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ORIGINAL PAPER

Impact of some biocontrol agents and essential oils on strawberry root rot caused by *Rhizoctonia solani*

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ABSTRACT

Rhizoctonia solani is one of the most important soilborne pathogens which threatens strawberries and may cause significant losses in yield. In this study,the pathogenicity test of ten isolates of *R. solani* isolated from soil and different plant species *i.e.* cucumber, mint, bean, pear, citrus, tomato and strawberry was carried out on strawberry plants and they were all pathogenic. Molecular characterization of *R. solani* isolates by sequencing conducted by the partial rDNA internal transcribed spacer (ITS) revealed 99% homology among the isolates. *R solani* isolates belonged to three different AG groups. The isolates sequences clustering supported the genetic basis of the anastomosis groups. Five isolates belonging to AG-G, four isolates belonging to AG-F and one isolate belonging to AG-3. *In vitro* investigation of ten isolates of suppressing *R. solani* mycelia growth revealed potential suppressive effects. *T. longibrachiatum* (T4) strain showed the highest suppressive effect (72.22 %).While,mint and clove oils at 0.5% concentration showed a reduction of *R. solani* 71.11 and 67.77%, respectively. In greenhouse experiments, *T. longibrachiatum* (T4) was the most effective in reducing root rot incidence followed by Mint, Clove oils at 1% concentration then *Pseudomonas stutzeri*, being 5.33, 8.33, 10.67and 14.33, respectively. Biochemical studies revealed that mint oil significantly increased peroxidase, polyphenol oxidase and chitinase activity in strawberry roots planted in infested soil.

Keywords: *R. solani, Trichoderma* spp. ,*Pseudomonas stutzeri*, Mint, Clove, Anastomosis groups**.**

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INTRODUCTION

In Egypt, strawberries (*Fragaria xananassa* Duch.) are one of the most significant vegetable crops. Strawberry plants are attacked by a number of soil-borne diseases causing significant losses, including *Rhizoctonia solani*, *Macrophomina phaseolina*, and *Fusarium* spp. (**Fahim et al., 1994 and El-Sharkawy, 2006).**These fungi cause black root rot, and wilt diseases (**Fang *et al*.,2011 and Fang *et al*.,2012**).

R. solani is a soil-borne pathogen that has the potential to severely damage a variety of crops (**Anderson,1982**). Currently, there are 13 groups of *R. solani* known as anastomosis groups (AGs) have been identified. Isolates from these separate groups do not anastomose with each other (**Carling,1996**).

Carling *et al*.,(2002) and Gonzalez, *et al*., (2006) reported that ribosomal DNA (rDNA) sequencing is used to reconstruct the phylogenetic relationships among different organisms of the form genus. Sequence data provided more effective means of genetic classifications within *Rhizoctonia* spp. Compared to previously utilized characteristics such as number of nuclei, plant host, or morphology. Sequence analysis of the genomic regions encoding the rDNA-ITS has become more common as sequences from various isolates have accumulated in databases,it is convenient for detecting AG. rDNA-ITS sequencing has been used to investigate the relationships among the AGs moreover to study the genetic diversity of *R. solani*, both with in and between the different AGs. (**Woodhall *et al*., 2007 and Lehtonen *et al*., 2008**).

The effectiveness of biocontrol agents as a green strategy has been demonstrated to manage plant diseases, promote plant

growth, performance productivity and boost output. Moreover, they can be used for controlling plant diseases by generating compounds that restrict fungi growth and stimulating plant defences against phytopathogens (**Wang *et al.*, 2018).**

During cultivation of crops, some beneficial fungi, such as *Trichoderma*, may be able to lower the density of pathogens. Numerous strategies are employed by this fungus, such as enhanced plant tolerance to abiotic stressors, pathogen competition, mycoparasitism, antibiosis, and activation of the pathogen defensive system. It also promotes plant growth. It can create a variety of biochemical chemicals, including siderophores that may facilitate biocontrol activities and both volatile and nonvolatile molecules (**Rubayet and Bhuiyan, 2023).**

Pseudomonas species stabilize atmospheric nitrogen levels, solubilize potassium and phosphate, and construct phytohormones, lytic enzymes, volatile organic compounds, antibiotics, and secondary metabolites under harsh conditions. **(Mehmood *et al.,* 2023).** *Pseudomonas* strains have a variety of characteristics that make them an excellent choice for use in agriculture as biocontrol and plant growth-promoting agents **(Kumudini *et al.,* 2017).** Although *Pseudomonas* strains can survive in severe settings and considered as a potential biocontrol agent for plant pathogens, *Phytophthora nicotianae*, *Fusarium solani*, *R.solani*, and *Pythium* sp. **(Saranraj *et al.*, 2023).** Therefore, alternatives to traditional fungicide control methods, such as biological control agents, resistant plant varieties, and essential oils, have been employed to manage plant fungal diseases, due to the detrimental effects of chemical fungicides on humans, the environment and all living organisms. **(Punja *et al.*, 2017)**

This study aims to identify *R. solani* isolates based on anastomosis groups and sequence analysis. Study pathogenicity and relative virulence of the isolates moreover, manage root rot disease using safe treatments under greenhouse conditions.

MATERIALS AND METHODS

1.Source of isolates

1.1.*Rhizoctonia solani* isolates:

Ten isolates of *R. solani* were used in this study, seven isolates of *R. solani* (Table 1) were obtained from Mycology Research and Diseases Survey Dept., (MRDSD) Plant Pathology Research Institute, ARC. Three isolates were obtained from The Identification of Microorganisms, Biological Control of Plant Diseases and Evaluation of Biofungicide Unit (IMPCPDEBU), Plant Pathology Research Institute, ARC.

1.2. Isolation and identification of *Trichoderma* spp.:

Soil samples were selected from the rhizosphere around healthy plants in naturally infested fields representing five governorates (Behaira, Gharbiya, Kafr-El Sheikh, Qaloubiya and Giza) to isolate different antagonistic microorganisms. *Trichoderma* isolates were identified based on the colony morphology and spores

Table 1: Sources of *R. solani* isolates.

No.	Code of isolates	Isolates	Host
1	R1	*R.solani* (MRDSD-138)	Cucumber
2	R2	*R.solani*(MRDSD -139)	Mint
3	R3	*R.solani*(MRDSD -140)	Soil
4	R4	*R.solani*(MRDSD- 46)	Bean
5	R5	*R.solani*(IMPCPDEBU-23)	Soil
6	R6	*R.solani*(MRDSD-141)	Pear
7	R7	*R.solani*(MRDSD-142)	Citrus
8	R8	*R.solani*(IMPCPDEBU-24)	Soil
9	R9	*R.solani*(IMPCPDEBU-25)	Tomato
10	R10	*R.solani* (MRDSD-143)	Strawberry

according to Barnett and Hunter's taxonomic criteria **(Barnett and Hunter, 1972).**

1.3. *Pseudomonas stutzeri* H2-MG738255*:*

The bacterial bio-agent *Pseudomonas stutzeri* strain (H2-MG738255)was obtained kindly from the Agricultural Microbiology Department. Soil, Water and Environment Research Institute (SWERI), Agricultural Research Center (ARC) Giza. Egypt.

