



## **Biological Control of *Sclerotium rolfii* by Using Indigenous *Trichoderma* spp. Isolates from Palestine**

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### **Abstract:**

The bioagent, *Trichoderma* species are known antagonists of other fungi, and have been shown to be very efficient biocontrol agents of several soil borne plant pathogenic fungi. Forty-seven local *Trichoderma* isolates were isolated from one hundred sixty nine soil samples from irrigated fields in the West Bank by using dilute plate techniques on *Trichoderma* selective media (TSM). The antagonistic potential of the local isolates against the phytopathogenic *Sclerotium rolfii* was investigated in dual culture and bioassay on bean plants. Application of testing isolates as a conidial suspension ( $3 \times 10^6$ ) greatly reduced the disease index of bean plants in different rates by a percentage of 67%. The results revealed that the variation of antagonistic potential between isolates was due to the variation in mycelium-coiling rate, sporulation, fungitoxic metabolites, induced growth response and temperature effect. The results showed that Jn14 and T36 were the most effective isolates at 25 °C and inhibited *S. rolfii* mycelial growth at a percentage of 79% due to fungitoxic metabolites production. Sporulation of the isolates reached a peak at 30 °C and decreased at 35 °C. The maximum absolute conidia production was  $1.5 \times 10^9$  conidia / ml by the isolate Jn14 at 30 °C. The Effect of *Trichoderma* on bean seedlings growth was obvious; height was nearly doubled (160% - 200%), while fresh and dry weight were increased in plant by 133% - 217% respectively. Germination of bean seeds treated with *Trichoderma* isolates occurred about four days earlier than those in untreated soil.

**Key words:** : Biological control, *Trichoderma*, *Sclerotium rolfii*.

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## المكافحة الحيوية لمرض العفن السكليريوسيني باستخدام عزلات فلسطينية من فطر التريكوديرما

### الملخص:

فطر التريكوديرما من الفطريات القاطنة في التربة، ينتمي إلى مجموعة الفطريات الناقصة (Deuteromycetes) الرمية. أوضحت الأبحاث العلمية إمكانية استخدامه في مكافحة الحويّة للأمراض النباتية القاطنة في التربة. في هذه الدراسة تم الحصول على 47 عزلة مختلفة من فطر التريكوديرما (*Trichoderma*) عزلت من 169 عينة تربة جمعت من الحقول الزراعية المروية والمزروعة بالمحاصيل المختلفة باستخدام طريقة (Dilute Plate Technique) على وسط غذائي خاص بفطر التريكوديرما.

تم دراسة مقدرة العزلات المختلفة من فطر (*Trichoderma*) لمكافحة مرض العفن الأبيض السكليريوسيني (*S. rolfsii*) باستخدام طريقة التداخل الفطري (Dual Culture) ومعاملة نباتات الفاصوليا الكاملة (Bioassay). أظهرت نتائج دراسة التداخل الفطري بين عزلات فطر التريكوديرما مع فطر (*S. rolfsii*) بأن اقوى العزلات كانت عزلة (J10) ومعدل نموها 3.25 ملم<sup>2</sup> / يوم. أظهرت نتائج استخدام عزلات التريكوديرما كمعلق يحتوي ابواغ الفطر بتركيز ( $3 \times 10^6$  /g soil) تم إضافتها الى التربة لمكافحة مرض العفن السكليريوسيني على نباتات الفاصوليا حيث أدت أقوى العزلات Jn14 إلى تقليل شدة الإصابة بالمرض بنسبة 67%، زيادة معدل نمو النباتات إلى الضعف وتبكير أنبات بذور الفاصوليا بأربعة أيام. وأوضحت نتائج دراسة تأثير درجات الحرارة على عملية التداخل بين أقوى العزلات والفطر الممرض بأن درجات الحرارة المثلى تتراوح بين 25-30 درجة مئوية. وكذلك تم دراسة آلية عمل فطر التريكوديرما على الفطر الممرض؛ ووجد بأن للفطر التريكوديرما المقدرة على التطفل على الفطر الممرض وأنتاج المثبطات الفطرية عند تربيته على بيئة البطاطا المغذية السائلة (PDB). وأظهرت النتائج بأن المثبطات المنتجة من العزلة Jn14 عند مزجها بنسبة 10% مع بيئة البطاطا المغذية الصلبة (PDA) قللت معدل نمو الفطر الممرض بنسبة 87%، أما المثبطات المنتجة من العزلة T36 قللت معدل نمو فطر الممرض بنسبة 94.7% على درجة حرارة 25م°. وكذلك أظهرت النتائج بأن عزلة التريكوديرما Jn14 لها مقدرة عالية على إنتاج الأبواغ ( $1.5 \times 10^9$  conidia/ml) على درجة حرارة 30م°.

