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Impact of Temperature and Culture Media on the Efficacy of local strain of *Trichoderma* spp. Against *Rhizoctonia solani*

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A B S T R A C T

This study aimed to identify the species of *Trichoderma* in potato fields in Nineveh Governorate and determine their effectiveness in controlling Rhizoctonia solani, the cause of potato stem canker disease . Six isolates of *Trichoderma* were isolated from potato roots, belonging to four species: *T. hamatum, T. atroviride , T. harzianum* , and *T. longibrachiatum*, and molecular identification was confirmed using ITS1 and ITS4 sequences. Different species of *Trichoderma* showed strong antimicrobial activity against the growth of the mycelium of R. solani , as the antimicrobial percentage ranged between 81.7% and 96.2%, and the strains *T. harzianum* and *T. atroviride* recorded the highest antimicrobial percentages. *Trichoderma* culture filtrate inhibits R. solani growth 53.7% and 67.4%. *T. harzianum* was superior in colony growth on different media, its ability to lower pH and produce more spores and biomass on (PDA, Czapek-Dox Agar, YEA) and at different temperatures (25, 15, 10 °C),

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Introduction

Rhizoctonia solani is a widespread plant pathogen with a wide host range. Among the important economic crops it infects and causes huge losses is potato[1].The widespread distribution of *R.solani* in soil and the lack of specific fungicides against this fungus make it difficult to control it , in addition to the environmental risks resulting from contamination with fungicides [2].An alternative strategy to control this fungus is to use resistant varieties, but the absence of these varieties, in addition to the wide host range and its ability to remain in the soil for long periods of time in the absence of its host, gives limited results in controlling this fungus [3]. Therefore, the appropriate alternative would be biological control. In this regard, the genus *Trichoderma*, which is one of the fungi of the Hypocreaceae family, which is found in all types of soil, has been raised. *Trichoderma* was first described in 1794 by Christiaan Hendrik Persoon who isolated it from soil and decomposing organic matter [4] .This fungus is widespread in various types of soils and tolerates relatively low soil moisture levels, its optimum temperature for growth ranges between 25-30 degreesCelsius. There are about 89 species belonging to this genus. These species show a great ability to use various materials as sources of carbon and nitrogen in addition to secreting different enzymes to decompose plant polymers into simple compounds to benefit from it as a source of energy and growth [5]. *Trichoderma* species are characterized by their rapid growth and high spore production, which has allowed the production of commercial preparations for this fungus to be included in fungus control programs [6]. The growth and production of spores in *Trichoderma* species are affected by several factors, including the crop and soil type, temperature, pH, and growth medium. These factors not only affect the survival of *Trichoderma* species, but also affect their ability to combat diseases. Where the type of crop and soil affects the availability of nutrients and the rhizosphere environment, which can either support or inhibit *Trichoderma* growth. Temperature extremes can limit their activity, while the soil's pH level affects nutrient availability and enzyme function, with *Trichoderma* generally favoring slightly acidic to neutral conditions. Additionally, a nutrient-rich growth medium enhances their ability to suppress pathogens, while a poor environment may reduce their effectiveness [7] . Temperature and pH are two major factors in the growth of *Trichoderma* and its ability to produce degradative enzymes and indicated that the optimum temperatures for maximum growth will generally be between 25°C and 30°C. *Trichoderma* amount of growth depends on the species and that the best growth is achieved between 37°C and 42°C. In addition, the pH of the medium is a determining factor for the enzymatic activity of *Trichoderma* fungi and its ability to produce secondary metabolites.[8,9 ,10 ,11] .The aim of this study was to evaluate different local species of *Trichoderma* isolated from the potato roots Rhizosphere under different media and temperatures.

Materials and Methods

Isolation of the pathogenic fungus

Isolation was carried out from samples of potato stems infected with stem canker collected from potato fields in Nineveh Governorate. The samples were placed under running water overnight and then washed with sterile water. The samples were cut into small pieces [1.5-2 cm]. The pieces were sterilized in 100% sodium hypochlorite solution for three minutes, the samples were washed several times with sterile water and dried with filter paper. The samples were transferred to Petri dishes (9 cm) containing the nutrient medium (PDA) supplemented with the antibiotic amoxicillin 100 mg. L-1. The dishes were incubated at 25 °C ± 2 for five days, then the dishes were examined for fungal growth. Pure fungal cultures were identified based on morphological characteristics according to [12].

