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ORIGINAL PAPER

The Competitive Potential of Different *Trichoderma* spp. to Control Rhizoctonia Root Rot Disease of Pepper (*Capsicum annuum* L.)

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ABSTRACT

Five isolates of *Trichoderma* spp. were investigated, in a preliminary study, for their efficiency in controlling Rhizoctonia root rot of pepper (*Capsicum annuum* L.). GC-MS analysis of most active *Trichoderma* spp. extracted secondary metabolites were studied. The major compounds identified by GC-MS analysis included hexadecanol, nonadecene, 1,2-benzenedicarboxylic acid, octadecenoic acid (z) and hexadecanol, 2-methyl. These compounds have been found to play an important role in controlling plant diseases. The results of antagonistic activity of three isolates proved higher efficiency in controlling *R. solani*. Browning of the *R. solani* mycelium was observed where mycoparasitism occurred. The parasitism of *R. solani* hyphal cells by *Trichoderma harzianum* was studied by light and scanning electron microscopy. Sequence data of *R. solani* isolates (R.1, R.2 and R.4) have been submitted to the GenBank under accession numbers (MZ267232, MZ267234 and MZ267689), respectively. The most effective isolates *i.e., T. asperellum, T. hamatum* and *T. harzianum* in addition to their mixture were formulated in different forms *i.e.,* suspension and powder, then tested at different concentrations under the greenhouse and protected cultivation conditions. Results of greenhouse studies indicated that all isolates and their mixture were effective in controlling pepper root rot disease and increasing percentage of survived plants. Moreover, results showed that suspension was effective than powder in reducing the disease. Higher bio-control efficiency at protected cultivation was obtained when the mixture of isolates was used at high concentration. Most of the *Trichoderma* spp. studied in the present work have been able to reduce severity of *R. solani*.

Keywords: Pepper, *Capsicum annuum*, Root rot, *Rhizoctonia solani*, *Trichoderma* spp., GC-MS

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INTRODUCTION

Many fungal diseases, including damping-off, root rot and wilt are common and serious in most of pepper (*Capsicum annuum* L.) producing regions, both in open field and protected cultivation, resulting in significant plant and crop losses. (Tuncer and Eken, 2013; Mannai *et al*., 2018). These diseases induced by several soil borne pathogens including *Rhizoctonia solani*, *Phytophthora capsici*, *Fusarium solani* (Abdel-Monaim *et al*., 2014; Mousumi *et al*., 2019). *R. solani* is an important soil born pathogen with a necrotrophic lifestyle (Anderson, 1982). This fungus has the potential to cause severe damage to many economically important agricultural and horticultural crops, as well as trees, all over the world (Kühn *et al*., 2009; Jabnoun-Khiareddine *et al*., 2015 and Anees *et al*., 2016). Furthermore, in Egypt, pepper is grown in short rotation with potato or tomato which are highly susceptible to Rhizoctonia diseases. However, yield losses continue to occur due to the pathogen's wide host range and the long-term survival of its resting structures (sclerotia) in the soil (Zachow *et al*., 2011).

Numerous approaches have been adopted to manage diseases caused by *Rhizoctonia solani*. Targeted specific fungal bio-agents are thought to be ideal for pathogen management strategies in crop protection, and they help to reduce the negative environmental impact of chemical pesticides (Mousumi *et al*., 2019). Considerable attention has been paid to the antagonistic effects of *Trichoderma* spp. against *R. solani* as a bio-agent for plant disease management (Howell, 2003; Muhanna *et al*., 2015). Among bio-agents, *Trichoderma* spp. can be considered as an ideal bio-agent for controlling plant diseases. This antagonist is very easy to be isolated and spreads rapidly on any organic staff (Papavizas *et al.,* 1982). *Trichoderma* spp. act through different modes of action, *i.e.,* mycoparasitism (Abd-El-Moity *et al*., 2003; Atia, 2005; Almeida *et al.,* 2007 and Kamel *et al.,* 2020). During mycoparasitic interactions, hydrolytic enzymes such as glucanase, chitinase and protease are produced by *Trichoderma* spp. (Mukhopadhyay and Kumar, 2020), production of antifungal

substances (Marques *et al.,* 2018), also it owns enzymatic system destroys the pathogens (Bhale and Rajkonda, 2012). In addition to these modes of action, *Trichoderma* spp. are widely reported as plant growth promoters (Vinalea *et al*., 2012 and Dabire *et al*., 2016). Many efforts have been made to make biological control as effective as pesticides in the suppression of fungal pathogens, such as formulation and dose increases (Ali *et al.,* 2012; Navaneetha *et al.,* 2015 and Mahmoud, 2017).

The present study evaluates the potential of five *Trichoderma* spp. against *R. solani* by examining the *in vitro* antagonism between them and the *in vivo* effect of different formulations at different concentrations, on the incidence of root rot caused by *R. solani* of pepper plants.

MATERIALS AND METHODS

1- Source of *R. solani* isolates:

Five isolates of *R. solani* were isolated from diseased pepper, tomato, and potato plants that showed root rot symptoms collected from farms located at four different Governorates (Table: 1). Affected roots were washed with running tap water, surface disinfected for 1 minute in 0.5% sodium hypochlorite and placed on autoclaved water agar plates containing 50 mg/l streptomycin sulphate (Demirci and Döken, 1993). After 48h incubation at 25°C, hyphae from the margin of each developing colony were placed on potato dextrose agar (PDA) medium and incubated at 25°C. The developed fungal colonies were examined using a light microscope, and only *R. solani* isolates were chosen and transferred to PDA slant. The purified fungal isolates were identified according to morphological characteristics as described by Sneh *et al*., (1991).

Table (1): *Rhizoctonia solani* isolated from rotted roots of five different vegetables used in this study.

Isolate	Host	Governorate
R.s 1	Sweet pepper	Al-Qaliobia
R.s 2	Bell pepper	El-Beheira
R.s 3	Chili pepper	Al-Ismailia
R.s 4	Potato	Al-Sharkia
R.s 5	Tomato	El-Beheira

2- Virulence evaluation of *R. solani* isolates:

Greenhouse experiments were carried out in the Central Lab of Organic Agriculture's greenhouse, Agricultural Research Center, Giza.

2.1. Plant material:

For greenhouse experiments, sweet pepper seeds (Gedion F1 hybrid) were used. Seeds were superficially disinfected for 2 minutes with 5% sodium hypochlorite, rinsed with sterile distilled water, and dried at room temperature. The seeds were then sown in 72 cell-trays containing peat moss that previously sterilized at 121°C (for one hour) and kept in a greenhouse for 30 days. Planting bags (30 cm) filled with 3 Kg clean peat moss were used. Sweet pepper transplants were transplanted (two transplants / planting bag). Each replicate contained twenty plants in ten planting bags. Three replicates were used for each treatment.