### Introduction:

Biological control of plant diseases has been the subject of extensive research in the last two decades. *Trichoderma* spp. is well documented as effective biological control agents of plant diseases (Harman et al. 1980, Sivan et al., 1984 and Coley-Smith et al., 1991). Control

of soil borne plant pathogens including *Sclerotium rolfsii* can be achieved by different fungicides, soil fumigants (Methyl bromide) and bioagents. Because of the concern regarding the toxicity of these compounds, there is a general trend to reduce the amounts

applied to soil. *Sclerotium rolfsii* is an economically important pathogen on numerous crops worldwide. It has an extensive host range; at least 500 species in 100 families are susceptible, the most common hosts are legumes, crucifers, and cucurbits, and commonly occurs in the tropics, subtropics, and other warm temperate regions (Punja, 1985). The biological control of the pathogen on bean plants by using *Trichoderma* spp and/or in combination with other techniques had been investigated (Hadar et al. (1979), Elad et al. (1980& 1981), Harman et al. (1980), Lewis and Papavizas (1984), Aziz et al (1997). The objective of the current research was aimed to isolate the bio-agent *Trichoderma* in Palestinian agricultural fields and to evaluate its potential in controlling the soil-borne pathogen *Sclerotium rolfsii*.

## Material and Methods:

### Bioagent Isolation:

One hundred sixty nine soil samples were collected from thirty locations at eight districts in Palestine-West Bank (Hebron, Jericho, Jenin, Tulkarim, Qalqelia, Ramallah, and Bethlehem) during summer 2000. One-liter soil samples were collected from each of irrigated vegetable fields and fruit-tree orchards at a depth of 5-10 cm of soil surface. The samples were collected from open, plastic covered field's solarized or chemically treated (with fungicides such as Dynone and/or Benlate or fumigated with methyl bromide in present or previous agricultural seasons). Each soil sample from the same

site was placed in a plastic bag and mixed thoroughly by external manipulation by hand through the bag. *Trichoderma* were isolated from soil samples, as follows: 25 g of soil samples was suspended in 225 ml of 0.1% agar water. Samples were shaken for 20-30 minutes on a rotary shaker at 250 rpm. Serial dilutions 10-1, 10-2, 10-3, and 10-4 were made for each soil sample and aliquot 0.1 ml of soil suspension dispensed onto *Trichoderma* - selective media (TSM) surface with a glass rod (Elad et al., 1981). The plates were incubated at 25 °C for 5-7 days. After incubation, *Trichoderma* were identified from other fungi based on color, size, shape, and appearance of colony on surface of TSM and then it was transferred to a potato dextrose agar (PDA) medium for purification and further identification. For each soil sample and suspension concentration, 5 plates were considered as replicates.

*Trichoderma* - Selective Media (TSM) consisted of the following components (g/l DW): magnesium sulfate heptahydrate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ), 0.2 ; potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ), 0.9; potassium chloride (KCl), 0.15; ammonium nitrate ( $\text{NH}_4\text{NO}_3$ ), 1.0; glucose, 3.0; chloramphenicol (Chloromycetin, Sigma Chemical Co., USA), 0.25; dexton (p-dimethylaminobenzenediazo sodium sulfonate 60% W.P, (Bayer, Germany), 0.3; pentachloronitrobenzene (Terracolor 75% W.P, Olin Chemicals, USA.) PCNB, 0.2; rose bengal (Tetrachlorotetradiodfluorescein, BDH Chemicals Ltd., England), 0.15 and agar, (Difco Laboratories, USA), 20 (Elad, et al., 1981)

