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**Evaluation of the antifungal activity of some
essential oils and *Trichoderma* spp against
Rhizoctonia solani, a bell pepper pathogen**

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Evaluation of the antifungal activity of some essential oils and *Trichoderma* spp against *Rhizoctonia solani*, a bell pepper pathogen

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Abstract:

This work aims to estimate the efficacy of four essential oils and three *Trichoderma* isolates for controlling *Rhizoctonia solani* in the laboratory (*in vitro*) and greenhouse (*in vivo*) conditions. *In vitro* tests indicated that all of the essential oils tested exhibited fungicidal activity against *R. solani* with different degrees of efficacy. Camphor and mustard oils were the best effective against *R. solani*. Obtained data revealed that the concentration used of Compher oil reduced the pathogenic fungal growth to 87.04, 90.47, and 97.27 % at concentrations 3, 6, and 9%, respectively. The antagonistic activity of three *Trichoderma* isolates demonstrated higher efficiency in controlling of *R. solani* . The most effective isolates were *T. asperellum* (72.34 %), followed by *T. harizianum* (63.02 %) compared to the control. Moreover, *in vivo* experiment showed that all tested agents were effective in reducing *Rhizoctonia* root rot incidence (post-emergence and Disease severity) and increasing some vegetative growth measurements of pepper plants (fresh weight of roots and plant height) in compensation to the infected (positive) control.

Keywords: Bell pepper, *Rhizoctonia solani*, Essential oils, *Trichoderma* spp.

Introduction:

Bell pepper (*Capsicum annuum* L.) is a nutritionally essential vegetable with medicinal properties (Sundaramoorthy *et al.*, 2012). It contains a variety of vitamins (vitamins C, A, E, B, and K), dietary fibers, minerals, and naturally occurring pigments that are beneficial to human health (Mishra *et al.*, 2017). It suffers from many fungal, bacterial, and viral diseases, causing significant crop losses under protected cultivation and open field (Abdel-Monaim *et al.* 2014). *Rhizoctonia solani* Kühn is a

highly pathogenic soil-borne pathogen that causes a variety of diseases in a wide variety of economically important crops under a variety of environmental conditions (Ogoshi,1987). *R. solani* causes a variety of different types of damage at various growth stages, including seed decline, root rot, pre-and post-emergence damping-off, and necrotic patches and wire stems on the tap root (Lopez *et al.*, 2009). Controlling this disease is conventionally dependent on chemical fungicides (Agrios, 2005). The intensive and unselective use of synthetic fungicides has caused health and environmental hazards when used irresponsibly (Dohroo *et al.*,1990). Natural product control of plant diseases has become critical because of their high efficacy, low cost, and safety for human and animal health and the environment. The application of essential oils can activate host defence mechanisms against phytopathogenic fungi (Prado *et al.*, 2013). Essential oils contain a complex mixture of monoterpenes and sesquiterpenes compounds with antimicrobial activity (Seema and Devaki, 2010). Antagonistic microorganisms used to control *R. solani* have been considered one of the different biological control methods. *Trichoderma* spp are the most widely used used biocontrol agents against fungal diseases of several plant crops (Papavizas *et al.*, 1982).

The present work aimed to estimate the potential of some essential oils and *Trichoderma* spp isolates as potential alternative biocontrol products in controlling *Rhizoctonia solani* under *in vitro* and *in vivo* conditions.

Materials and Methods:

The pathogen isolate:

Bell pepper (cv. balady) plants with root rot symptoms have been selected from various locations around Menoufia governorate, Egypt. Small sections of roots with lesions were sterilised in 0.5 percent NaOCl for two minutes. Rinsed many times with sterilised water and dried with sterilised Whatman filter paper. After sterilising the plant sections, they were placed in Petri dishes with Potato Dextrose Agar (PDA) media and incubated at 25 °C for 3-7 days before being sterilised using the hyphal tip procedure. *Rhizoctonia solani* isolates obtained from pepper roots were identified based on their microscopic and morphological characteristics as stated by (Meyer *et al.*, 1998). Identification was confirmed by the Botany Department, Faculty of Agriculture, Menoufia University, Egypt.