2.2. Fungal inoculum and inoculation:

Inocula of five *R. solani* isolates were prepared by growing the fungi on corn meal sand medium, 1:3 w: w and 40% water, enrich with 2% peptone (Abd El-Moity, 1985). Bottles containing the medium were inoculated with equal disks 5 mm of *R. solani* from 5 days old cultures. Each bottle was inoculated with one *R. solani* isolate. Inoculated bottles were incubated horizontally for 15 days at 25°C. Loaded fungal growth was mixed with the soil at the rate of 10 g/ kg soil (each gm contains 6×10^5 CFU). Infested planting bags were irrigated periodically and left 7 days to the adequate distribution of the inoculums. Planting bags containing non infested soil were supplied with the same amount of autoclaved corn meal-medium.

To test the virulence of five *R. solani* isolates to cause root rot disease, thirty days old pepper seedlings were transplanted in planting bags inoculated with different *R. solani* isolates, each planting bag was inoculated with one *R. solani* isolate. Planting bags containing infested soil with *R. solani* isolates 1,2,3,4 or 5 were prepared as described previously. Three replicates were used for each isolate. All treatments were evaluated sixty days after transplantation, and the percentage of dead plants was calculated to determine the virulence of each isolate under test:

$$\textbf{Dead plants (\%)} = \frac{\textbf{Number of dead plants}}{\textbf{Total number of plants}} \times 100$$

Pepper plants were uprooted and washed at the end of the experiment to remove the adhering peat moss. The disease severity was rated based on the extent of the root system browning on a 0-5 scale as described by Mannai *et al.,* (2018), Where 0 = clean roots, 1 = 1-25 %, 2 = 26-50%, 3 = 51- 75%, 4 = 76-100% of root browning, and 5= dead plant.

$$\% \text{ Disease severity (DS)} = \frac{\Sigma\, n \times v}{N \times V} \times 100$$

Where:

n= number of plants in each category.
v = numerical value of each category.
N= total number of plants in sample.
V = the highest category.

2.3. Identification of tested *R. solani* by molecular technique:

The DNA of the fungal isolate was extracted using the Genomic DNA preparation kit. For molecular identification, genomic DNA from different *Rhizoctonia* isolates were extracted and PCR amplification was done using ITS region according to the DNeasy Plant Mini Kit (Qiagen), following the instructions of the manufacturer. The samples were kept at -20°C. The PCR amplification of the complete ITS region was done using universal primers ITS-1: 5′-TCC GTA GGT GAA CCT GCG G-3′, and ITS-4: 5′-TCC TCC GCT TAT TGA TAT GC-3′ (White *et al*., 1990). PCR products were separated on 2 %agarose gel electrophoresis. The DNA ladder (50 bp, GeneDireX) was also loaded in the gels to estimate the proper band size of amplified products. PCR cleanup was carried out to the PCR product, using GeneJET™ PCR Purification Kit (Thermo K0701). The purified fragments were sequenced at the Macrogen facility in Korea using the BigDye Direct Cycle sequencing kit (ABI Applied Biosystems). The obtained sequences were compared to those in the public database using Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nih.gov) to determine similarity to sequences in the GenBank database and having accession number. Three isolates of *R. solani* (R. 1, R.2 and R.4) which showed higher pathogenic capabilities were chosen to identify their Anastomosis group (AG).

3- Isolation, purification and identification of bio-agents:

Five different *Trichoderma* spp. were isolated from roots of healthy plant *i.e.,* pepper, bean, strawberry, garlic and tomato plants by the method described by Abd El-Moity, (1976). The purified fungal isolates were identified using a light microscope according to Rifai, (1969). Morphological identification was confirmed by comparison of ITS sequences of nuclear ribosomal DNA. For molecular identification, genomic DNA of three different *Trichoderma* isolates (T.1, T.3 and T.4) were extracted and PCR amplification was done using ITS region as mentioned with *Rhizoctonia* isolates. Also, similarity with sequences in the GenBank database and having accession number for *Trichoderma* isolates were done.

4- *In vitro* antagonism assay

The antagonistic effect of *Trichoderma* isolates against three *Rhizoctonia solani* isolates was studied. Trichoderma isolates were evaluated *in vitro* using dual culture test (Rahman *et al*., 2009) for their antagonism and mycoparasitic potential against *R. solani*. Petri-dishes (9.0 cm in diameter) each contain 15 ml of gliotoxin fermentation medium (GFM) developed by Brain and Hemming (1945) were prepared. Each plate was inoculated at one side with a disk (0.5 cm in diameter) of *R. solani* from the periphery of 5 days old culture. The opposite side of each plate was inoculated with a disc (0.5 cm in diameter) of one of *Trichoderma* spp. obtained from 4 days old culture. Three plates were used for each *Trichoderma* isolate. Plates inoculated only with *R. solani* were served as control. All inoculated plates were incubated at 25°C. When mycelial growth covered all medium surfaces in the control plates, all plates were then examined and percentages of reduction in *R. solani* mycelial growth were calculated using the formula descript by Mayo *et al.,* 2015 as follows:

$$\text{Growth inhibition} = \frac{(r1 - r2)}{r1} \times 100$$

Where:

r1: growth of pathogenic fungus in control plates
r2: growth of pathogenic fungus in treated plates with the antagonist

Light and scanning electron microscopy (SEM) were used to investigate the parasitism of *R. solani* hyphal cells by *Trichoderma harzianum*. The plate cultures were examined seven days after incubation to confirm the interaction under a light microscope at 40X in an optical microscope (CHS, Olympus optical Co. Ltd.). The interaction sites were marked and $1cm^2$ agar block was removed for SEM preparation using JEOLJSM-5200, Tokyo, Japan at the Faculty of Agriculture, Cairo University.

5-Identification of potentially bioactive compounds of *Trichoderma* spp. by Gas chromatography–mass spectrometry analysis:

Three *Trichoderma* spp. were screened for their abilities to produce antimicrobial secondary active metabolites. GC-MS analysis was used to characterize the bioactive compounds from the

most promising isolates. The chemical composition was performed using Trace GC-TSQ mass spectrometer (Thermo Scientific, Austin, TX, USA) with a direct capillary column TG–5MS (30 m × 0.25 mm × 0.25 µm film thickness). The column oven temperature was initially held at 50°C and then increased at a rate of 5°C /min to250°C hold for 2 min. increased to the final temperature 300°C by 30°C /minutes and kept for 2 minutes. The injector and MS transfer line temperatures were kept at 270, 260°C respectively; Helium was used as a carrier gas at a constant flow rate of 1 ml/min. The solvent delay was 4 min., and diluted samples of 1 µl were injected automatically using Auto sampler AS1300 coupled with GC in the split mode. EI mass spectra were collected at 70 eV ionization voltages over the range of m/z 50-650 in full scan mode. The ion source temperature was set at 200°C. The components were identified by comparison of their mass spectra with those of WILEY 09 and NIST 14 mass spectral database as described by Abd El-Kareem *et al.*, (2016).