2. Pathogenicity tests*:*

Ten isolates of *R. solani* were tested under greenhouse conditions at The Identification of Microorganisms, Biological Control of Plant Diseases and Evaluation of Biofungicide Unit (IMPCPDEBU), Plant Pathology Research Institute, ARC. The inoculum of each *R. solani* isolate was grown on sorghum grain sand medium (**Ahmed, 2013**). The percentages of disease incidence (D.I) of root rot disease were determined post-transplanting (49 days from transplanting) according to the method described by **Ahmed (2005)** and **Ahmed (2013)** as follows: -

$$\text{Disease incidence (D.I) of root rot \%} = \frac{\text{Number of root} - \text{rotted plants}}{\text{Total number of plants}} \times 100$$

3- Determination of anastomosis groups and phylogenetic analysis of *R. solani* isolates.

3.1 - Anastomosis group of *R. solani* isolates

Anastomosis grouping (AG) of ten *R. solani* isolates was studied. Isolates were paired using mycelial discs in all possible combinations among them were done. Mycelial discs (5 mm diameter) taken from the edge of an actively growing colony (7 days old) of each isolate were placed approximately 3 to 4 cm away from the other isolate. Three isolates were paired in one dish and each isolate was self-paired as a control. A set of 3 replicates was used for each pairing. Dishes were incubated at 25°C for one week. The pairing of the isolates was evaluated and the combinations were classified according to **Macnish *et al.*, (1997)** hyphal anastomosis reaction is indicative of compatibility or incompatibility **(Anderson, 1982).**

3.2. Slide technique

Anastomosis of hyphae was determined using a modification of the microscope-glass slide technique according to **(Kronland and Stanghellini, 1988)**with some modifications. 5-mm diameter disks from the growing margins of young colonies (7 days old) on PDA from all isolates were placed 2-3 cm away from the other isolate on a sterilized glass slide covered with agar film. Slides were placed on glass bars in 15cm diameter Petri dishes and incubated for 24-48 h at 25°C. When the hyphal tips of two isolates were in contact with each other, methylene blue was used to distinguish hyphae anastomosis. Microscopic examination was carried out to record the compatible and incompatible between isolates. This experiment was repeated twice to ensure accurate results.

3.3.DNA isolation

R. solani cultures were grown on PDA medium (5-mm-diameter discs, taken from a 7-day-old culture), were transferred into a 250 ml flask containing 100 ml of potato-dextrose broth and incubated at 25ºC for 4 days, the grown mycelia were harvested and filtrated through filter paper then washed with sterile deionized water, frozen in liquid nitrogen, freeze-dried and stored at – 20ºC.DNA extracted from 50 mg of freeze-dried mycelial using a Qiagen DNA extraction Kit, was dissolved in 100 µl of elution Buffer. PCR amplification, the internal transcribed spacer (ITS) region of the isolate was amplified using the 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'). The ITS region of the rDNA and part of the DNA template were amplified by PCR reaction: for a total volume of 20µl: 10µl Master Mix, 2µl DNA template, 2µl Primer, 6µl deionized H_2O. The amplification protocol using PCR was carried out as follows: Denaturation at 94ºC for 2min. 35 cycles, each consisting of the following

steps: Denaturation at 94ºC for 30s. Annealing at 52ºC for 30s.Extension at 72ºC for 1min. Final extension at 72ºC for 10min. Amplicon was sent to Macrogene Korea and was run on an automated DNA sequence (ABI 3700 capillary sequencer, Macrogene, Korea). The obtained sequences were submitted to GenBank to acquire accession numbers. The sequences were analyzed with BLAST® (**Altschul*et al.*, 1997**) against the National Center for Biotechnology Information (NCBI) sequence Database (http://blast.ncbi.nlm.nih.gov).

4. Laboratory experiment:

4.1. Antagonistic effect of bioagents against *R. solani* under laboratory conditions

The antagonistic effects of ten *Trichoderma* isolates, *Pseudomonas stutzeri*, against the radial growth of the most aggressive pathogenic isolate of *R. solani* were screened according to (**Bertani, 1951and Rahman *et al.*, 2009).** The percentage of *R. solani* mycelial growth reduction was calculated using the formula suggested by (**Karaoglu-Alpay *et al.*, 2018**):

$$\text{Percentage Inhibition of Radial Growth} = \frac{(R1 - R2)}{R1} \times 100$$

(R1 = Control colony of radius, R2 = *Trichoderma*-treated colony radius).

4.2. Molecular characterization of most effective*Trichoderma* isolate:

The most effective *Trichoderma* isolate was identified by sequence and submitted to GenBank. DNA was extracted using the Dellaporta procedure (**Dellaporta *et al.*, 1983**). The internal transcribed spacer region (ITS) of rRNA was amplified using primer ITS4 and ITS5 (**White *et al.*, 1990**). The PCR reaction was carried out in a 20µL reaction volume with 10 µL of PCR Master Mix (*amaROne*PCR, GeneDireX, Inc.), 1.5µL of each primer, and 2µL of template DNA. The PCR amplification conditions were carried out following **Haouhach *et al.* (2020).**The amplicon was sent to sequencing (ABI 3700 capillary sequencer, Macrogene, Korea)**.** To assign taxonomy, the BLASTn algorithm was performed using the NCBI GenBank database, comparing the queries to type specimens.

4.3. Analysis of *Pseudomonas stutzeri*

A- Chitinase Activity:

For qualitative determination of the Chitinase enzyme, the colour change on Petri dishes from yellow colour to red colour was recorded as an indication of chitinolytic the method explained by **(Monreal and Reese, 1969).**

B-Estimation of hydrogen cyanide(HCN):

For the production of HCN, bacterial cultures were streaked overnight on Luria-Bertani (LB) plates that had 4.4 g/L of glycine. According to **(Lorck, 1948),** the filter paper's colour changing from yellow to brown was noted as a sign of cyanogenic production.

C-Production of Poly-β-Hydroxybutyrate (PHB):

Accumulation of PHB was estimated according to the method adopted by **Law and Slepecky (1961)**.Samples were taken after 48h and a cell pellet of 5ml culture was collected by centrifugation (4000 rpm for 20min.). The pellet was suspended in 5ml of 0.2% (w/v) sodium hypochlorite, for 1h at 37°C to allow the total lyses of the cell suspensions. Granules of PHB were collected by centrifugation (2000rpm for 10min.), washed with distilled water, acetone and ethanol, and dissolved in chloroform. After getting rid of chloroform by evaporation, the residue was hydrolyzed and dehydrogenated with 10 ml concentrated sulfuric acid and heated for l0 min at 100°C in the water bath to be converted to crotonic acid. The solution was cooled, then a sample was transferred to a cuvette and the absorbance at 235nm was measured against a sulfuric acid blank. A standard curve was obtained using standard crotonic acid (Sigma Chemical Co, quantitative conversion of poly-β hydroxybutyric acid to crotonic acid).

4.4. Effect of essential oils on the radial growth of *R. solani* in *vitro*

The essential oils of clove (*Syzygium aromaticum*), mint (*Mentha cervina*) and sesame (*Sesamum indicum*) at concentrations of 0.25, 0.5 and 1.0% (v/v)were prepared and tested, incubation conditions and growth measurements and calculations were followed as stated before against the linear growth of *R. solani in vitro*. The tested oils were obtained from EL Captain Company for Extracting Natural Oils from Plants, Cairo-Egypt. (**Fahiem, 2010**).