***In vitro*: Evaluation of the antifungal activity of essential oils in controlling *R. solani*:**

Marketable essential oils of Camphor, Carnation, Garlic, and Mustard were bought from the Chemical Manufacturing Development Company (CID) in Egypt and used for bioassay tests. Three concentrations of each essential oil [EO] (3, 6, and 9%) were prepared by adding an appropriate quantity of each EO to melted PDA medium followed by addition of Tween-20 (1%, v:v) to disperse the EO in the medium, then the mixed dispensed in sterilized petri dishes (Abdel-Kader *et al.*, 2012). Three replicated plates for each treatment were maintained and the results were recorded when the control plate was full of fungal growth. The fungitoxicity was carried out in terms of percent mycelial growth inhibition against the tested fungus growth, and was calculated using the following formula:

$$PI = \left[\frac{C - T}{C} \right] \times 100$$

Wherever, PI: is the inhibitory percentage over control, C: is mycelial radial growth in control plate, T: is mycelial radial growth in treatment (Shivapratap *et al.* 1996).

***In vitro*: Evaluation of the antifungal activity of *Trichoderma* spp isolates in controlling *R. solani*:**

Rhizosphere soil selected from soil round healthy pepper roots from diverse locations in Monoufia governorate. Warcup soil plate and dilution plate method were conducted using PDA medium. After that, the plates were incubated at 25°C for seven days and examined daily (Ammar 2003). The samples were isolated mostly based on physiological and morphological properties (Gerlach and Nirenberg 1982). The antagonistic ability of three tested isolates of *Trichoderma* spp. was assessed against *R. solani* according to the method described by (Fokkema, 1973). Three-day-old cultures of *Trichoderma harzianum*, *T. koningii*, and *T. asperellum* were used as sources of antagonistic inocula. A disc of each of the tested *Trichoderma* isolates (4mmØ) was placed 20 mm away from the edge of the PDA plates (9 cmØ). A pathogen disc was put in the Petri plate's centre (Devi *et al.*, 2015). Three replicated plates for each treatment were incubated at 25°C until the growth of the control plate completely covered the check plates.

***In vivo*: Evaluation of tested essential oils and *Trichoderma* spp in controlling *R. solani*:**

Soil sterilization and infestation:

Plastic pots (15 cm in diameter) used in this experiment were sterilized by dipping them in 5% formalin for 5 minutes and leaving them for a week until the formalin evaporated. Clay loam soil and sand (2:1 v:v) was twice autoclaved at 121°C for 20 minutes. The isolated *R. solani* and *Trichoderma* spp, were individually grown in bags on sterilized barley medium (75 g barley grains + 25 g sand + 100 mL water). The bags were incubated for 14 days at 25°C. Sterilized soil was infested with isolates at the rate of 3% of soil weight. The infested soil was irrigated every day for a week to allow the fungus to spread throughout the soil before sowing (Mannai *et al.*, 2018). The second experiment was carried out by dipping pepper seedlings (cv. balady) roots for 30 min in tested essential oils at the concentration (9 %) and then left to air dry. Treated seedlings were transplanted in pots infested with *R. solani* isolate (Seema and Devaki, 2010). Uninoculated pepper seedling in sterilized soil used as negative control (N.C). Inoculated and untreated pepper seedlings were planted in infested soil with *R. solani* only and used as a positive control (P.C). The pots plant were placed in a greenhouse (24±2 °C) and were watered on a daily basis until the final estimation was determined. Seven days after transplanting, post-emergence damping-off (%) was calculated by dividing the number of plants demonstrating disease signs by the total number of emerged seedlings. Sixty days after transplantation, disease severity and fresh root weight, and plant height (plant growth parameters) were evaluated. On a scale ranging from 0 to 5, the severity of the disease was determined by the density of *R. solani* lesions on the collar and roots, where 5 = death of the plant, 4 = pronounced wilting and necrosis, 3 = slight wilting and necrosis, 2 = slight wilting with pronounced chlorosis, 1 = chlorosis of lower leaves and 0 = no symptoms according to the following formula equation:

$$\text{Disease severity DS(\%)} = \left[\frac{n \times V}{N \times V} \right] \times 100$$

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Where: n = degree of infection rated on a scale of 1-5, v = number of plants in a category, N = highest degree of infection rate, and V = total number of plants screened (EPPO, 1997).