6- Preparation and Formulation of different *Trichoderma* spp.:

Trichoderma isolates were grown in flasks (250 ml) each contained 200 ml liquid gliotoxin fermentation medium (GFM). Flasks were inoculated with 5 mm fungal discs obtained from the periphery four days old culture of each isolate. Inoculated flasks were incubated at shaking incubator with 170 rpm at 25°C for 12 days (Ali *et al.*, 2012). Different *Trichoderma* spp. have been formulated in two different forms *i.e.,* suspension and powder. The mycelium mats and broth of *Trichoderma* isolates were mixed in the blender. The suspension was prepared by homogenizing fungal growth to contain 30×10^6 CFU/ml plus 5 % Arabic gum (Ali, 2013). For powder preparation, sterile water (10 ml) was added to the cultured Trichoderma isolates, and the surface was lightly scraped with a sterile transfer loop. The resulting suspension has been filtered through sterile muslin (Navaneetha *et al.,* 2015). The conidia suspension was adjusted for homogenizing fungal growth to be 30×10^7 CFU/ml, and then 10 ml was thoroughly mixed with 90 g talc powder plus 5% Arabic gum (thickeners amendment).

7- Transplants treatment under artificial and natural infection:

In vivo bio-control trials were carried out to evaluate the ability of tested *Trichoderma* spp. to reduce Rhizoctonia root rot disease. Suspension or powder formula was diluted in water to be containing 2×10^5, 3×10^5 and 6×10^5 CFU/ ml. Transplants were immersed in suspension for 10 min. before transplanting.

8. Effect of different concentrations from different forms of *Trichoderma* spp. and their mixture for controlling Rhizoctonia root rot of pepper in a greenhouse experiment:

Pepper transplants were dipped in a suspension of each Trichoderma isolate at concentration of 2×10^5, 3×10^5 and 6×10^5 CFU/ ml for 10 min. Pepper transplants dipped in water acted as control. Seedlings were transplanted into planting bags filled with peat moss previously infested with the highest virulence *R. solani* isolate, which prepared as previously described. Percentage of survived plants were recorded sixty days post transplanting to evaluate the efficacy of using *Trichoderma* spp. at different forms and concentrations in controlling Rhizoctonia root rot disease of pepper.

% Survived plants =

$$\frac{\textbf{Number of survived plants}}{\textbf{Total number of plants}} \times 100$$

9- Evaluation of different forms of different isolates of *Trichoderma* spp. and their mixture for controlling root rot diseases of pepper in protected cultivation:

Sweet pepper seedlings with three true leaves were transplanted, on September 28. Natural infested sandy soil under a commercial plastic house conditions at the County of El Ayat, Giza, Egypt was used to study the effect of three *Trichoderma* spp. on controlling root rots in sweet pepper. Three replicates were used for each treatment. Each treatment (replicate) contains 100 pepper plants (each replicate equal 33.3 m^2, 3 plants/ m^2). Pepper transplants were dipped in different concentrations of *Trichoderma* spp. forms at the same time. Treated seedlings were then transplanted in naturally infested soil. Pepper transplants without any treatment acted as a control. All treatments were examined periodically till sixty days from transplanting time, percentage of survived plants were recorded as mentioned previously.

10-Statistical analysis:

Experiments were conducted according to a completely randomized design. Data were subjected to statistical analysis of variance according to procedures outlined by (Snedecor and Cochran, 1980) using SAS software, version 2004. The means of treatments were compared by Duncan’s test at 0.05 level of probability.

RESULTS AND DISCUSSION

The work aims to increase the efficacy of *Trichoderma* spp**.** for controlling plant disease. To achieve this aim, the efficiency of three *Trichoderma* spp**.** and their mixture with two different formulations at three concentrations were investigated.

1- Virulence evaluation of *R. solani* isolates:

This study aimed of determines the pathogenic capabilities of the five tested *R. solani* isolates against sweet pepper transplants. Results in Fig. (1) showed that, all tested *R. solani* isolates increased disease incidence compare with control treatment. *R. solani* isolates showed variability in respect of pathogenicity as well as morphological characteristics (Fig. 2). *R. solani* (1) which isolated from the Qaliobia governorate was the most virulent isolate (Fig. 3) and caused 60% damping off and 86% disease severity. On the other hand, *R. solani* (5) was the least pathogenic fungus and recorded only 26.7% disease incidence and 35% disease severity was recorded. The other isolates fall in between. Rhizoctonia isolates differed among themselves in severity of the infection, although similar conditions, this is an indication of the role in different virulence (Rubio *et al.,* 1996). Abdel-Sattar *et al.,* (2017) stated that virulence of isolates in pathogenicity attributed to the difference between *R. solani* Anastomosis groups (AGs).

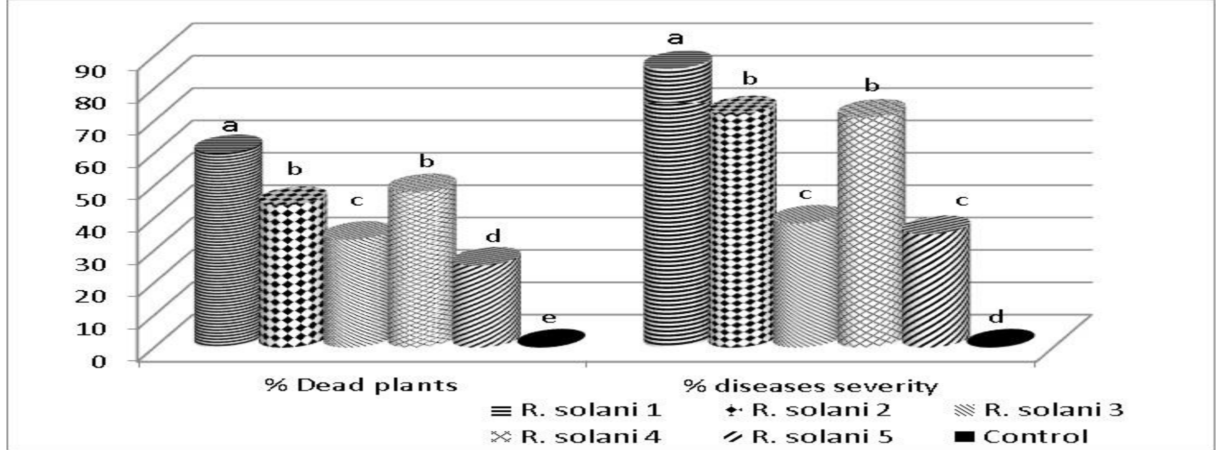

Fig. (1): Pathogenicity of five *R. solani* isolates on sweet pepper transplanting (Means with the same letters, in each column are not significantly different).