## Mycelial growth rate:

Mycelial growth rate of *Trichoderma* isolates was determined as follows: Four Petri dishes (90mm diameter) containing PDA were centrally inoculated with a 5-mm of agar plugs from 7-day-old PDA cultures of each *Trichoderma* isolate to determine the average linear growth of each isolate. Plates were incubated at 25 °C under continuous light and inspected daily for three consecutive days. Radial mycelial growth was recorded every 24 hours during this period. The colony diameter was measured as the mean of two perpendicular diameters measured at the third day minus the diameter at first day. Average linear growth rates (ALG) were calculated by using formula:

$$\text{ALG (mm /day)} = [C3 - C1] / T$$

Where C3: colony diameter in mm after three days, C1: colony diameter in mm after one day of incubation and T: the difference in time (day).

The experimental design used was a completely randomized with four replicates (plates) for each treatment.

The effect of temperatures on linear growth for most effective isolates (H2, J8, Jn14, Jn21, Q27, T33, T36, N38, R42, and Egy.52) on PDA supplemented with Chloramphenicol (250 mg/L) under continuous fluorescent light was evaluated. Ninety mm diameter Petri dishes were inoculated with 5mm agar plugs from 5-day-old PDA cultures of isolates mentioned above. Plates were randomly incubated at different temperatures including 10, 15, 20, 30, 35, and 40 °C with four plates (replicates) per each isolate. Radial

mycelial growth was measured as the mean of two perpendicular diameters after 48 hours minus that after 24 hours of mycelial growth and calculated in cm<sup>2</sup>/day. At the end of mycelial growth study, the plates of all isolates were incubated for 1 more week and used for the assessment of conidia production. Conidias were harvested by flooding the plates with 10 ml of distilled water and then agitated with a glass rod. The resulting suspensions were filtered through a layer of sterile filter papers and conidia concentrations from the four plates/ temperature were counted using a haemocytometer under microscopic power of 40X (Zhang & Watson, 1997).

## Evaluation of antagonistic potential

### Dual culture interaction:

Dual culture interaction between the pathogenic fungi, *S. rolfsii* which isolated locally from bean plants and the *Trichoderma* isolates (47 isolates) were studied. A 5-mm diameter mycelial block 7-days-old cut from the margin of each *Trichoderma* isolates and of pathogen colonies placed 3 cm apart on the PDA medium surface. Five Petri dishes were used for each isolate. The plates were incubated at 25 °C under continuous light and inspected daily for approximately 8-9 consecutive days for mycoparasitism. The fungi colony margins would meet 2-3 days after inoculation. The area interaction of mycoparasite and the host was measured every 24 hours after contact (Dennis & Web-

ster, 1971 a&b). The experiment was conducted as completely randomized design with five replicate (Petri dishes) for each isolate.

The most effective isolates (H2, J8, Jn14, Jn21, Q27, T33, T36, N38, R42, and Egy.52) and *S. rolf sii* was repeated studied based on the method of Prasun & Kanthadai (1997). Four replicates of 90 mm diameter PDA plates were inoculated with 7mm mycelial discs of 7-day-old *Trichoderma* isolates in addition *S. rolf sii* at a distance of 3 cm apart from each other. The plates were incubated at 15, 20, 25, 30, and 35 °C for 3-5 days in closed polyethylene bags lined with moist tissue papers (to prevent desiccation of the media), and observed regularly for ability of one fungus to restrict the growth, or to overgrow the other. The experimental design was completely randomized with four replicates (plates) for each treatment.