The antifungal activity of the investigated bioagents was assessed in this experiment at the Faculty of Agriculture, Menoufia University, Egypt, under greenhouse conditions.

Statistical analysis

All data were analyzed using one-way analysis of variance (ANOVA) following by LSD test for mean separation. Statistical significance was defined as P value <0.05 (CoStat-statistic software, CoHort software)

Results:

***In vitro*: Evaluation of essential oils against *R. solani*:**

The data showed clearly that all tested essential oil concentrations were effective in inhibiting fungal growth of *R. solani* in all experimental trials in petri dishes. It was clear that increasing the concentration of any tested extract reduced fungal growth more effectively, as shown in Table (1) and Fig(1). Camphor oil was most active in inhibiting the growth of fungal growth than the other essential oils (97.27%) at 10 % concentration, followed by Mustard oil (87.04%), Garlic oil (85.22%), and Carnation oil (82.05%), respectively.

Table (1): Antifungal activity of some essential oils against mycelium growth of *R. solani* using *in vitro* assay.

Essential oils	Conc. (%)	Growth of mycelium (mm)	Growth inhibition (%)
1- Camphor	3	11.24 ⁱ	87.04 ^c
	6	8.26 ^j	90.47 ^b
	9	2.03 ^k	97.65 ^a
2- Carnation	3	36.74 ^d	57.65 ^h
	6	24.32 ^f	72.35 ^f
	9	15.55 ^g	82.05 ^e
3- Garlic	3	40.66 ^c	53.13 ⁱ
	6	28.15 ^e	67.55 ^g
	9	12.82 ^h	85.22 ^d
4- Mustard	3	52.41 ^b	39.48 ^j
	6	23.73 ^f	72.24 ^f
	9	11.24 ⁱ	87.04 ^c
Control		86.77 ^a	00.00 ^k
L. S. D. 0.05		0.9215	0.8477

Means within the same column followed by the same letter(s) are not significantly different ($P \geq 0.05$, LSD test)

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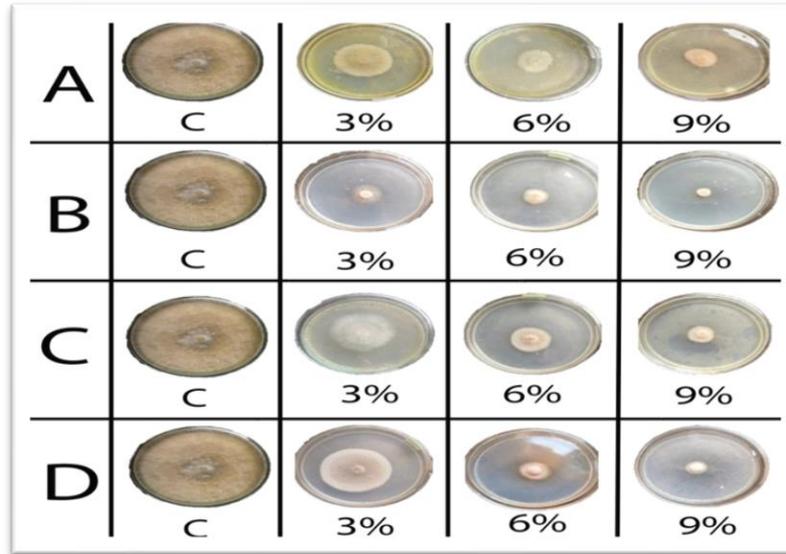


Fig (1): Inhibitory effect of different essential oil concentrations on *Rhizoctonia solani* growth on PDA medium, **A**: Carnation oil treatment **B**: Camphor oil treatment **C**: Garlic oil treatment **D**: Mustard oil treatment.