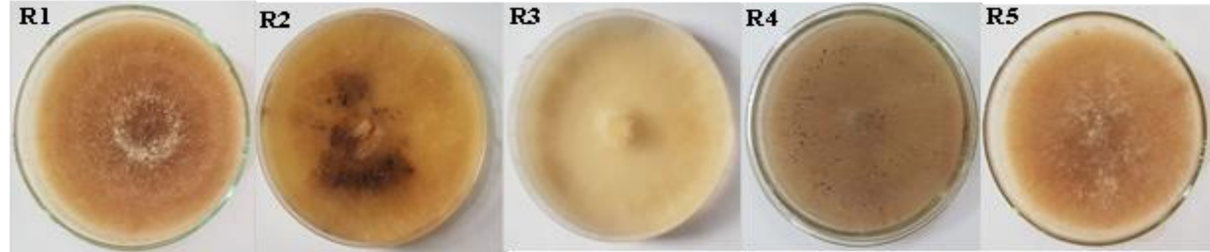

Fig. (2): Petri plates showing cultural differences among the five *R. solani* isolates.

Fig. (3): *R. solani* (1), the most virulent isolate, infection symptoms on sweet pepper plants compared to control treatment.

2- Identification and molecular characterization of *R. solani* isolates:

Five *R. solani* isolates were isolated and identified using morphological and microscopical characteristics. Three *R. solani* isolates (R.1, R.2 and R.4) were the most virulent isolates among five isolates on pathogenicity test trial. PCR amplification with the primers (ITS-1/ITS-4) resulted in an approximately 620-bp product within the internal transcribed spacer (rDNA-ITS) region of the tested isolates (Fig.4). This result agrees with the findings of Abdel-Sattar *et al.,* (2017). The sequence of the isolates

was submitted to the National Center for Biotechnology Information (NCBI), GenBank and gave accession numbers (MZ267232, MZ267234 and MZ267689), respectively.

Nucleotide identity between the three *R. solani* sequences was determined for anastomosis group identification. Three sequences of *R. solani* were belonged to AG-5, AG-4 and AG3, which GenBank accession numbers are JF701707, MH172675 and JX050235 were used as references. The comparison of sequences registered in GenBank and sequences of tested *R. solani* isolates revealed that the isolates had 98.92% to 99.86% sequence homology with the referent isolates. The resulting data of sequences which analyzed with BLAST compared to the sequence database GenBank (NCBI) to detect similar sequences of known AGs, showed that the three tested *R. solani* isolates R.1, R.2 and R.4 are belonged to AG-5, AG-4 and AG3, respectively. Abdel-Sattar *et al.,* (2017) mentioned that specific PCR-based methods or DNA sequencing of the ITS-rDNA were useful for detecting and identifying AGs. Significant differences in virulence were observed among Rhizoctonia AG isolates. (Tuncer and Eken, 2013 and Yang *et al*., 2015). Basbagci *et al.*, (2019), stated that ITS sequencing is a powerful tool for understanding and determining the relationship between *R. solani* anastomosis groups. However, grouping of the isolates was not related to their geographic origins or virulence pattern.

3- Isolation, purification, and identification of bio-agents:

Five different *Trichoderma* spp. were isolated from roots of healthy plant species; pepper, bean, strawberry, garlic, and tomato. The purified fungal isolates were morphologically identified using a light microscope. Morphological identification was confirmed by comparison of ITS sequences of nuclear ribosomal DNA. For molecular identification, genomic DNA of for the most effective Trichoderma isolates (T.1, T.3 and T.4) were extracted and PCR amplification was done using ITS region. Gel electrophoresis after PCR reaction, the presence of amplified products was tested by running an agarose gel. DNA bands were approximately 700 base pairs long (Fig.4). This result agrees with Shahid *et al.,* (2013). Molecular identification of Trichoderma isolates based on the sequence analysis of the nuclear ribosomal regions, isolates were identified as *Trichoderma asperellum* strain (Tr-21), *T. hamatum* strain (Th-21) and *T. harzianum* strain (Ts-21). Sequence data of the *Trichoderma* spp. have been submitted to the GenBank under accession numbers (MW965676, MW965776 and MW965792), respectively.

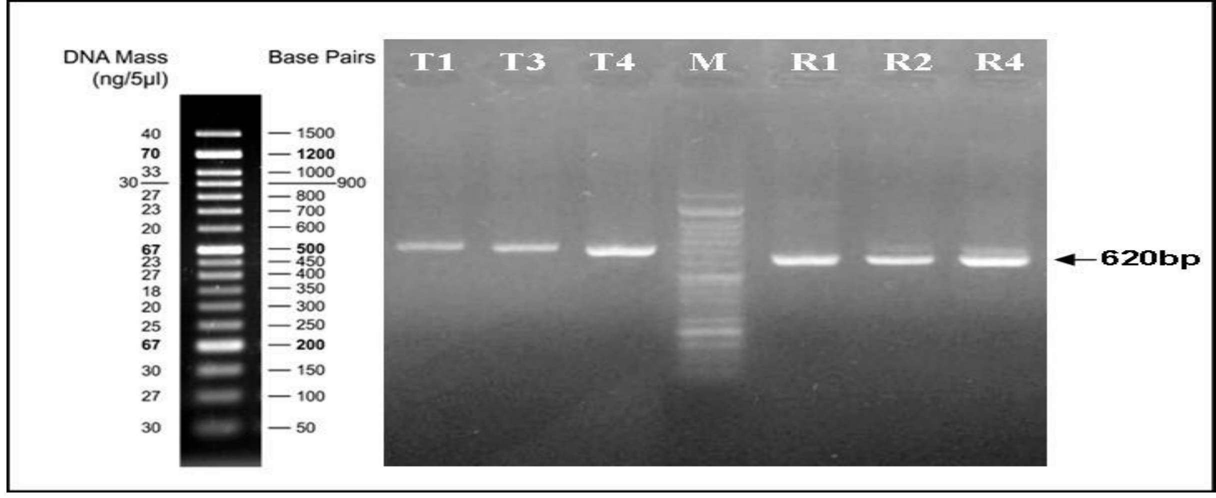

Fig. (4): Gel electrophoresis after PCR reaction to identify *R. solani* and *Trichoderma* spp. (M: The DNA ladder 50 bp, (GeneDireX)).