***Trichoderma* spp.**

Trichoderma species were isolated from the rhizosphere of potato roots collected from potato fields in Nineveh province. The isolation process was carried out by serial dilutions. The plates were incubated for 72 h at 25 ± 2 °C. The plates were examined for fungal growth and the fungal isolates were purified and then identified based on morphological characteristics [13,14].

Molecular Diagnosis

DNA Extraction

DNA was extracted according to the instructions of [15].The ITS region of *Trichoderma* isolates was amplified using the universal primer ITS1 and the reverse primer ITS4.

Polymerase Chain Reaction.The PCR products were directly sequenced in both directions. The nucleotide sequences were aligned and compared with the sequences of other *Trichoderma* species available in the NCBI database using BLAST.

Dual culture technique (DCT)

Reciprocal culture was performed to evaluate the antimicrobial activity of *Trichoderma* species against the pathogenic fungus *R. solani*. Discs of 0.5 cm diameter of *Trichoderma* species colonies were transferred to Petri dishes (9 cm) at a distance of 5 cm from 5 mm discs of *R.solani* colonies. The control treatment included discs of *R.solani* only. The dishes were incubated at 25 °C ± 2 for five days. Then the antimicrobial capacity of *Trichoderma* species was calculated based on the area of the *R. solani* colony using ImajeJ program. The growth inhibition of *R.solani* was evaluated using the following equation:

Existing *R. solani* inhibitors = (RC –RT)/ RC ×100
Where RC = *R.solani* colony area in control treatments, RT = *R.solani* colony area in treated treatments [16].

Efficacy of *Trichoderma* culture filtrate as antifungal

Trichoderma spp. were grown in 100 ml of PSB media and incubated at 25 ± 2 °C for 10 days. *Trichoderma* spp. culture filtrate was obtained by filtration through double layers of muslin and then centrifuged at 10,000 rpm for 15 min. culture filtrate was further filtration through Millipore filters (45 and 22µm).PDA medium supplemented with 5% *Trichoderma* spp. culture filtrate was spread in Petri dishes (9 cm) inoculated at the center with 0.5 cm discs of R.solani colony. The control treatment included PDA medium only, with 0.5 cm discs of R.solani colony. All plates were incubated at 25 ± 2 °C for 5 days. The growth inhibition of *R.solani* was calculated as described in 2.4. [17].

Biomass estimation of *Trichoderma* spp.

A liquid PDA medium was prepared in 250 ml conical flasks with 100 ml. flask. It was sterilized in an autoclave at 121°C and 1.5 bar pressure for 20 minutes. It was inoculated with a 0.5 cm diameter disk taken from the edge of a five-day-old *Trichoderma* spp. culture and incubated at 25°C ± 2 for 10 days .The cultures were filtered after the end of the incubation period through filter paper type No. 1 Whatman, which was previously weighed to separate the biomass of *Trichoderma* sp. The filter papers were dried at 70°C until the weight stabilized. The results were taken according to the following equation: Biomass weight = weight of filter paper with biomass - weight of filter paper.

Estimation of pH *Trichoderma* spp.

Culture filtrate resulting from the growth of *Trichoderma* spp. on PDA medium was used to estimate the pH in *Trichoderma* spp. culture filtrate using a pH meter.

Effect of different culture media on the growth of *Trichoderma* spp.

Three types of culture media PDA Czapec (dox) Agar Yeast extract Agar were used to study the mycelium growth of *Trichoderma* spp. Petri dishes (9 cm diameter) containing the three types of culture media were used in this test, where the center of each dish was inoculated with a 0.5 cm diameter disk taken from the edge of a five-day-old *Trichoderma* spp. colony. The results were taken by calculating the colony area of *Trichoderma* spp. using Image J program. After 24, 48, 72hours and 7 days of incubation.

Effect of different incubation temperatures on the growth of *Trichoderma* spp.

Three temperature degrees (10,15,25) were used to study the effect of incubation temperature on mycelium growth of *Trichoderma* spp. Petri dishes (9 cm diameter) containing PDA media were used in this test, where the center of each dish was inoculated with a 0.5 cm diameter disk taken from the edge of a five-day-old *Trichoderma* spp. colony. The results were taken by measuring the colony area of *Trichoderma* spp. using the Image J program after 7 days of incubation.

