasm resistant to *S. sclerotiorum.* Although

application of fungicides is still the main tool for controlling fungal diseases, nevertheless fungicides have many undesirable attributes (Bastos 1996). Biological control, the use of specific microorganisms that interfere with plant pathogens, is a nature-friendly, ecological approach to overcome problems caused by the standard chemical methods of plant protection. Novel biocontrol agents have therefore been looked for, and *Trichoderma* isolates have been the preferred choice (Boland & Hall 1994.). Biological control agents are perceived to have specific advantages over synthetic fungicides, including fewer non-target and environmental effects, efficacy against fungicide-resistant pathogens and reduced probability of resistance development (Harman 2000; Cook 1988; Anonymous 1999; Tsror *et al*. 2001). Besides other modes of action, enzymes responsible for cell-wall degradation such as chitinases and glucanases have been associated with the ability of *Trichoderma* to control plant pathogens (Papavizas 1985).

The objective of this work was to obtain and test bioagents for controlling *S. sclerotiorum*. Out of 30 isolates of *Trichoderma*, three were selected on the basis of their high level of chitinase and/or glucanase activity along with rapid growth rate *in vitro*. There are many reports demonstrating that chitinases and β-1,3 glucanases are effective features associated with the ability of *Trichoderma* to control plant pathogens (Brimner & Boland 2003; Zeilinger *et al*. 1999; Haran *et al*. 1996; Kubicek *et al*. 2001; Harighi *et al*. 2007; Wang *et al*. 2003).

According to our results, colonies of *T.harzianum, T.atroviride* and *T.longibrachiatum* always grew faster than *S.sclerotiorum* in single or mixed culture. Rapid growth of *Trichoderma* is an important advantage in competition with plant pathogenic fungi for space and nutrients (Cook & Baker 1989; Deacon & Berry 1992). In dual culture, these isolates inhibited the radial growth of *S.sclerotiorum* S1 and S2, with the greatest growth reduction of 93% and 85% respectively caused by *T.atroviride*. These *Trichoderma* species form coiled structures around the hyphae of *S.sclerotiorum*. This coiling is characteristic of the interaction between mycoparasitic and phytopathogenic fungi, leading to penetration of the cell wall (Calvet *et al*. 1989). The breakdown of chitin by production of chitinase and gluconase enzymes is achieved at the site of *Trichoderma* penetration (Zeilinger *et al*. 1999). After penetration, the release of antibiotics permeates the affected hyphae and inhibits resynthesis of the phytopathogenic cell wall (Lorito *et al*. 1996).

Among *Trichoderma* species, therefore, *T.harzianum*-8, *T.atroviride* PTCC5220 and *T.longibrachiatum* PTCC5140 showed the highest biocontrol activity against the two strains of phytopathogenic *S.sclerotiorum* (S1 and S2) tested. These organisms can therefore be used for assessment of field biocontrol against *S. sclerotiorum*.

Acknowledgements

This research was funded by a grant (Project No. 247) from National Institute for Genetic Engineering and Biotechnology (NIGEB), Tehran, P.O.Box 14155-6343, I.R. of Iran

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الملخص العربى

تأثير المستخلصات النباتية لثلاثة أنواع من نبات تريكويرما على فطر سكليروتينيا سكليروتيوم المسبب لمرض تعفن جذور النباتات

ماترويدى س – زمانى م ر – موتاليبى م
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يعتبر مرض تعفن سيقان نبات الكانولا (براسيكا نابيس) والذى يسببه فطر سكليروتينيا سكليروتيوم واحد من الآمراض الخطيرة التى تواجه النباتات فى إيران. ولدراسة كيفية مقاومة هذا المرض، تم إستخدام ثلاث عزلات مختلفة من بين 30 عزلة من المستخلصات النباتية لثلاثة أنواع من نبات تريكويرما: تريكويرما هارزيانوم – تريكويرما تروفيريدى – تريكويرما لونجيبراشاتم،والتى ثبت إحتواها على معدلات عالية من إنزيمر الكاينيز و/أو إنزيم الجلوكيناس بالاضافة إلى سرعة معدل نموها تحت الظروف المعملية مقارنة بالأنواع الآخرى.
أثبتت الدراسة أن المستخلص النباتى للنوع تريكويرما تروفيريدى كان له تأثير مثبط وفعال ضد نمو بعض العزلات لفطر سكليروتينيا سكليروتيوم (س1 و س2) والتى وصلت نسبة تثبيطها إلى حوالى 85-93%. وكانت ميكانيكية عمل المستخلص النباتى من خلال تشكيل تراكيب حلزونية والتى تمكنت من اختراق جدار وشعيرات الفطر. أيضا يمكن القول بأنه من المفضل دراسة تأثير هذا المستخلص النباتى الهام فى المقاومة الحيوية لفطر سكليروتينيا سكليروتيوم تحت الظروف الحقلية.