4- The antagonistic effect of *Trichoderma* spp. on *Rhizoctonia solani* isolates:

To screen the efficacy of five *Trichoderma* isolates, a laboratory experiment was carried out to study effect of these isolates on the growth rate of three *R. solani* isolates. Data presented in Fig (5) indicate that Trichoderma isolates differed in their efficacy in reducing mycelium growth of *R. solani* isolates. Isolate number one was the most effective one followed by isolates three and four, respectively. Meanwhile isolates two and five were clearly less effective. The pathogenic fungal isolates of *R. solani* also showed different degrees of reaction against *Trichoderma* spp. *In vitro* bioassays revealed that the Trichoderma isolates completely outgrew the pathogen, causing brownish discoloration at the initial point of interaction. Mousumi *et al*., (2019) stated that,

among tested Trichoderma isolates, *T. harzianum* and *T. asperellum* were found to be potent biocontrol agents, as observed by inhibiting the radial growth of phytopathogens. Hence, the possible mycoparasitism between both Trichoderma isolates and *R. solani* was attributed to hyphal interactions followed by the production of chitinase enzyme which lead to degradation of *R. solani* cell walls. The effect of chitinase enzymes on the *R. solani* cell wall has been shown to be involved in biological control (Haran *et al*., 1996 and El-Tarabily *et al.*, 2000).

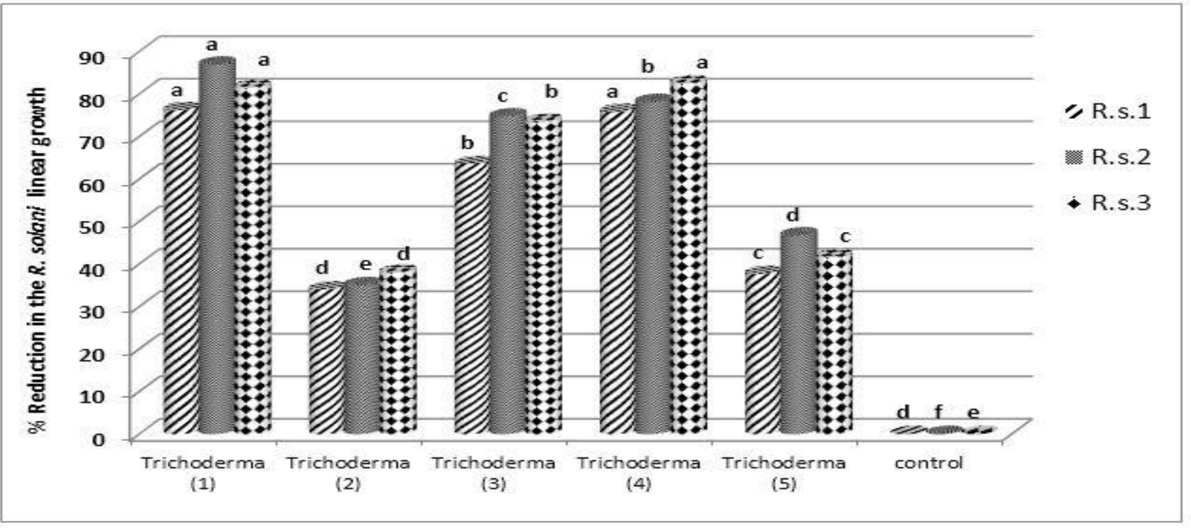

Fig. (5): The antagonistic effect of five *Trichoderma* isolates on three *R. solani* isolates (Means with the same letters, in each column are not significantly different).

5-Light and scanning electron microscope observation of *T. harzianum* myco-parasitism:

Macroscopic observations of *in vitro* bioassays revealed that *T. harzianum* was chosen for visualization via light and scanning electron microscope (SEM). The results showed a complete colonization of *R. solani* with *T. harzianum*. The parasitic hyphae invaded the host hyphae and grew on the surface with coiling before penetrating the cell wall. *T. harzianum* antagonistic effect resulted in the breakdown of pathogen hyphae due to the presence of extracellular enzymes. Because of the high concentrations of chitinase which are produced by *Trichoderma* spp. (Abbas *et al.,* 2017), these enzymes cause the pathogen's cell wall degradation. *R. solani* invaded hyphae appeared to be disintegrated. The parasitized hyphae frequently collapsed. (Fig. 6). Mycoparasitism is an important and complex process in which *Trichoderma* spp. extends lytic enzymes at its host and attaches to and coils around the fungal hyphae, sometimes penetrating them (Parizia *et al.*, 2012). Cell wall lysis was observed, indicating that lytic enzymes were secreted in response to contact with the host pathogen (Yobo *et al*., 2004).

6-Identification of potentially bioactive compounds of three *Trichoderma* spp. by GC-MS:

Mass analysis and chromatogram of *T. asperellum, T. hamatum* and *T. harzianum* revealed the presence of 35, 13 and 9 compounds, respectively (Fig., 7). The chemical identification of the bioactive compounds presents in the ethyl acetate extracts of *T. asperellum, T. hamatum* and *T. harzianum* was performed by GC-MS based on the retention time, Percentage area, molecular weight, formula, biological activities and structure of the compound (Table, 2 and Fig.,8). This work focused on the main secondary metabolites produced by different *Trichoderma* spp. Most of the identified compounds have been reported to possess interesting biological activities and their roles in controlling plant pathogens. The major compounds identified by GC-MS analysis include 2,5-Cyclohexadiene-1,4-dione, 2,6-bis (1,1-dimethylethyl), Hexadecanol, Nonadecene, 1,2-benzenedicarboxylic acid, octadecenoic acid (z)-, 1-Docosene, tert-Hexadecanethiol, 9-octadecenoic acid (z) methyl ester and Hexadecanol, 2-methyl. From this analysis, a total of eleven compounds have been detected and identified mainly as fatty acid esters, phenols, alkenes and aromatic chemicals. Six compounds namely 2,5-Cyclohexadiene-1,4-dione, 2,6-bis (1,1-dimethylethyl), 1-Hexadecanol, 1-Nonadecene, 1-Docosene and 1-Hexadecanol 2- methyl were found to be antimicrobial, antifungal and antibacterial.

Another 4 compounds work as antioxidant namely 2,2-Dideutero Octadecanal; 9,12-Octadecadienoic acid (Z, Z); 9-octadecenoic acid (z)-methyl ester and 1-Docosene, 1-Hexadecanol, 2-methyl. While 1-Methoxy-2-propyl acetate act as solvent. Several studies have been attributed to antifungal, antibacterial and antioxidant effect of these compounds (Asghar *et al*., 2011; Hema *et al*., 2011; Senthilkumar *et al*., 2011; Belakhdar *et al*., 2015; Zeilinger *et al*., 2016 and Shafiques *et al*., 2019).