Statistical

Anova was used to show differences between treatments, Compare means was done by L.S.D test

Results and Discussion

Pathogen Diagnosis

Colony characteristics match those of Rhizoctonia solani, where colony colors ranged from white at the beginning, gradually turning to dark brown in varying degrees. Mycelium has many branches that are right angles from the Mycelium. microsclerotia form from Barrel-shaped cells clustered in chains.

***Trichoderma* species**

Isolation results from potato root rhizosphere showed the appearance of six isolates belonging to four species of *Trichoderma* based on the taxonomic keys prepared by Refai and on sequences of ITS1 and ITS4, molecular identification which are *T. hamatum, T. atroviride, T. harzianum*, and *T. longibrachiatum*. The species accession numbers were obtained from Gen Bank.

which are PP849533.1:1-467 *T.ibrachiatum* strain BN6, PP849414.1:1-460 *T.longibrachiatum* strain BN5, PP848184.1:1-464 *T. harzianum* strain BN3, PP814628.1:1-469 *T. atroviride* strain BN2 , PP799246.1:1-485 *T.longibrachiatum* strain BN1, PP849384.1:1-464 *T. hamatum* strain BN4.

***Trichoderma* spp. antagonistic activity**

Table (1) shows the result of Trichoderma spp. antagonistic activity species against R.solani mycelium growth. antagonism percentage ranged between 81.7 – 96.2. *T. harzianum* strain BN3 and *T. atroviride* strain BN2 record the higher significant antagonism percentage against *R.solani* mycelium growth over the rest of the species, reaching 96.2% and 91.2% for each followed by the rest of *Trichoderma* spp.*Trichoderma* spp. antagonistic activity Table (1) shows the result of *Trichoderma* spp. antagonistic activity species against *R.solani* mycelium growth. antagonism percentage ranged between 81.7–96.2.*T. harzianum* strain BN3 and *T. atroviride* strain BN2 record the higher significant antagonism percentage against *R.solani* mycelium growth over the rest of the species, reaching 96.2% and 91.2% for each, followed by the ability of *Trichoderma* species culture filtrates the rest of *Trichoderma* spp. Results also showed to inhibit radial growth of *R.solani* Inhibition percentage ranged between 53.7% to 67.4%, the highest significance percentage of inhibition recorded in *T. harzianum* strain BN3 at 64.7% and the lowest in the *T. hamatum* strain BN4 at 53.7%. followed by the rest of *Trichoderma* spp.

Table 1 In vitro assays of antagonistic *Trichoderma* species

Trichoderma species	%antagonistic activity	%antagonistic activity
T. longibrachiatum strain BN6	55.70	82.4
T. longibrachiatum strain BN5	64.44	84.5
T. harzianum strain BN3	67.4	96.2
T. atroviride strain BN62	65.55	91.2
T. longibrachiatum strain BN1	61.85	81.7
T. hamatum strain BN4	53.7	83.6
L.S.D.	3.21	5.48

Study of the growth of *Trichoderma* spp. on different nutritional media.

Tables (2,3, and 4) show the *Trichoderma* spp. colonies area grew on PDA, Czapek – Dox Agar, and YEA, media during 24, 48, 72 hours, and 7 days, incubation periods .In PDA medium Table (3) The highest average colony area (26.22 cm^2) over the incubation period was recorded in *T. harzianum* strain BN3 and the lowest average colony area (21.32 cm^2) was recorded in *T. longibrachiatum* strain BN1.The highest average colony area (47.61 cm^2) was recorded in all species after seven days of incubation. While, the highest average colony area was recorded in *T. harzianum* strain BN3 after seven days of incubation at 53.89 cm^2 which did not differ significantly from the average of *T. longibrachiatum* strain BN6 and *T. longibrachiatum* strain BN5 at 48.40 and 48.11 cm^2 respectively. IN Czapek – Dox Agar table (3) no significant differences were recorded between *Trichoderma* spp. The largest average colony area over the incubation period was recorded in *T. harzianum* strain BN3 at 22.18 cm^2 The largest average colony area was recorded for all species after seven days of incubation at 40.95 cm^2, while the largest average colony area was recorded in *T. harzianum* strain BN3 after seven days of incubation at 46.01 cm^2

Table 2. *Trichoderma* spp. colonies of area of on PDA medium estimated in cm^2 over several incubation period