5. Effect of bioagents and essential oils in strawberry plants planted in infested soil with root rot pathogens under greenhouse conditions:

An *in vitro* experiment was conducted using the most effective bioagent and essential oil, *i.e. T. longibrachiatum*, *P.stutzer*i, mint and clove oil (1%) to study their efficiency in suppressing the most pathogenic isolate *R. solani* (R3). The experiment was performed in 3 replicates (**Askim *et al.,* 2021**). Plants were subjected to the recommended agricultural practices in the clay soil under greenhouse conditions at (IMPCPDEBU), ARC, Giza, Egypt. The percentages of disease incidence of root rot diseases were determined 7 weeks post-transplanting (49 days from transplanting) according to the method described by **Ahmed (2005)** and **Ahmed (2013).**

6. Determination of oxidative enzymes and hydrolytic enzymes in strawberry roots.

Samples of strawberry roots collected from the most effective treatments for decreasing disease such as *T. longibrachiatum*, *P.stutzeri*, clove, mint and Rhizolex were chosen for assaying the different oxidative enzymes (Peroxidase activity and Polyphenol oxidase) and hydrolytic enzymes (Chitinase), in addition to, untreated plant roots as control. Root tissue samples from each treatment at 14 and 21 days from treatment were collected and homogenized immediately using liquid nitrogen (**Ojha and Chatterjee 2012**). The crude extract was used to estimate the Peroxidase, Polyphenol oxidase and Chitinase, activities (**Anand *et al.,* 2007**). The activity of Polyphenol oxidase activity (PPO), Peroxidase activity (PO) and Chitinase were assayed according to **(Abou-Zeid, *et al.* 2018).**

7. Effect of inoculation with the treatments on NPK content in strawberry shoot dry biomass:

Plant samples were collected after 20 days and analyzed, ground, and digested, determination of plant N, P and K contents was carried out as described by **Van Schouwenburg (1968).**

8. Statistical analysis:

Data were statistically analyzed for ANOVA according to **Snedecor *et al.,* (1989)** where mean values were compared using L.S.D at P>5 %.

RESULTS AND DISCUSSION

1. Isolation of *Trichoderma* spp.:

Ten isolates of *Trichoderma* were purified and identified according to their morphological features by using a light microscope **(Barnett and Hunter1972).** Fungal isolates were maintained on a PDA medium and kept in a refrigerator at 6°C. (**Table, 2**).

Table 2: Sources of *Trichoderma* spp isolates:

No.	Code	Isolates	Location
1	T1	*Trichoderma* sp.	Kafr-El Sheikh
2	T2	*Trichoderma* sp.	Behaira
3	T3	*Trichoderma* sp.	Gharbiya
4	T4	*Trichoderma* sp.	Giza
5	T5	*Trichoderma* sp.	Kafr-El Sheikh
6	T6	*Trichoderma* sp.	Giza
7	T7	*Trichoderma* sp.	Giza
8	T8	*Trichoderma* sp.	Qaloubiya
9	T9	*Trichoderma* sp.	Behaira
10	T10	*Trichoderma* sp.	Gharbiya

2. Pathogenicity tests:-

Data in Table (3) reveal that the 10 isolates of *R. solani* were aggressive in causing the highest disease incidence of root rot. All the isolates developed black root rot disease. Isolate R3 gave the highest D.I (86.7), followed by isolate R8 which

gave D.I (70.4). However, isolate R10 caused the lowest D.I and recorded (30.7). Our results are in agreement with those reported by **Errifi *et al*., (2019)** who found that the pathogenicity test revealed significant differences in disease severity among tested isolates. *R. solani* isolate (H1) gave 60% disease severity followed by *R. solani* isolate (R1) which gave 50% while the least level of severity was 40% of *R. solani* isolate (G1). Whereas, **Asad-Uz-Zaman *et al*. (2015)** evaluated four isolates of *R. solani* for their pathogenicity to strawberry plants and found that one isolate of them gave the highest virulence (95.47% mortality) characteristics.

Table (3) Pathogenicity of different isolates of *R. solani* on strawberry seedlings

No. of isolates	Disease incidence (%)	Plant survival (%)
R1	45.4	54.6
R2	34.4	65.6
R3	86.7	13.3
R4	34.4	65.6
R5	54.4	45.6
R6	33.7	66.3
R7	34.4	65.6
R8	70.4	29.6
R9	55.7	44.3
R10	30.7	69.3
Control	0.0	100
L.S.D. at 5%	4.46	4.12

3-Determination of anastomosis groups and phylogenetic analysis of *R. solani* isolates

3.1-Anastomosis group of *R. solani* isolates

R. solani isolates were classified by anastomosis groups (AGs) based on the hyphal fusion reaction (Table 4). Anastomosis occurs by the formation of fusions between the isolates hyphae in the form of bridges A and H shape (Fig 1). Fig (2) shows the pairings of three isolates of *R. solani* from three different AGs showing the development of incompatible reactions (clearing zones) in the region of mycelial contact. Pairing between isolates of *R. solani* shows compatibility with intermingled mycelium in the zone of mycelial contact.

Data in Table (5) reveal that according to mycelial compatibility, three anastomosis groups (AGs) were found among the isolates.AG-1 is composed of five isolates and identified as AG-G, while AG-2 is composed of four isolates assigned to AG-F and AG-3 contained one isolate, identified as AG-3. AG 1 and AG2 are composed of binucleate isolates while, AG3 is a unique group that has only one isolate, a multinucleate isolated from strawberry. In this study, R10 (AG-3) was incompatible with all the tested isolates.

There were 100 pairings of 10 isolates and only showed compatibility in 30 combinations reactions (30%). The vegetative compatibility was clear within the same group but was compatible or incompatible with isolates from other groups (Fig.3).

3.2. DNA isolation

Identification of the AGs groups of 10 *R. solani* isolates, previously isolated from different crops based on the number of nuclei, PCR amplification sequences of the rDNA-ITS region (ITS1 and ITS4) are presented in Tables (6 and 7). Blast analysis for the rDNA-ITS sequences of the 10 isolates at NCBI database showed that all the isolates were *R. solani* with sequence identity up to 99% with that in the database which revealed that these isolates come from one ancestor,but it failed to categorize them into separate and distinct groups based on AGs. Nine isolates were identified as binucleate (BN) and one isolate was identified as Multinucleate (MN). The remaining BN isolates were assigned to AG-G (5 isolates), AG-F (4 isolates), MN was assigned to AG-3 (one isolate) and AG-G (R3) isolate gave the highest disease incidence.Genetic relationships among *R. solani* isolates were inferred from Maximum likelihood analysis of the aligned ITS1, ITS4 region sequence in MEGA X compared with 46 sequences isolates available in GenBank. The rDNA-ITS region sequences of the *R solani* isolates

Table (4): Anastomosis group of *R. solani* isolates on PDA medium after 7 days

Isolates	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10
R1	+	-	+	+	-	-	+	-	-	-
R2	-	+	-	+	-	-	-	-	-	-
R3	+	-	+	+	-	+	+	-	-	-
R4	+	+	+	+	-	-	+	-	-	-
R5	-	-	+	-	+	-	-	+	+	-
R6	-	-	+	-	-	+	-	-	-	-
R7	+	-	+	+	-	-	+	-	-	-
R8	-	-	-	-	+	-	-	+	+	-
R9	-	-	-	-	+	-	-	+	+	-
R10	-	-	-	-	-	-	-	-	-	+