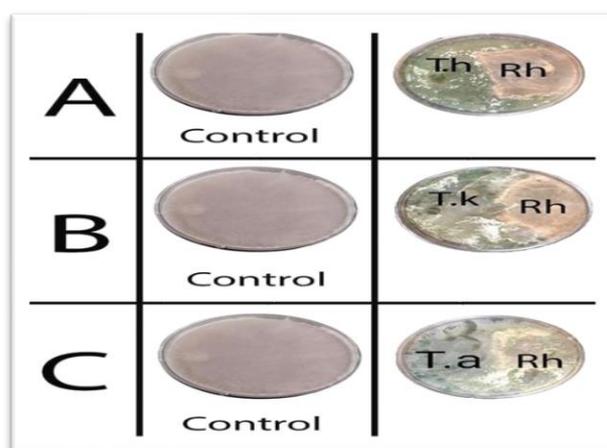
***In vitro*: Evaluation of different *Trichoderma* spp isolates against *R. solani*:**

Trichoderma harzianum, *T. koningii*, and *T. asperellum* were isolated from healthy pepper rhizosphere were tested as biocontrol agents. The results in Table (2) and Fig.(2) clearly demonstrate that all tested *Trichoderma* spp inhibited *R. solani* mycelium growth. *T. asperellum* and *T. harzianum* were the most effective in reducing growth compared to control (74.51 and 71.15 %), respectively. While, *T. koningii* gave the least efficiency in reducing growth (67.35%).

Table (2): Antifungal activity of some *Trichoderma* spp against mycelium growth of *R. solani* using *in vitro* assay.

Treatments	Growth of mycelium (mm)	Growth inhibition (%)	Mode of action	
			O. G * (mm)	I.Z** (mm)
<i>T. harizianum</i>	25.03 ^c	71.15 ^b	+	-
<i>T. koningii</i>	28.33 ^b	67.35 ^c	+	-
<i>T. asperellum</i>	22.11 ^d	74.51 ^a	+	-
Control	86.77 ^a	0.00 ^d	-	-
L. S. D. 0.05	1.3716	0.7873		

Means within the same column followed by the same letter(s) are not significantly different ($P \geq 0.05$, LSD test)



Fig(2): The effect of the tested *Trichoderma* spp isolates on *Rhizoctonia solani* grown on PDA medium, **A:** *T. harizianum* treatment **B:** *T. koningii* treatment **C:** *T. asperellum* treatment

***In vivo:* Evaluation of tested essential oils against *Rhizoctonia solani*.**

In comparison with the positive control, all tested essential oils significantly decreased post-damping off pepper seedlings inoculated with *R. solani*. Results in Table (3) and Fig. (3) showed that Camphor oil was the most effective causing 91.67 decrease in Post-emergence damping-off, followed by Garlic oil (88.3%), Mustard oil (83.3%) and carnation oil (66.67%). The severity of Rhizoctonia root rot was evaluated 60 days after transplanting and found to be significantly lower between the treatments tested and the positive control (Table 3). Maximum decrease was achieved by camphor oil (62.97 %) followed by mustard (51.86 %), garlic (48.15%), and carnation (33.34%) compared to positive control. The data also showed that all tested essential oils significantly ($P < 0.05$) enhanced plant height of treated plants compared to positive control (Table 3), plants treated with camphor oil achieved the maximum height (33.86 cm) compared to positive control (29.68 cm) while, carnation oil- treatment achieved the lowest plant height (31.91 cm). In addition, the fresh root weight mean was significantly increased in camphor oil-treatment (5.11 g) compared to positive control (3.08 g) (Table 3) at the same time, it did not differ significantly between the other treatments tested (Table 3).

***In vivo*: Evaluation of tested *Trichoderma* spp against *Rhizoctonia solani*:**

Data present in Table (3) indicate that all *Trichoderma* spp isolates improved post emergence percentage of *R. solani*-inoculated seedlings as compared to pathogen-inoculated and untreated control. This improvement reached 75 % using *T. asperellum* isolate and 66.67% by *T. harizianum* isolate and 50% using *T. koningii* isolate. Also, data in Table (3) show that *T. asperellum*, *T. harizianum* and *T. koningii* reduced disease severity by 48.15 % , 44.45 and 29.63 respectively. Three *Trichoderma* spp increased the height plants- inoculated and treated plants compared to pathogen-inoculated and untreated control ones (Table 3). Indeed treatment with *T. asperellum* increase plant height (32.67cm) compared to positive control (29.68 cm) followed by *T. harizianum* (32.00 cm) and *T. koningii* (31.44 cm). Fresh weight of roots, notes 60 days after transplanting, did not differ significantly between tested *Trichoderma* spp.