Trichoderma species	Species x incubation period effect				Species effect
	24h	48h	72h	7 dyes	
T. longibrachiatum strain BN6	3.44	16.22	25.04	48.11	23.20
T. longibrachiatum strain BN5	3.89	16.97	25.29	48.40	23.64
T. harzianum strain BN3	4.03	17.93	29.03	53.89	26.22
T. atroviride strain BN62	3.22	15.41	23.78	45.70	22.03
T. longibrachiatum strain BN1	3.08	14.92	23.04	44.24	21.32
T. hamatum strain BN4	3.31	16.07	22.77	45.32	21.86
incubation period effect	3.50	16.25	24.83	47.61	

L.S.D. Species x incubation period effect :6.25 | incubation period effect : 6.42 | Species effect 6.12

Table 3. *Trichoderma* spp. colonies of area of on Czapek – Agar medium estimated in cm^2 over several incubation period

Trichoderma species	Species x incubation period effect				Species effect
	24h	48h	72h	7 dyes	
T. longibrachiatum strain BN6	2.86	14.11	21.76	41.83	20.14
T. longibrachiatum strain BN5	2.77	13.84	21.33	41.00	19.73
T. harzianum strain BN3	3.24	15.53	23.94	46.01	22.18
T. atroviride strain BN62	2.66	13.41	20.68	39.74	19.12
T. longibrachiatum strain BN1	2.52	12.98	20.03	38.48	18.50
T. hamatum strain BN4	2.54	13.05	20.14	38.68	18.60
incubation period effect	2.76	13.82	21.31	40.95	

L.S.D. Species x incubation period effect :4.45 | incubation period effect : 8.12 | Species effect 6.11

Table 4. *Trichoderma* spp. colonies of area of on YEA medium estimated in cm^2 over several incubation period.

Trichoderma species	Species x incubation period effect				Species effect
	24h	48h	72h	7 dyes	
T. longibrachiatum strain BN6	2.41	12.56	19.37	41.83	17.89
T. longibrachiatum strain BN5	2.23	11.95	18.41	41.00	16.99
T. harzianum strain BN3	2.77	13.82	21.31	46.01	19.70
T. atroviride strain BN62	2.14	11.54	17.82	39.74	16.44
T. longibrachiatum strain BN1	2.14	11.63	17.91	38.48	16.53
T. hamatum strain BN4	2.52	13.01	19.64	38.68	18.21
incubation period effect	2.37	12.42	19.08	40.95	

L.S.D. Species x incubation period effect :2.85 | incubation period effect : 6.12 | Species effect 2.01

Effect of incubation at 25,15,10˚C on the growth of *Trichoderma* spp.

Table (5) show the effect of incubation (25,15,10˚C) on the growth of *Trichoderma* spp . for seven days. Where the highest average colony area was recorded in *T. harzianum* strain BN3 48.85 cm^2, which did not differ significantly from the average colony area of T. longibrachiatum strain BN5 and *T. longibrachiatum* strain BN6 (43.29 and 42.74 cm^2), respectively. The highest average colony area was recorded for all types of *Trichoderma* when incubated 25°C after seven days of incubation (47.61 cm^2).Which did not differ significantly from the average colony area of other *Trichoderma* spp. when incubated (15 °C 44.44 cm^2), while the largest average colony area was recorded in *T. harzianum* strain BN3 when incubated 25 °C after seven days of incubation (53.89 cm^2).

Table 5. Effect of incubation temperature 25,15,10 °C on the growth of colonies of *Trichoderma* spp.

Trichoderma species	Incubation Species x temperatures effect			Species effect
	10	15	25	
T. longibrachiatum strain BN6	35.85	44.26	48.11	42.74
T. longibrachiatum strain BN5	36.46	45.01	48.40	43.29
T. harzianum strain BN3	41.47	51.20	53.89	48.85
T. atroviride strain BN62	33.32	41.13	45.70	40.05
T. longibrachiatum strain BN1	35.12	43.36	44.24	40.90
T. hamatum strain BN4	33.77	41.69	45.32	40.26
incubation temperatures effect	36.00	44.26	48.11	

L.S.D. Species x incubation period effect :6.85 incubation period effect : 4.92 Species effect 6.47

Biomass

Table (6) shows the variation of biomass of *Trichoderma* species .The highest average biomass was (0.62 g 100 ml^{-1})in *T. harzianum* strain BN3 and this species did not differ significantly from the two species *T. longibrachiatum* strain BN6 and *T. atroviride* strain BN62(0.54, 0.49g100ml^{-1}) respectively.

pH

Table (6) also show the high ability of *Trichoderma* to reduce pH, especially the species *T. atroviride* strain BN62, *T. harzianum* strain BN3 and *T. hamatum* strain BN4 with rates of (5.14, 5.34 , 5.23) respectively.