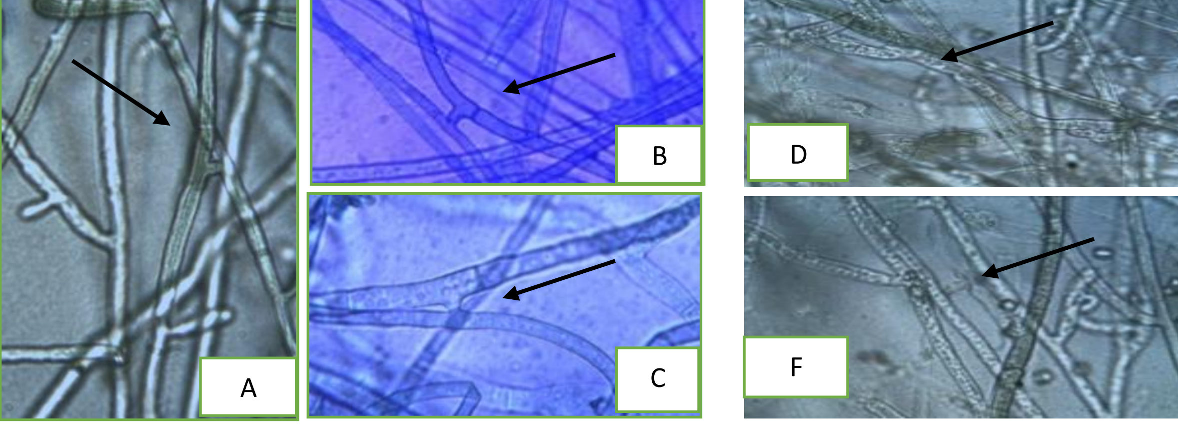

Fig (1): Anastomosis (A,B and C) between two compatible isolates showing H and A shape.(D and E) Pairings of two isolates of *R. solani* showing the development of incompatible reactions (lyses between hyphae).

Fig (2): Pairings of three isolates of *R. solani* from three different AGs. A and B show mycelial compatibility reactions between different isolates of *R. solani* C and D show the development of incompatible reactions (clearing zones) in the region of mycelial contact and show intermingled or produced a knitted ridge between mycelium in the zone of mycelial contact. This photograph was after one week of growth on PDA media.

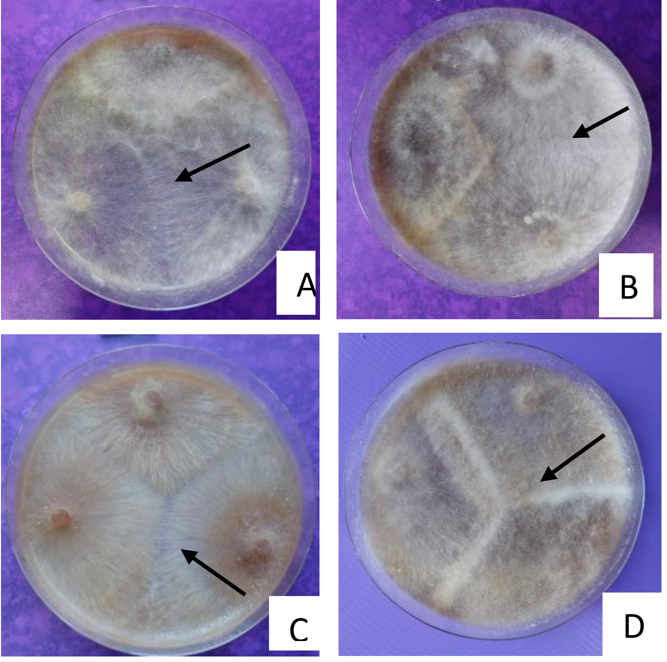

Table (5): Anastomosis group of *R. solani* isolates according to the dendrogram.

No.	Isolates
AG1	R1-R2-R3-R4 and R6
AG2	R5-R7-R8 and R9
AG3	R10

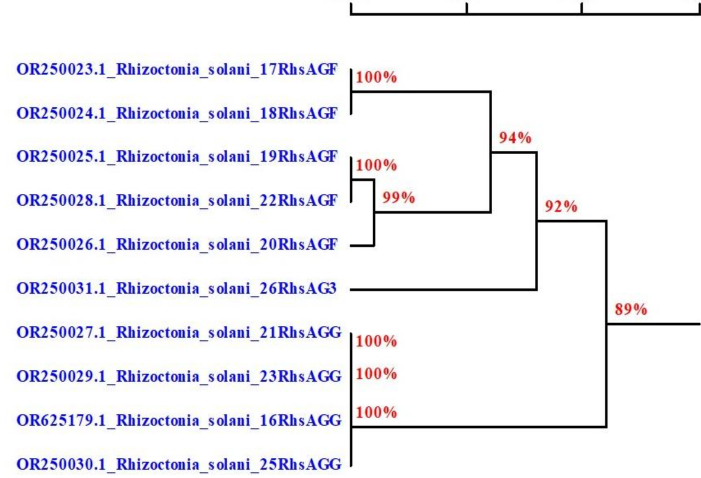

Fig (3): Dendrogram for 10 isolates of *R. solani*

showed high sequence homology (identity 100%) of AG-G isolates R1-R2-R3-R4 and R6, AG-3R5-R7-R8 and R9 showed (99 – 100% identity) and AG-F isolate R10 showed (99.2-99.4% identity), Figs 4 and 5.

For most combinations it was possible to detect the presence of anastomosis, so can concluded that these isolates belong to the same anastomosis group. On the other hand, those combinations that did not present anastomoses were the same ones in which the mycelium compatibility was not observed. Moreover, results showed that *Rhizoctonia* comprises both multinucleate and binucleate species which are divided into anastomosis groups(AGs).

Table (6): Accession number of *R. solani* in GenBank.

No. of isolates	Isolates	Host	Colony color	AG Group	GenBank accession number
R1	*R. solani*	Cucumber	brown	AGF	OR250023
R2	*R. solani*	Mint	brown	AGF	OR250024
R3	*R. solani*	Potato/ Soil	brown	AGF	OR250025
R4	*R. solani*	bean	brown	AGF	OR250026
R5	*R. solani*	Soil	Off white	AGG	OR250027
R6	*R. solani*	pears	Pale brown	AGF	OR250028
R7	*R. solani*	citrus	off white	AGG	OR250029
R8	*R. solani*	Pepper/ Soil	off white	AGG	OR625179
R9	*R. solani*	tomato	off white	AGG	OR250030
R10	*R. solani*	strawberry	Dark brown	AG3	OR250031

Our results are in agreement with those reported by **Yang *et al*., (2015), Erper *et al*., (2016) and Yang *et al*., (2017)** who classified most combinations between *R. solani* isolates, as hyphal fusion, and or hyphae of the isolates overlapped one on top of the other to determine which anastomosis group belongs, It is essential to classify the *R. solani* isolate as part of an identified group and then pairing with the other isolates or use of molecular methods with specific primers to determine the anastomosis group. **Sneh *et al*. (1996),** reported that the anastomosis group classified the perfect fusion as one in which the hyphae cell does not die after fusion,

Table (7): Details of reference sequences that were retrieved from the GenBank database to evaluate the phylogenetic relationships of the 10 *R.solani* isolates in this study.