Table (3): Post-emergence damping-off incidence, growth parameters and disease severity on pepper cv. balady plants inoculated with *R. solani* and

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treated with some essential oils and *Tricoderma* spp after 7 and 60 days, compared to controls.

Measurments Treatment	Post damping-off (%)	Disease severity (%)	Plant height (cm)	Root weight (g)
Camphor oil	5 ^f	1.0 ^c	33.86 ^a	5.11 ^a
Carnation oil	20 ^c	1.8 ^{bc}	31.91 ^b	4.08 ^{ab}
Garlic oil	10 ^e	1.4 ^{bc}	32.60 ^{ab}	4.25 ^{ab}
Mustard oil	10 ^e	1.3 ^{bc}	33.11 ^{ab}	4.82 ^a
<i>T. harizianum</i>	20 ^c	1.5 ^{bc}	32.00 ^b	4.52 ^{ab}
<i>T. koningii</i>	30 ^b	1.9 ^{ab}	31.44 ^{bc}	4.11 ^{ab}
<i>T. asperellum</i>	15 ^d	1.4 ^{bc}	32.67 ^{ab}	4.47 ^{ab}
Negative control (N.C)	0.00 ^g	0.0 ^d	34.13 ^a	5.67 ^a
Positive control (P.C)	60 ^a	2.7 ^a	29.68 ^c	3.08 ^b
LSD _{0.05}	1.5669	0.8654	1.8255	1.7309

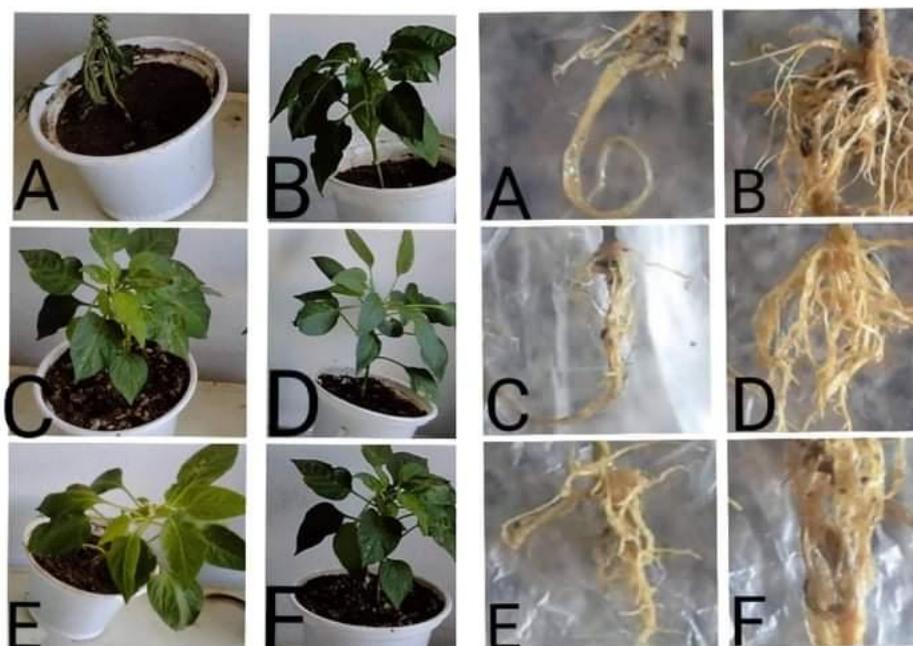


Fig. (3): Post-emergence damping off, disease severity and root rot of a pepper cv. balady seedling inoculated with *R. solani* and treated with some essential oils compared to control A: Positive control (P.C), B: Negative control (N.C), C: Carnation oil treatment D: Camphor oil treatment, E: Garlic oil treatment, F: Mustard oil treatment.