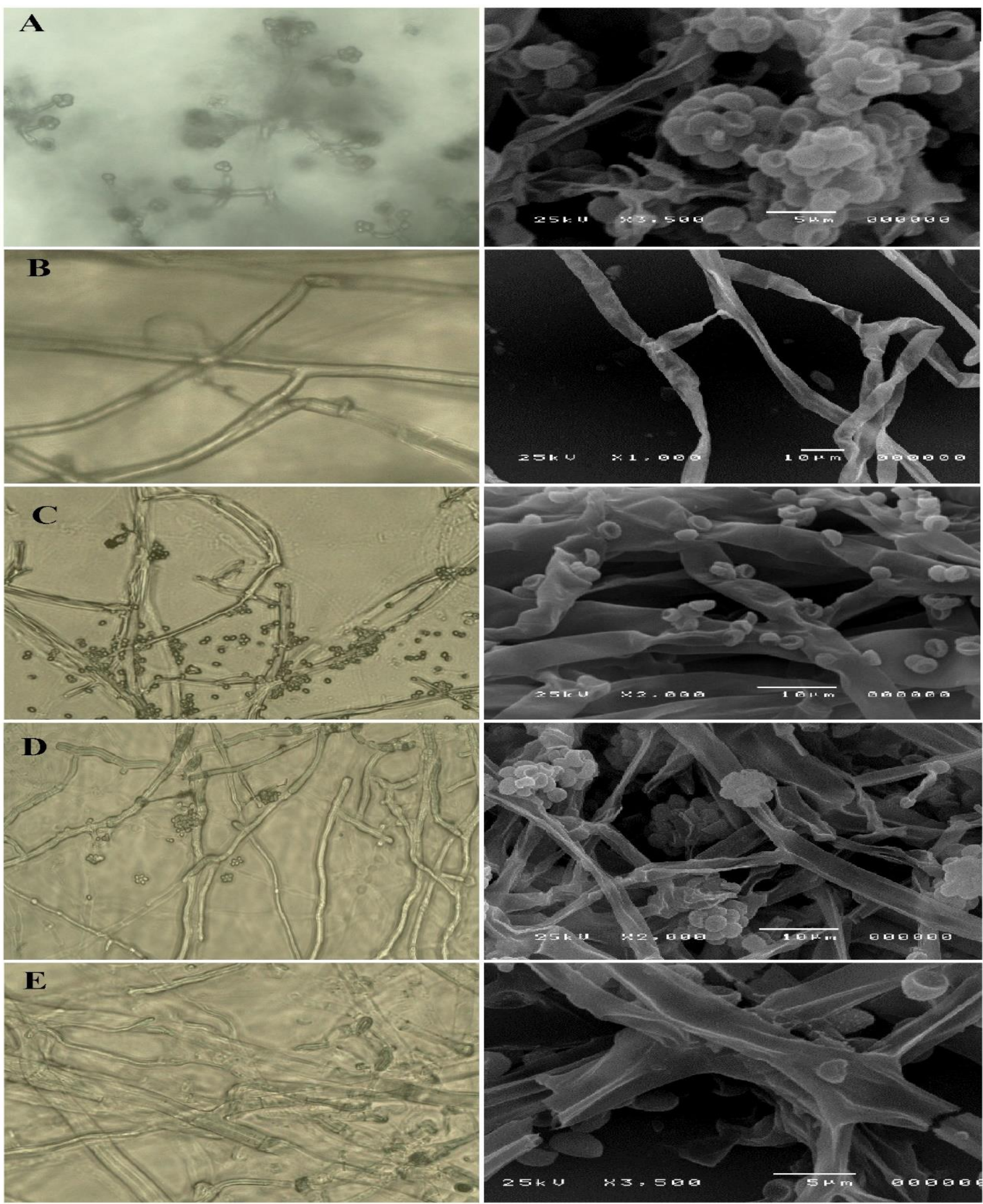

Fig. (6): Light and scanning electron micrographs of hyphal interactions between *Rhizoctonia solani* and *T. harzianum*. (A) Culture of *T. harzianum*. (B) Normal *R. solani* mycelium. (C) Early stages of mycoparasitism by coiling and spore formation without penetration of *T. harzianum* in the mycelium over *R. solani*. (D, E) Spore formation of *T. harzianum* over *R. solani* mycelia and cell walls breakdown of *R. solani*. (Light microscope at 40X in an optical microscope).

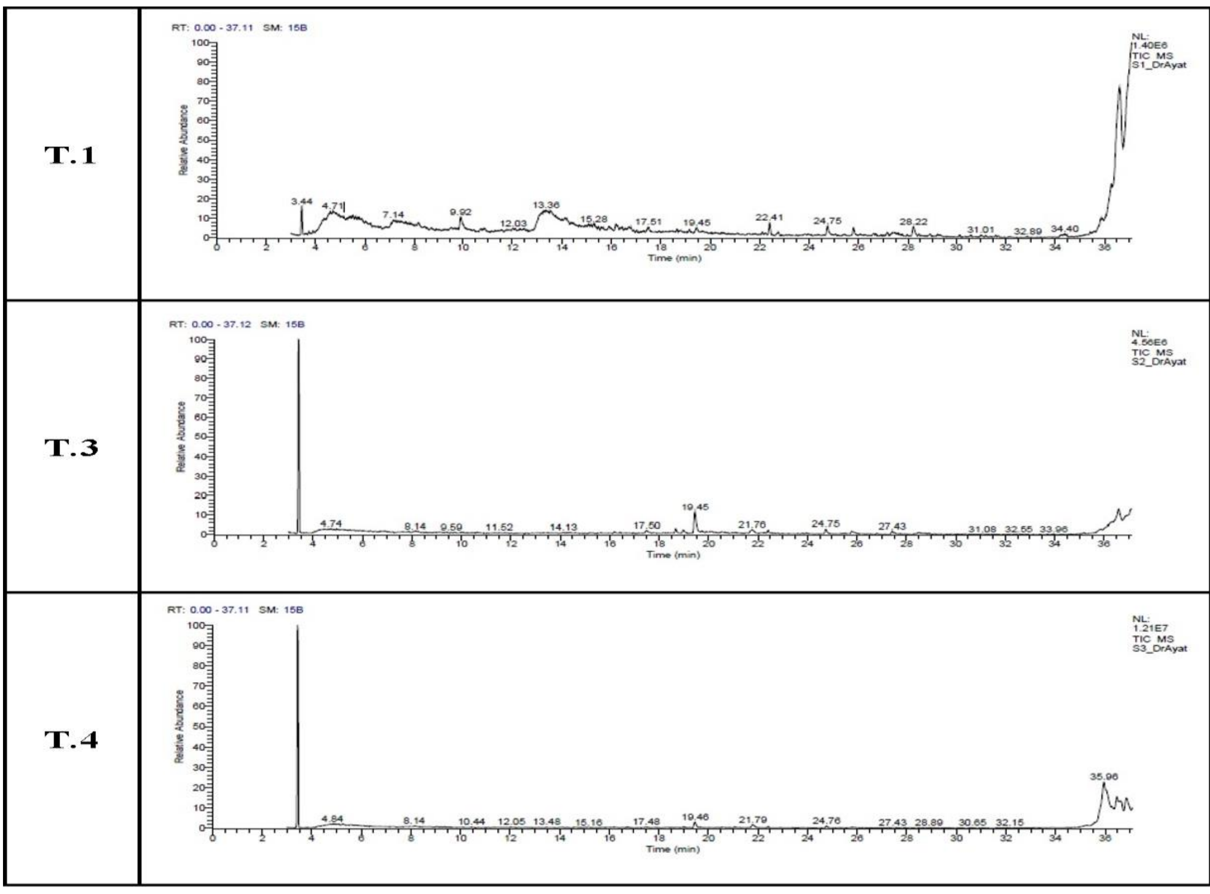

Fig. (7): Total ion chromatogram of the three *Trichoderma* spp. (T.1 = *T. asperellum*, T.3 = *T. hamatum*, T.4 = *T. harzianum*).