GenBank accession number	Anastomosis group	Host plant	Geographic origin	Isolate
OR250023.1	17RhsAGF	Cucumber	Egypt	17RhsAGF
OR250024.1	18RhsAGF	Peppermint - Mentha	Egypt	18RhsAGF
OR250025.1	19RhsAGF	Soil	Egypt	19RhsAGF
OR250026.1	20RhsAGF	Bean	Egypt	20RhsAGF
OR250027.1	21RhsAGG	Soil	Egypt	21RhsAGG
OR250028.1	22RhsAGF	Pears	Egypt	22RhsAGF
OR250029.1	23RhsAGG	Citrus	Egypt	23RhsAGG
OR625179.1	16RhsAGG	Soil	Egypt	16RhsAGG
OR250030.1	25RhsAGG	Tomato	Egypt	25RhsAGG
OR250031.1	26RhsAG3	*Fragaria x ananassa*	Egypt	26RhsAG3
DQ102403.1	AG-A	*Fragaria x ananassa*	USA	Am1
FJ440196.1	AG-A	Corn	China	YWK-83
AF354091.1	AG-B(o)	Sweetpotato	Japan	SIR-2
AF354092.1	AG-A	Soil	Japan	C-662
AY927315.1	AG-A	*Fragaria x ananassa*	Italy	R2
MF070679.1	AG-A	Potato	China	SHX-DDJJ-3
MZ569567.1	AG D	*Triticum aestivum*	USA	62C
KJ012010.1	AG-DI	wheat	China	QZ1-2-1
KX831960.1	AG-E	*Lupinus angustifolius*	Poland	HS30 AG-E
MN160708.1	AG-K	corn	USA	C2359
FJ492158.1	AG-K	*Beta vulgaris*	USA	F523
AB196652.1	AG-K	Soil	Japan	SH-10
DQ102397.1	AG-G	*Fragaria x ananassa*	USA	Gm2
AY738627.1	AG-G	*Fragaria x ananassa*	Italy	R1
AB196658.1	AG-G	*Rosa odorata*	Japan	4Wak-600
AY927325.1	AG-G	*Fragaria x ananassa*	Italy	R18
AB196647.1	AG-G	*Arachis hypogaea*	Japan	Su-1
MZ396073.1	AG-H	*Triticum aestivum*	USA	C271
DQ102430.1	AG-B(o)	*Fragaria x ananassa*	USA	RU18-1
AB219143.1	AG-B(o)	*Fragaria x ananassa*	Japan	C-302
AB286930.1	AG-Ba	*Oryzae-sativae*	Japan	Scl-2
AJ000192.1	AG-Bb	*Oryzae-sativae*	United Kingdom	IMI 062599
AB196650.1	AG-I	Artemisia	Japan	AV-2
DQ102443.1	AG-I	*Fragaria x ananassa*	USA	Im1
AB290023.1	AG-I	Sugar beet	Japan	55D21
AJ419932.1	AV-2	*Pinus sylvestris*	Finland	AV-2
DQ102441.1	AG-F	*Fragaria x ananassa*	Israel	Str47
JQ676880.1	AG2-1	Canola	Canada	R049
EU730809.1	AG2-2	lupin	Canada	R37
MW999160.1	AG-3	*Daucus carota*	Sweden	RhCaES-64
MW999167.1	AG-3	*Daucus carota*	Sweden	RhCaES-131
MZ379606.1	AG4 HGII	*Triticum aestivum*	USA	C321
MZ379598.1	AG4 HGII	*Triticum aestivum*	USA	C111
AF478452.1	AG-5	Bean	Turkey	Bean-Ankara
AB000011.1	AG-8	Wheat	Australia	A68
AF153802.1	AG-11	Soil	Australia	ZN667

The multi-nucleate species *R. solani* Kühn (teleomorph: *Thanatephorus cucumeris* (Frank) Donk.) is divided into 14 anastomosis groups: AG-1 to AG-10, AG-BI (**Sneh *et al*., 1991**), AG-11 (**Carling *et al*., 1994**), AG-12 (**Carling *et al*., 1999**), and AG-13 (**Carling *et al*., 2002**), while binucleate *Rhizoctonia* spp. (teleomorph: *Ceratobasidium* rogers) isolates are grouped into AG-A to AG-S (**Sneh *et al*., 1991).** Some *R. solani* isolates are multinucleate and fall under the

anastomosis groups AG1–AG3 3 which also differ in their virulence.

(**Kucharska *et al*., 2018**). Similarly, **(Fang *et al*.,2013**) demonstrated that some isolates of *R. solani* associated with strawberry root rot showed considerable genetic diversity and also variation in their virulence.