Discussion:

The root rot produced by *Rhizoctonia solani* in the world has become a serious problem to be solved. The use of fungicides to control this disease has been limited due to environmental pollution, fungicide resistance and restricted for use in organic agriculture (Zhansheng *et al.* 2019). The present study investigated the antifungal activity of some essential oils and three *Trichoderma* spp as alternative biocontrol agents against *R. solani*. Four essential oils namely Camphor, Carnation, Garlic and Mustard have shown promising results against *R. solani*. The results confirmed that four essential oils have antifungal properties on mycelial growth (*in vitro*). It is well recognized that some plant oils contain compounds up to inhibit the microbial growth (Naqui *et al.* 1994). These plant compounds can be of diverse structures and diverse mode of action when compared with

antimicrobials conventionally used to control the microbial growth and survival (Nascimento *et al.* 2000). Potential antimicrobial properties of plants had been related to their ability to synthesis, by the secondary metabolism, some chemical compounds of relatively different structures with antimicrobial activity, including alkaloids, Tannins, flavonoids, glycosides cumarins, terpens, organic acids and phenylpropanes (Nychas 1996). Several studies on the fungicidal activities of essential oils have indicated that many of them have the power to inhibit fungal growth. Compher and thyme oils were established to be highly efficient fumigants fungicide against a range of the soil-borne fungi (*Fusarium solani*, *Fusarium oxysporum*, , *Rhizoctonia solani* and *Pythium* spp)(Abdel-Kader *et al.*, 2012).

The ability of essential oils to increase the fungicide efficacy by acting on/in the fungal cell has been established in investigations. In general, natural compounds limit mould growth by causing cytoplasmic membrane rupture, cytoplasm granulation, and inhibition of extracellular and intercellular enzymes. These biological activities may occur sequentially or simultaneously, culminating in the suppression of mycelium germination (Campo *et al.*, 2003). Additionally, we observed a reduction in post-emergence damping-off on pepper seedlings treated with four essential oils when compared to pepper seedlings inoculated with *R. solani* and left untreated. These results correspond to those of (Seema and Devaki, 2010), who showed that cinnamon oil was the most efficient method of preventing *R. solani* infection in tobacco produced in greenhouses and fields. Carnation, Camphor, Garlic, and Mustard were also used to reduce the severity of disease on pepper plants this reduction reached 51.86% with Mustard oil, 62.97% with Camphor oil, 48.15% with Garlic oil, and 33.34% with Carnation. Our findings corroborate those of (Elsheshtawi *et al.*, 2012), who indicated that seed treatment and root soaking in Citronella, Cinnamon, Onion, and Mint oils resulted in a reduction in disease severity ranging from 22.35 to 52.22 %. Microbial compounds depended on antagonistic microorganisms signify an environmental friendly method to control numerous soil-borne diseases on numerous crops (Haas and Defago, 2005). Fungal species belonging to the genus *Trichoderma* are worldwide in occurrence and easily isolated from the soil. The use of *Trichoderma* species as biocontrol agents against various plant diseases has been reported by several workers (Coley-Smith, *et al.*, 1991).

Trichoderma can indirectly biocontrol phytopathogens by three mechanisms, i.e. competing for space and nutrients (Sid Ahmed, *et al.*, 1999), through production of antibiotic metabolites, whether volatile or diffusible metabolites (Chet, *et al.*, 1997) and direct parasitism on pathogen (Yedidia, *et al.*, 1999). On the other hand, mycoparasitism is considered a direct biocontrol mechanism and in addition, they could have a stimulatory effect on plant growth (Naseby, *et al.*, 2000).

The results presented here detail the biological control activity of three *Trichoderma* spp isolates. *In vitro* and *in vivo* studies confirmed that selected *Trichoderma* spp can successfully be used to restrict or control *R. solani* under controlled conditions. Results indicated that the dual culture contacts between *Trichoderma* isolates and *R. solani* occurred after 3 days of growth. All *Trichoderma* isolates grew over the *R. solani* colonies and degraded its mycelium. The study further revealed that *T. harizianum*, *T. koningii* and *T. asperellum* parasitized the hyphae of *R. solani*. Similar results were obtained earlier on *T. harzianum* and *T. asperellum* against *Botrytis cinerea* (Barakat, *et al.*, 2006). The bioassays showing positive extracellular chitinase enzyme activity for three isolates supported this mode of action. Hence, the possible mycoparasitism between *Trichoderma* isolates and *R. solani* was attributed to hyphal interactions followed by the production of chitinase enzyme leading to degradation of *R. solani* cell walls (ELad, *et al.*, 1982).