Table (2): Identification of potentially bioactive compounds of three *Trichoderma* spp. by GC-MS analysis

RT	Percentage area			Molecular formula	Molecular weight	Compound name	Biological activity
	T.1	T.3	T.4				
3.42	67.01	4.97	56.6	$C_6H_{12}O_3$	132	1-Methoxy-2-propyl acetate	Solvent
16.10	0.8	2.33	-	$C_{14}H_{20}O_2$	220	2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)	Active antifungal compound
18.60	4.33	0.64	2.63	$C_{16}H_{34}O$	242	1-Hexadecanol	Antifungal
22.32	7.12	2.93	-	$C_{19}H_{38}$	266	1-Nonadecene	antibacterial, antifungal
22.43	0.47	-	-	$C_{16}H_{34}S$	258	tert- Hexadecanethiol	Antioxidant and antibacterial
23.72	1.27	2.77	-	$C_{26}H_{42}O_4$	418	Phthalic acid, butyl tetradecyl ester	Antioxidant
25.32	1.91	-	-	$C_{22}H_{34}O_4$	362	1,2-Benzenedicarboxylic acid, butyl 8-methylnonyl ester	Antimicrobial, antifouling
25.70	5.80	1.27	-	$C_{22}H_{44}$	308	1-Docosene	Antifungal
27.22	0.41	-	-	$C_{18}H_{34}D_2O$	270	2,2-DIDEUTERO OCTADECANAL	Antioxidant
27.33	8.68	-	2.71	$C_{19}H_{34}O_2$	294	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	Antioxidant
27.42	4.74	-	13.31	$C_{19}H_{36}O_2$	296	9-octadecenoic acid (z)-, methyl ester	Antioxidant
31.63	0.9	2.31	8.18	$C_{17}H_{36}O$	256	1-Docosene, 1-HEXADECANOL, 2-METHYL-	Anti-microbial

T.1 = *T. asperellum*, T.3 = *T. hamatum*, T.4 = *T. harzianum*.

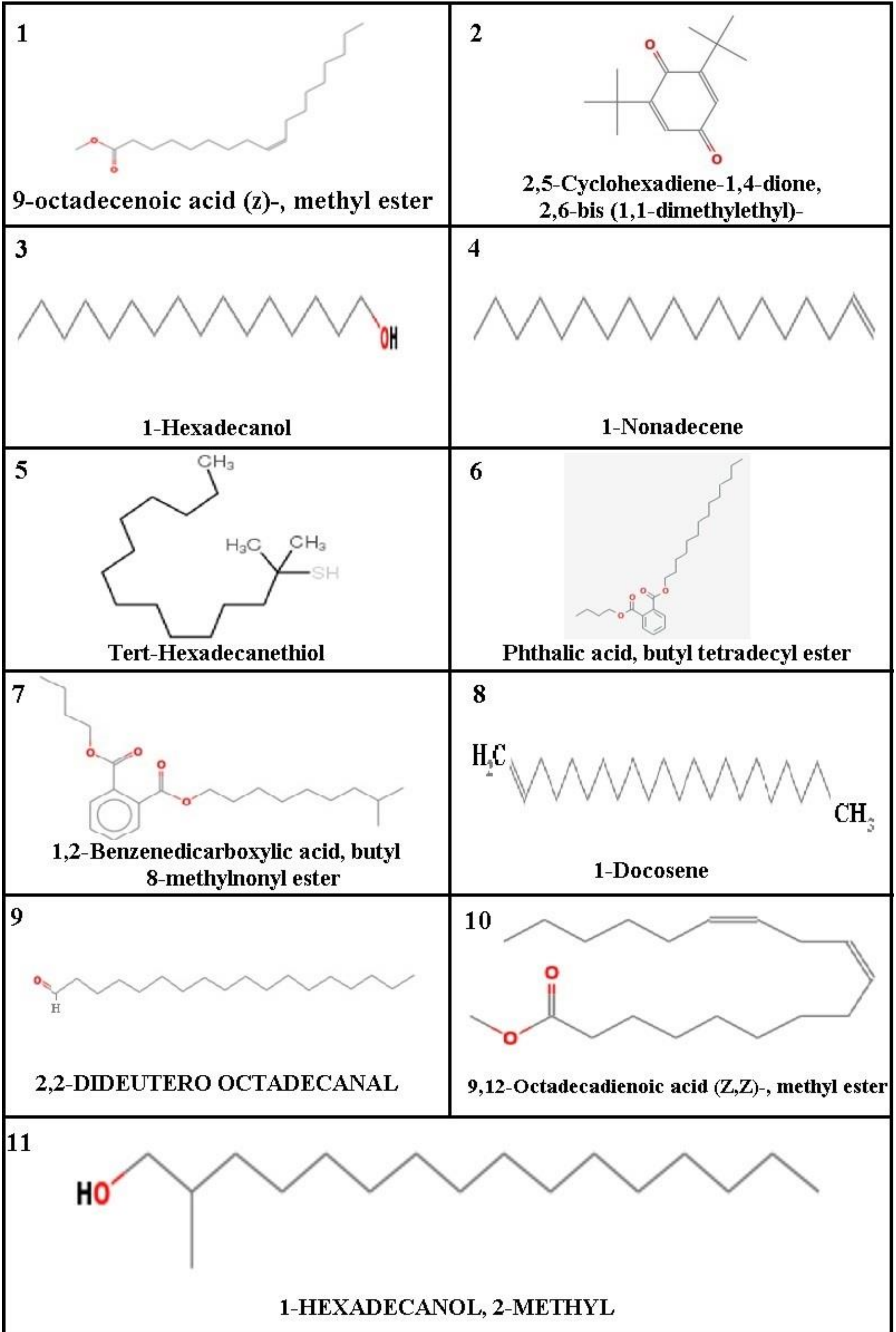

Fig. (8): Identified structure of phytochemical compounds of *Trichoderma* spp. using GC-MS.