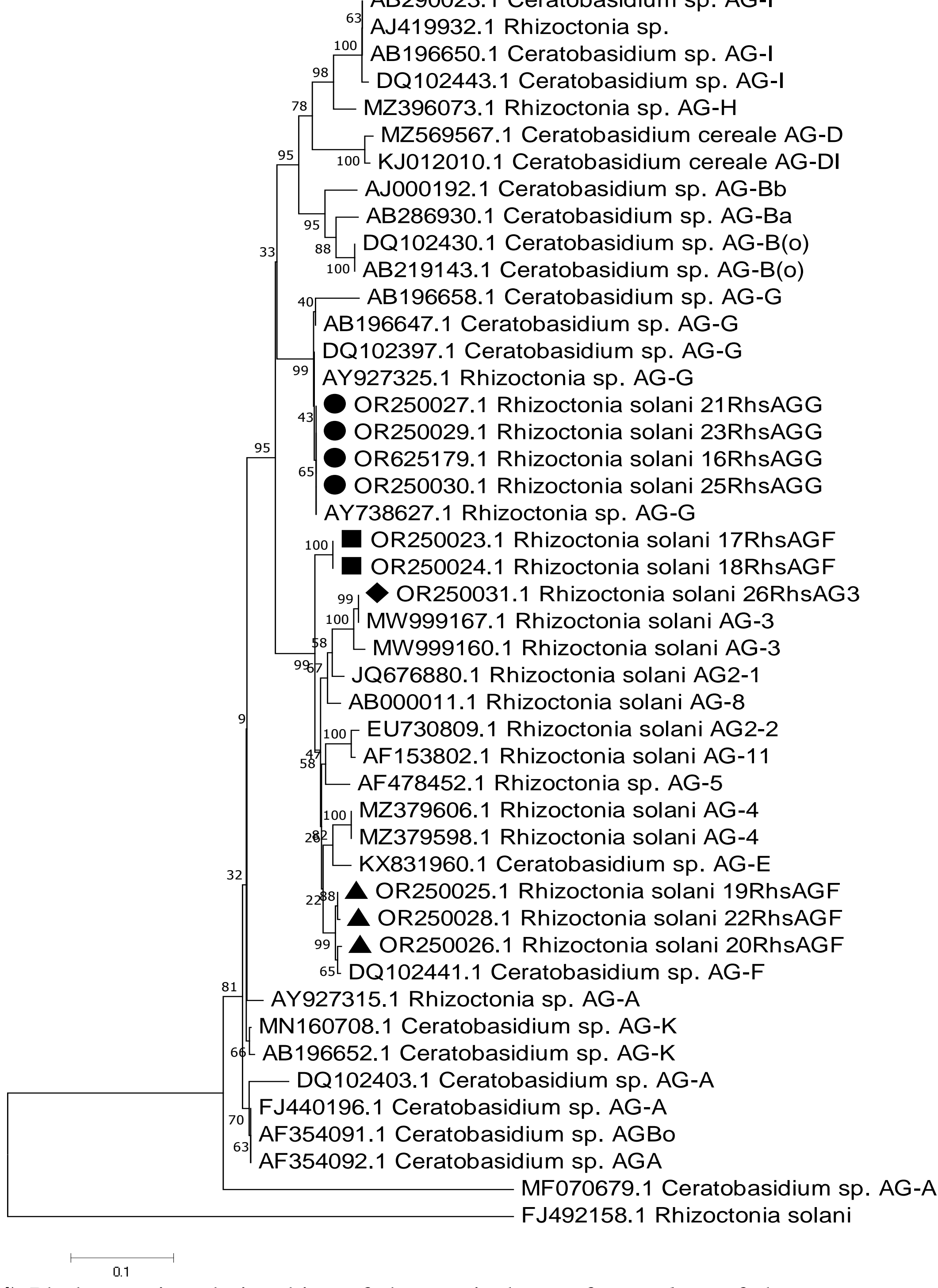

Fig (4): Phylogenetic relationships of the ten isolates of *R. solani* of the present compared with 46 reference isolates from across the world available in GenBank inferred by Maximum Likelihood analysis of the aligned ITS1, ITS4 region sequence in MEGAX.The bootstrap support from 1000 replication is indicated on the branches. Symbols indicate the location of our isolates.

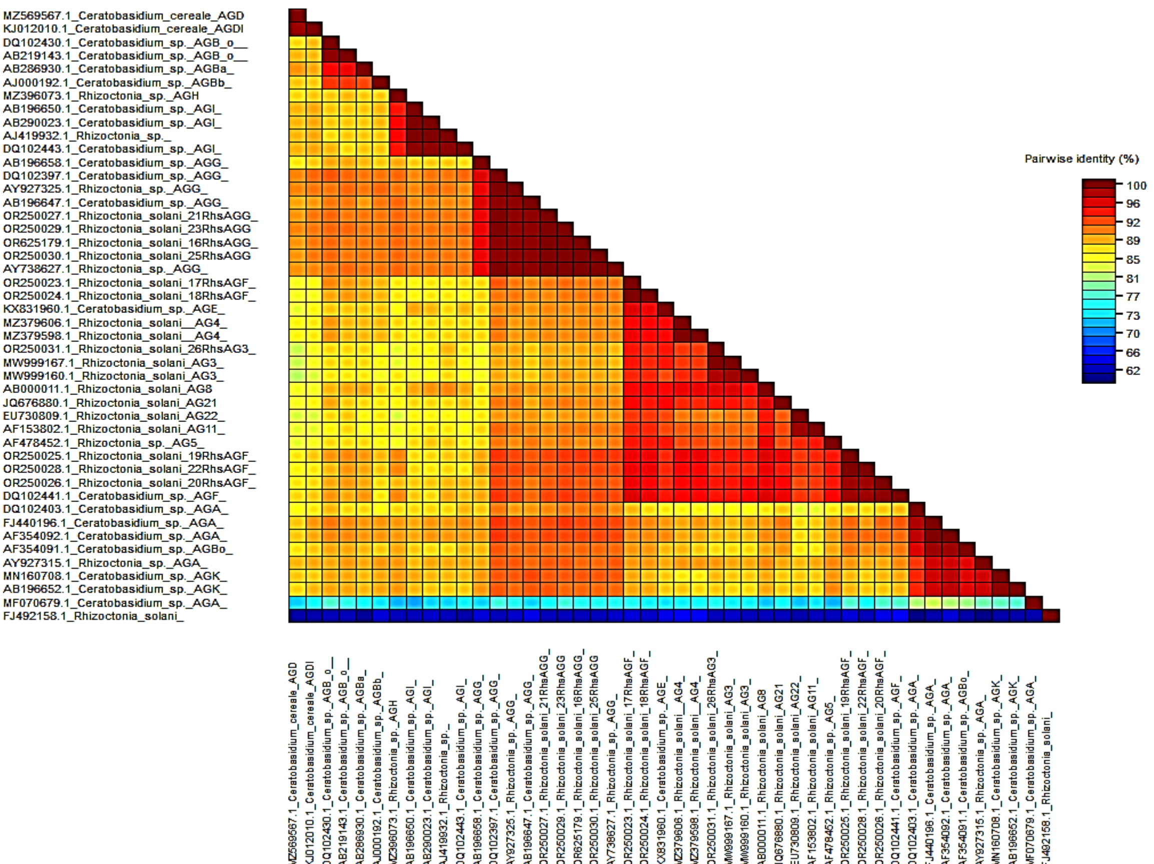

Fig (5): Genetic relationships among ten *R. solani* isolates in this study compared with 46 reference isolates from across the world available in GenBank based on the internal transcribed spacer sequences and AG group.

Data in Table (8) show that the pathogenicity test and anastomosis group were carried out to investigate the virulence of *R. solani* isolates. Results show that isolates of *R. solani* belonging to AG-G ranged between 86.7 to 33.7%, AG-F ranged between 70.4 to 34.4% and AG- 3 was 30.7%. Results show that isolates of *R. solani* belonging to AG-G and AG-F were more aggressive than AG-3 isolate.

Table 8:Pathogenicity test and Anastomosis group of *R. solani* isolates

Isolates	AG group	Binucleate (BN)/ Multinucleate (MN)	Disease incidence (%)
R1	AG-G	Binucleate (BN)	45.4
R2	AG-G	Binucleate (BN)	34.4
R3	AG-G	Binucleate (BN)	86.7
R4	AG-G	Binucleate (BN)	34.4
R5	AG-F	Binucleate (BN)	54.4
R6	AG-G	Binucleate (BN)	33.7
R7	AG-F	Binucleate (BN)	34.4
R8	AG-F	Binucleate (BN)	70.4
R9	AG-F	Binucleate (BN)	55.7
R10	AG-3	Multinucleate (MN)	30.7

4. Laboratory experiment:

4.1. Antagonistic effect of bioagents against *R. solani* under laboratory conditions: Data in Table (9) show variations in antagonism among the isolated *Trichoderma* isolates and *P. stutzeri* against *R. solani* (R3). The highest reduction in the growth of *R. solani* was recorded by

isolates T4 and T6 (72.22% and 71.11%), respectively, followed by isolate T1 which gave a 70.0% compared to the control, meanwhile, *P. stutzeri* recorded 67.77%. Many researchers demonstrated that *Trichoderma* spp. degraded the cell wall of pathogens due to the production of lytic enzymes such as chitinase, peroxidase, polyphenol oxidase and glucan 1-3 *ß*-glucosidases (**Mausam *et al.*, 2007 and Ahmed, 2013**). *Trichoderma* spp. have been widely used as an antagonistic fungal agents against several pathogenic fungi. *Trichoderma* spp., coiled around the pathogen hyphae for penetration, causing malformation and destruction for the parasitized mycelium **(Akrami and Yousefi, 2015)**. The application of *Trichoderma* spp. demonstrated the highest increase in total phenols, total nitrogen percentage and total chlorophyll of strawberries compared to the control (**Ahmed, 2017).**

Table (9): Effect of different antagonistic bioagents on the growth of *R. solani* in PDA plates 7 days after inoculation.