Sporulation

Number of spores produced by *Trichoderma* (Table 6).The species *T. harzianum* strain BN3 outperformed in the number of spores produced, as the logarithm of spores reached 7.81. cm^2.it was followed by *T. longibrachiatum* strain BN5 and *T. longibrachiatum* strain BN6, where the logarithm of spores reached 7.45 and 7.52. cm^2 . respectively.

Table 6. Biological characteristics of *Trichoderma* spp.

Trichoderma species	Biomass g dry weight.100 ml^{-1}	pH	*Log of spores.cm^{-2}
T. longibrachiatum strain BN6	0.54	6.19	7.52
T. *longibrachiatum* strain BN5	0.45	6.17	7.54
T. harzianum strain BN3	0.62	5.34	7.81
T. atroviride strain BN62	0.49	5.14	7.33
T. longibrachiatum strain BN1	0.46	5.37	7.29
T. hamatum strain BN4	0.42	5.23	7.19

L.S.D. Biomass 1.14 pH 0.18 Log of spores 0.28

The effect of different temperatures and culture medium on the ten *Trichoderma* spp. isolates was observed. Excellent growth of *Trichoderma* was found at temperatures between 25-30 °C. Growth and sporulation were evaluated. Ten *Trichoderma* isolates were tested on four different media to evaluate growth and reproduction. Maximum mycelial weight was found on potato dextrose medium (276.00 mg100 ml^{-1}) and less on Czapek-Dox medium (96.00 mg100 ml^{-1}). [18,19].The effect on the growth and sporulation of fungi depends on several factors. Temperature, light, micro and macro chemical components of the growth medium under laboratory conditions .*Trichoderma viride* was able to grow on all tested media. Malt yeast extract agar was the most suitable medium for growth. Moreover, yeast extract acted as a growth promoter .The results showed that the stationary growth phase of *T. viride* occurred after 9 days with the onset of the declining phase after 11 days of incubation .It is necessary to consider the growth of fungi separately as they exhibit different growth patterns or modes. Generally, the maximum growth ranges between 10-35°C and the optimum between 20-30°C. [20]. When evaluating the growth of *T. harzianum* in different culture media potato dextrose agar, modified potato dextrose agar, water agar, carrot agar, and corn flour agar. The linear growth was

recorded at 24 h intervals after inoculation and the average growth rates were calculated. Wet weight and dry weight.[21] The highest linear growth, fresh weight and dry weight were recorded in potato dextrose agar and the lowest growth was recorded in water agar. This was followed by modified potato dextrose agar which was statistically similar to carrot agar which was different, followed by cornmeal agar.[22,23].The increase in biomass indicates the ability of *Trichoderma* spp. to exploit the environment well. Which enhances the chances of its survival in its biosphere, growth and sporulation .It also enhances its chances of parasitism, antagonism, and analysis of organic materials and increases the availability of nutrients and activates plant growth regulators by increasing the number of plant cells that it inhabits.[7,24].The decrease in the pH value is due to the ability of *Trichoderma* spp. to produce organic acids. Especially in environments where the carbon source is simple sugars. This is of great importance in increasing the availability of some nutrients in the soil, especially phosphorus, as these acids work to dissolve it and increase its availability and then facilitate it for the plant directly through its absorption from the roots or indirectly through *Trichoderma* sp. and then obtain it after the death of fungal cells in the rhizosphere or the colonization of the plant root cells. The acidic environment created by *Trichoderma* spp. in the rhizosphere increases the efficiency of ionic and cationic transport of elements necessary for plant growth, in addition to the fact that low pH inhibits the growth of pathogenic bacteria in the roots [25 ,26] The high sporulation of *Trichoderma* spp. gives a good advantage to the biological resistance agent in increasing the chances of success of its uses in biological control by increasing its spread and long duration of survival .[27] (Hariharan *et al.,* 2022)

Conclusion

The study showed the effectiveness of local *Trichoderma* isolates, especially *T. harzianum* BN3, in inhibiting *R.solani* growth, the isolates varied in growth and spore production under the influence of different media and incubation temperatures.

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