7- Effect of different concentrations from different forms of *Trichoderma* spp. and their mixture for controlling Rhizoctonia root rot of pepper in a greenhouse experiment:

The most effective isolates 1,3, 4 and their mixture were prepared as suspension and powder, each of these were applied in a greenhouse experiment at three concentrations, *i.e.,* 2×10^5, 3×10^5 and 6×10^5 CFU/ ml. Results in Table (3) indicate that all tested forms were effective in reducing the disease with all tested isolates and all concentrations. In the present work suspension form was better than powder in controlling the disease. Concerning the concentration of conidia/ unit, it was found that increase the concentration of *Trichoderma* spp. propagules give rise increasing in controlling the disease. As for the efficiency of different single isolates and their mixture in controlling the diseases, it was found generally, that mixture of isolates was the most effective treatment. The highest percentage of survived plants was obtained when *T. harzianum* was formulated as suspension (95%) or powder (90%) at concentration $6x10^5$ CFU. The lowest effective treatment was when *T. asperellum* was used at low density ($2x10^5$) in powder form where 81.5% survived plants was recorded. When a mixture of *Trichoderma* spp. was used, the highest percentage of survived plants (97%) was achieved compare with only 60 % in control treatment. Use of an isolate of Trichoderma that has more than one mechanism against the pathogen increases the potentiality of biocontrol (Howell, 2003 and Mousumi *et al*., 2019). Moreover, using more than one isolate increase the possibility of occurring a synergistic effect of different mechanisms against the pathogen (Schisler *et al.,* 1997 and Navaneetha *et al*., 2015). *Trichoderma* spp. are the potential bio-agents which inhibit *R. solani* by confrontation through mycoparasitic or antibiosis or competition as well as inducing plant defense responses (Lorito *et al.,*1993 and Abbas *et al.,* 2017). Combining effective isolates of bio-agents was also studied in this respect, Cook (1993) stated that several strains of Trichoderma should be included in a formulation to widen the range of control. Application of Trichoderma formulations with mixture strains perform better than individual strains for the diseases management and plant growth promotion (Kumar *et al*., 2014). These findings are consistent with other studies in which reduction of *R. solani* damping-off was achieved with formulations of various Trichoderma isolates (Lewis and Lumsden, 2001).

Table (3): Effect of different formulations and concentrations of different Trichoderma isolates on the percentage of survived pepper plants grown in artificially infested soil with *Rhizoctonia solani.*

Formulation	Concentration CFU (10^5)	Percentage of survived pants			
		T.1	T.3	T.4	Mix
Suspension	2	82.70^c	82.00^c	85.70^{de}	90.00^c
	3	88.47^a	90.33^{ab}	93.33^{ab}	95.33^{ab}
	6	91.00^a	92.70^a	95.00^a	97.00^a
Powder	2	81.50^c	82.00^c	83.00^e	86.00^d
	3	83.70^{bc}	86.00^{bc}	88.70^{cd}	92.77^{bc}
	6	87.00^{ab}	88.33^{ab}	90.00bc	95.00^{ab}
control	--	60.00^d	60.00^d	60.00^f	60.00^e

Values in each column with the same letter not significantly different according to Duncan's Multiple Range test (P= 0.5); T.1 = *T. asperellum*, T.3 = *T. hamatum*, T.4 = *T. harzianum*.; Control: artificially infested with *R. solani* without adding the bio- agent.

8-Evaluation of different forms of different isolates of *Trichoderma* spp. and their mixture for controlling root rot diseases of pepper in protected cultivation:

The two aforementioned formulations of most effective isolates and their mixture were applied in protected cultivation at three concentrations. Results in Table (4) indicate that all forms of *Trichoderma* spp. were effective in reducing disease with all tested isolates (single isolates or their mixture) and all concentrations. Suspension formulation was better than powder in reducing the disease. Results also confirmed that increase of concentration, of different forms, increased the efficacy in controlling the disease. According to the obtained results, it was found that the highest percentage of protection (88 % survived plants) was noticed when *T. harzianum* was used at high concentration (6×10^5) of pepper transplants compared with only 40% in the control treatment. The lowest effect was detected when *T. asperellum* with powder preparation was used at

the rate of (2×10^5), where only 70% survived plants was recorded. As for the efficiency of different single or mixture isolates in controlling the disease. It was found generally that mixture of isolates was performed better than single isolate, these results were in agreement with Abd El-Moity, (1985) and Singh and Singh, (2014). The beneficial effects of *Trichoderma* spp. have been attributed to many factors such as antagonism, mycoparasitism, production of secondary metabolites, promoting of plant growth and plant resistance (Papavizas and Lumsden, 1980; Harman, 2006; Almeida *et al.,* 2007; Vinalea *et al*., 2012 and Dabire *et al*., 2016). This wide range of effect is very promising for producing commercial biocide. In addition, a correlation was reported between concentration of the bio-agent propagules and the efficacy of biocontrol (Navaneetha *et al.,* 2015). Because the concentration of conidia required is relatively high, mass production of the antagonist to obtain the required conidia through a rapid efficient and inexpensive fermentation is a critical issue requirement. Fungal formulations should become a standard in biocontrol applications in order to increase efficacy (Herrera *et al.,* 2020).

Table (4): Effect of different formulations and concentrations of different Trichoderma isolates on the percentage of survived pepper plants grown under natural infection.

Formulation	Concentration CFU (10^5)	Percentage of survived pants			
		T. 1	T. 3	T. 4	Mix
Suspension	2	72.00^{c}	73.00^{c}	83.00^{bc}	86.00^{d}
	3	76.00^{b}	80.70^{b}	84.00^{abc}	92.00^{ab}
	6	82.00^{a}	86.00^{a}	88.00^{a}	95.00^{a}
Powder	2	70.00^{c}	72.00^{c}	80.00^{c}	84.00^{d}
	3	73.00^{bc}	75.00^{c}	84.00^{abc}	87.00^{cd}
	6	80.00^{a}	81.00^{b}	86.00^{ab}	90.00^{bc}
control		40.00^{d}	40.00^{d}	40.00^{d}	40.00^{e}

Figures in each column with the same letter not significantly different according to Duncans Multiple Range test (P= 0.5); T.1 = *T. asperellum*, T.3 = *T. hamatum*, T.4 = *T. harzianum*.; Control: naturally infested soil without adding the bio- agent.

CONCLUSION

Both morphological and molecular approaches are important methods to identify *Trichoderma* spp. Biocontrol mechanisms of various *Trichoderma* spp. have been studied. Furthermore, the Trichoderma isolates displayed a strong antagonistic activity against *R. solani*. *In vitro* and *in vivo* studies confirmed that the selected *Trichoderma* spp. can successfully be used to control *R. solani*. Current research suggests that using a mix of *Trichoderma* spp. at high concentration may lead to the development of strategies for pathogen control.

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