Moreover, the damping-off caused by *R. solani* was greatly decreased in pepper plants in *in vivo* studies. When compared to the *R. solani*-inoculated control in pots, *T. asperellum* treatment reduced post-emergence damping-off by 75%. Lewis & Lumsden (2001) found that four strains of *R. solani* controlled damping-off on cucumbers in a soilless mix employing *T. hamatum* by 71-81%. However, plant stands of 42, 77, and 48% were observed on pepper when *T. virens*, *T. hamatum*, and *T. harzianum* were used, respectively. *Trichoderma* spp. treatments were also proven to reduce the severity of disease on pepper plants. In comparison to the *R. solani*-inoculated and untreated control, this reduction was 48.15% with *T. asperellum*, 44.45% with *T. harzianum*, and 29.63% with *T. koningii*. Our findings are in agreement with those of Sid Ahmed *et al.*, (2003), who found that seed treatment and soaking pepper roots in *T. harzianum* reduced root rot induced by *Phytophthora capsici* by % and reduced root rot caused by *R. solani* by 38%. We also reported an increase in the pepper plant's cv's Balay

height and fresh root weight in the present work. In pot and cell trays experiments, Mannai *et al.*, (2018) found that treatment with *G. virens* increased plant height by 12.13 % and 27.32% compared to the *R. solani*-inoculated control. Similarly, when tomato plants were infected with *T. harzianum*, *T. viride*, or *G. virens*, their fresh root weights increased by more than 50% relative to *V. dahliae*-inoculated and untreated controls (Jabnoun-Khiareddine, 2009).

Conclusion:

The present study demonstrated that, based on *in vitro* and *in vivo* assays, the tested essential oils *Trichoderma* spp treatments were found to be effective against *R. solani* in this investigation. Our results showed that some of the tested biocontrol agents, applied at different pepper growth stages, were able to suppress disease and to advance plant growth. Their effectiveness will be further evaluated under field conditions, in naturally infected soils, and against the other pepper phytopathogenic fungal species.

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تقييم فاعلية بعض الزيوت الأساسية و *Trichoderma spp* ضد فطر *Rhizoctonia solani* أحد مسببات أمراض الفلفل الحلو

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الملخص العربي:

الهدف من هذا العمل هو تقدير فاعلية أربعة زيوت أساسية وثلاث عزلات من *Trichoderma* في مقاومة فطر *Rhizoctonia solani* تحت ظروف المعمل والصوبة الزجاجية وأشارت نتائج الإختبارات المعملية إلي أن جميع الزيوت الأساسية التي تم اختبارها أظهرت نشاطا مبيدا ضد فطر *R. solani* بدرجات مختلفة من الفعالية وكانت زيوت الكافور والخردل هي الأفضل فاعلية ضد هذا الفطر وأظهرت النتائج المتحصل عليه أن التركيزات المستخدمة لزيت الكافور قللت من الفطر إلي (87.04, 90.47, and 97.27%) بتركيزات (3, 6, and 9%) علي التوالي. أظهر النشاط المضاد لثلاث عزلات من فطر *Trichoderma* كفاءة في مقاومة هذا الفطر وكانت العزلات الأكثر فاعلية هي (*T. asperellum* (72.34 %) يليها *T. harizianum* (63.02 %) مقاومة بالكنترول علاوة علي ذلك أظهرت النتائج التي أجريت تحت ظروف الصوبة الزجاجية أن جميع العوامل المختبرة كانت فعالة في تقليل حدوث مرض عفن الجذور الريزوكتوني (موت البادرات بعد الظهور فوق سطح التربة وشدة المرض) وزيادة بعض قياسات النمو الخضري لنبات الفلفل (الوزن الطازج للجذور – طول النبات مقارنة بالكنترول المصاب).