Treatments	Location	Linear growth, cm	Reduction %
T1	Kafr-El Sheikh	2.7	70.00
T2	Behaira	2.8	69.11
T3	Gharbiya	3.8	57.77
T4	Giza	2.5	72.22
T5	Kafr-El Sheikh	2.95	67.22
T6	Giza	2.6	71.11
T7	Giza	3.5	61.11
T8	Qaloubiya	3.8	57.77
T9	Behaira	3.3	63.33
T10	Gharbiya	2.71	67.66
Pseudomonas stutzeri	Behaira	2.9	67.77
Control		9.0	0
L.S.D. at 5%		0.7	2.7

4.2. Molecular characterization of the most effective *Trichoderma* isolate:

The most effective *Trichoderma* isolate was identified by the sequence in GenBank (T4) as *Trichoderma longibrachiatum* accession No. OM757833.

4.3. Analysis of *Pseudomonas stutzeri*:

A-Chitinase activity

Reliable chitinase activity assays must be carried out to identify *Pseudomonas stutzeri* that generate chitinase for usage in biocontrol against some plant diseases. We herein outline some of the more popular approaches. Adding chitin to a solid media and then watching as halos form around the colonies as a result of chitin degradation are some of the most basic techniques. **Kumar *et al.*,(2022)** documented that the bacterium *P. stutzeri* PSIISS-1 co-inoculated with the pathogen *R. solani* had significantly inhibited the sheath blight disease with an efficacy of 76% in susceptible paddy MTU-7029.

B-Estimation of hydrogen cyanide(HCN)

Results showed that *P. stutzeri* can release hydrogen cyanide. The positive reaction indicated that the formation of HCN was authenticated by a colour change from yellow to dark brown. The change in the colour of the filter paper from orange to brown after incubation was considered as microbial production of HCN following incubation and was interpreted as an indication of microbial production of HCN.

Discolouration of the filter paper from orange to brown after incubation was considered microbial production of HCN (Fig 6). These results are in agreement with **Wang *et al.* (2018)** who reported that *P. stutzeri* OX1 has the antagonistic behaviour against *R. solani* and displayed various plant growth-promoting (PGP) characteristics, including the synthesis of siderophores, hydrocyanic acid (HCN), and ammonia, in addition to phosphate

solubilization and the production of indole acetic acid. Among the isolates, there was a significantly higher percentage of IAA production but the isolates were moderate producers of HCN.

C-Production of Poly-β-Hydroxybutyrate (PHB)

Results show that the *P. stutzeri* strain produced the amount of PHB reaching up to 1.83 mg ml^{-1}.

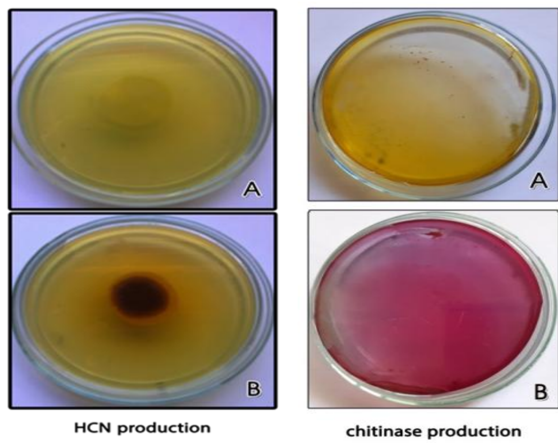

Fig (6): Production of some biocontrol substances by *P. stutzeri* strain (A: negative reaction, B: positive reaction)

4.4. Effect of essential oils on the radial growth of *R. solani in vitro*.

Data in Table (10) and Fig (7) indicate that the tested plant oils of clove and mint reduced the radial growth of *R. solani* isolate. The high concentration of mint and clove oil (1.0 %) showed a 100% and 67.77% reduction of *R. solani* growth, respectively. On the other hand, sesame oil did not show any effect on the growth of *R. solani*. These findings are in agreement with those obtained by **Arora and Kaur (1999)** who found that essential oils could be used in plant disease control as antimicrobial compounds against a range of damping-off diseases**.** The mode of action of essential oils on the fungal cell to promote the fungistatic or fungicide effect (**Juglal *et al.,* 2002).** The antifungal activities of clove essential oil at various concentrations (0, 0.5, 1, 2, 4% v/v) were evaluated in vitro against root rot and wilt pathogens. **(Thabet and Khalifa 2018**).

Table (10): Effect of three essential oils on the radial growth of *R. solani* under laboratory conditions.

Plant oils	Conc. (%)	Radial growth	Reduction%
Clove	0.25	6.7	25.55
	0.5	5	44.44
	1	2.9	67.77
Mint	0.25	3.2	64.44
	0.5	2.6	71.11
	1	0	100
Sesame	0.25	9	0
	0.5	9	0
	1	9	0
Control		9	0
L.S.D. at 5%		0.519	1.540

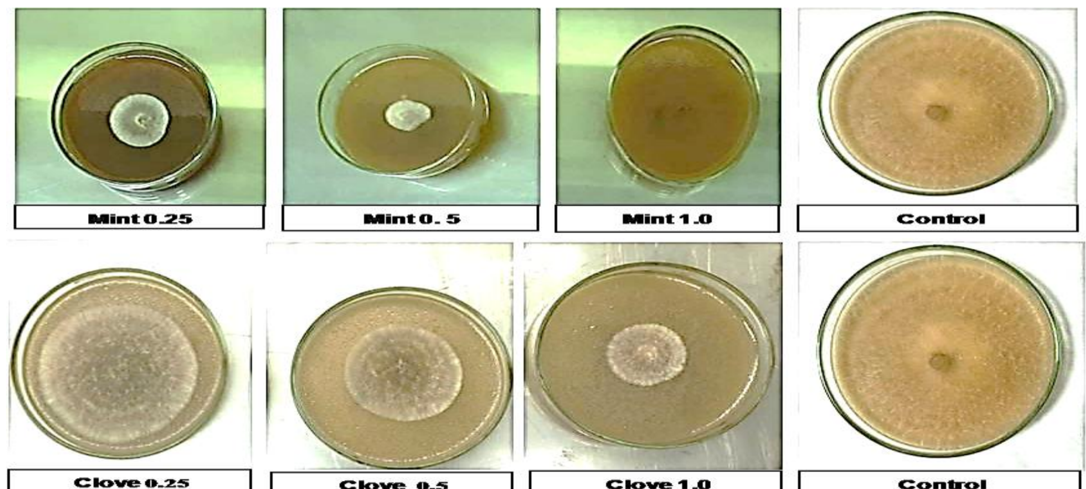

Fig (7): Effect of different concentrations of Mint and Clove oils on the linear growth of *R. solani*

5. Effect of bioagents and essential oils on strawberry roots planted in infested soil by the causals of root rot diseases under greenhouse conditions:

Results in Table (11) show that all treatments were effective in decreasing root rot disease. *T. longibrachiatum*(T4) was the most effective in decreasing disease incidence followed by mint, clove oils and *P. stutzer*i, being 5.33, 8.33, 10.67and 14.33%, respectively. Meanwhile, fungicide (Rhizolex) gave the highest effectiveness in controlling strawberry root rot, being 3.67%.

Table (11): Effect of bioagents and essential oils on strawberry infection by root rot diseases under greenhouse conditions

Treatments	Disease incidence (%)	Plant survival (%)
T. longibrachiatum (T4)	5.33	94.67
P. stutzeri	14.33	85.67
Clove oil	10.67	89.33
Mint oil	8.33	91.67
Rhizolex	3.67	96.33
Control (infected)	100	0
Control(uninfected)	0	100
L.S.D at 5%	1.525	1.9

These results may be due to the ability of some bioagents to produce growth regulators and antioxidants, which play a clear role in improving plant physiology and metabolism (**Hernandez *et al.*, 2011and Karlidag *et al.*, 2012**). **Abdenaceur *et al.*, (2022)** showed, the plant growth-promoting potential of *Trichoderma* spp. and *Pseudomonas* sp. *in vitro*, through their production of phosphatases, siderophores, hydrogen cyanide (HCN), and ammonia (NH3). In addition, a colorimetric assay was utilized to quantitatively determination of plant growth phytohormones, such as gibberellic acid and indole-3-acetic acid (IAA). *Trichoderma spp.,* react to the presence of other competitive pathogenic organisms, thereby preventing or obstructing their development. Stimulation of every process involves the biosynthesis of targeted metabolites like plant growth regulators, enzymes, siderophores, and antibiotics. (**Sood *et al.*, 2020**). Moreover *Trichoderma* spp act as mycoparasitic (**Matei and Matei, 2008**) or by the production of antifungal compounds such as endo chitinase, ß-glucosidase-1,3-glucanase or by competing for root space or nutrients (**Balode, 2009**). Whereas, plant essential oils can inhibit the pathogenic fungi by their chemical compounds which include phenolic

compounds, due to these compounds sensitize the phospholipid bilayer of the microbial cytoplasmic membrane causing an increase in the permeability and un-availability of vital intracellular constituents (**Juven *et al.,* 1994**).

6. Determination of oxidative enzymes and hydrolytic enzymes in strawberry roots.

6.1.Peroxidase activity and Polyphenol oxidase activity

Data in Table (12) indicate that the maximum increase in peroxidase activity and polyphenol oxidase activity was recorded in infected roots at 14 days with Mint oil followed by *T. longibrachiatum*, Clove oil and *P. stutzer* treatments.

Meanwhile, at 21 days after transplanting the maximum increase in both peroxidase and polyphenol oxidase activity was recorded with Mint. These results are in agreement with **Abd-El-Khair *et al.,* (2011) and Surekha *et al.,* (2014)** who found that *Trichoderma* spp. and *Pseudomonas* spp. promote the activation of defence enzymes, chitinase, peroxidase and polyphenol oxidase, which play an important role in plant defence mechanisms against pathogens infection in treated bean plants.Oxidative enzymes such as peroxidase and polyphenol oxidase enhance the formation of lignin, and oxidation of phenols to more toxic quinones, while other oxidative phenols contribute to the formation of defense barriers for reinforcing the cell structure (**Seo *et al.,* 2012**). The activity of oxidative enzymes, particularly peroxidase and polyphenol oxidase, promotes the production of lignin and the oxidation of phenols into more toxic quinones.

6.2.Chitinase activity

Data in Table (13) reveal that all treatments increased the chitinase activity after 21 days more than after 14 days. The best increase in chitinase activity was recorded at 21days by *T. longibrachiatum*. These results are in line with **Schlumbaum *et al.*, (1986), Ham *et al.*, (1991), Leah *et al.*, (1991) and Velazhahan *et al.*, (2003)** who reported that chitinases hydrolyze chitin which is the major component of fungal cell walls, leading to direct inhibition of growth of several fungi.

Table (12): Effect of peroxidase activity and polyphenol oxidase activity in roots infected with *R. solani* at 14 and 21 days after transplanting under greenhouse conditions.

Treatment	Enzyme activity			
	Peroxidase activity		Polyphenol oxidase activity	
	14days	21 days	14 days	21 days
***T. longibrachiatum* (T4)**	5.346	4.811	4.982	4.754
P.stutzeri	4.891	4.403	4.543	3.101
Clove oil	5.100	4.692	4.971	3.582
Mint oil	6.160	5.822	5.216	4.319
Rhizolex	3.415	3.112	2.100	1.067
Control (infected)	2.834	2.473	1.781	0.410
Control(uninfected)	1.637	0.531	0.610	0.372
L.S.D at 5%	0.203	0.219	0.174	0.070

Table (13): Effect of chitinase activity in roots infected with *R. solani* at 14 and 21 days after transplanting under greenhouse conditions

Treatment	chitinase activity	
	14days	21 days
***T. longibrachiatum* (T4)**	0.0224	0.0654
P.stutzeri	0.0211	0.0492
Clove oil	0.0352	0.0638
Mint oil	0.0312	0.0551
Rhizolex	0.0171	0.0212
Control (infected)	0.0202	0.0282
Control(uninfected)	0.0126	0.0236
L.S.D at 5%	0.003	0.013

7. Effect of inoculation with the tested treatments on NPK content in strawberry shoot dry biomass.

Results in Table (14) explain a maximum total plant N_2content of 54.24 mg. plant^{-1} was recorded in the inoculum of *P. stutzeri* followed by *T. longibrachiatum* with 49.9 mg, also this inoculum resulted in maximum phosphorus and potassium–contents of 15.90 and 25.81 mg. plant^{-1} for *P. stutzeri,* followed by *T. longibrachiatum* with 13.35 and 25.09 mg.plant^{-1} , respectively. It was noted that there are no significant differences between treatments *P. stutzeri* and *T. longibrachiatum* compared with the control. The results showed that there are no significant differences between the uses of oil treatments compared to control. These results are in agreement with **Mehmood *et al.,* (2023**) who clarified that numerous *Trichoderma* spp and *Pseudomonas* species use both direct and indirect methods to effectively manage diseases and control plant pathogens. Under stress, conditions were fix atmospheric nitrogen (N), solubilize potassium (K) and phosphate (p), and create secondary metabolites, lytic enzymes, phytohormones, volatile organic compounds, and antibiotics that cause inhibition of pathogen development and promote plant growth, this is inconsistent with the obtained results of **Ke *et al.,* (2019)** who reported that endophytic *P. stutzeri* is considerd as nitrogen-fixing bacteria , improved plant growth and plant nitrogen content in addition to enhancing crop yields.

Table (14) NPK contents in 45-days old strawberry plants grown in infested soil under greenhouse conditions.

Treatments	**N_2 content (mg plant^{-1})**	**P content (mg plant^{-1})**	**K content (mg plant^{-1})**
T. longibrachiatum	49.9	13.3	25.0
Ps stutzeri	54.2	15.9	25.8
Clove oil	44.4	12.1	19.7
Mint oil	44.7	11.6	20.3
Rhizolex	34.3	11.0	21.8
Control (infected)	32.1	10.6	17.1
Control (uninfected)	47.8	13.0	9.8
L.S.D at 5%	12.4	3.1	2.0

CONCLUSION

The present study shows the identification of *R. solani* isolates by sequencing the partial rDNA internal transcribed spacer revealing 99% homology between the strains. The isolate sequences clusters supported the genetic basis for the three anastomosis groups AG-G, AG-F and AG-3. *T. longibrachiatum*, Mint, Clove oils and *Pseudomonas stutzeri* were the most effective in reducing root rot disease respectively. Enzyme activities were increased with all treatments

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