### Bioassay:

The screening for antagonistic potential was studied using the method of Mihuta and Rowe (1986). Forty-seven local isolates of *Trichoderma* and another five isolates (three isolates from Egypt and two isolates from the Hebrew University) were tested in growth chamber for antagonisms to *S. rolf sii*. They were grown on PDA medium at 25°C under continuous fluorescent light. After 10 days of incubation, conidia were harvested from cultures by flooding the plates with 10 ml of sterile distilled water and removed by agitation with sterile bent glass rod and poured into sterile test tubes and agi-

tated for 15 sec. with vibrating agitator. The resulting suspensions were filtered through a layer of sterile tissue papers and conidial concentration in the suspension was determined with a haemocytometer and sterile distilled water was added to bring concentration to  $3 \times 10^7$  conidia / ml. Four milliliters of each suspension was added to pots containing a 0.5-kg sandy soil previously autoclaved at 121°C for 1 hr on three successive days. The inoculated sandy soil was incubated for seven days at 25 °C and then mixed thoroughly 0.1g sclerotia of *S. rolf sii*. The sclerotia were collected from the plates and dried for three days in incubator at 30°C. Each plastic pot (10 cm diameter) filled with non-autoclaved sandy soil to 2/3 of pot volume and then seeded with six bean seeds. The final mixture containing pathogens and bioagent was filled the last 1/3 volume of pots which were previously seeded with bean seeds. The experimental design used was completely randomized with five replicates (pots) for each *Trichoderma* isolate. The control soil containing pathogen without the bioagent (*Trichoderma* isolates). Plants were harvested after three weeks of seeding in growth chamber at 25 °C under a 12-hr photoperiod. All seedlings were uprooted, and hypocotyls were evaluated for infection by *S. rolf sii* on a scale from 1 to 4: (1= symptomless, 2= small lesions with no hypocotyl constriction, 3= large lesions with some hypocotyl constriction, and 4= hypocotyl girdled).

These ratings were converted to a disease index (DI) value for each pot by using the formula:

$$DI = 30 * (1A + 2B + 3C + 4D) / N$$

where A, B, C, and D represent the number of seedlings rated as 1, 2, 3, or 4 respectively; 30 – the number of seeds planted; and N – the number of seedlings rated after three weeks.

## Taxonomy identification:

*Trichoderma* isolates were grown on PDA in 90-mm Petri dishes, and plates incubated at 25 °C for 7 days. Isolates were placed into groups according to colonial morphology, conidiation color, and color of reverse colony. Sterile glass cover slips, 50 x 25 mm, were held with forceps and immersed in autoclaved melted water agar (20g Difco agar / 1L distilled water) at about 45°C for 1-2 second, and allowed to drain. The cover slip was then laid singly on the surface of 2% solidified water agar in center of 90-mm diameter Petri dishes, so that a thin film of agar set on the upper surface (Laing & Deacon, 1991). Each plate was inoculated with a 5 mm plug cut from the edge of 7 days old growing colonies of each *Trichoderma* isolates on potato dextrose agar (PDA). The plug is then placed 2cm apart of the cover slip placed on the agar surface, so that *Trichoderma* colony would grow across the coated cover slips. Plates containing coated cover slips were incubated at 25 ± 2 °C and inspected daily for four days for *Trichoderma* mycelium growth. Each cover slip was removed carefully without damaging the fungus mycelium, and then it was inverted on sterile microscopic slide (24.4 x 76.2) and sealed by nail varnish

to prevent drying. The growing isolates were studied using fresh direct mounts in Lactephenol cotton blue under medium and high magnifications, x20, and x40, respectively. The top of the cover slip was cleaned, and microscopic observations were made throughout the coated cover slip and thin film of agar. Three replicates were used for each *Trichoderma* isolate.

## Mode of Action:

### A) Antibiosis:

The ability of *Trichoderma* sp. to inhibit the mycelial growth of *S. rolfii* through production of fungitoxic metabolites at different temperatures was tested according to the method mentioned by Dennis & Webster (1971 a&b). Fifty ml of potato dextrose broth (PDB) pH 6 in 250 ml Erlenmeyer flasks was inoculated with 7mm-agar disk from 7-day-old PDA cultures of three *Trichoderma* isolates J8, Jn14, and T36 and incubated at 25°C without shaking. After 10 days of incubation, the cultures were filtrated through Millipore membrane filter (0.45 µm) and were autoclaved at 121°C for 15 minutes. The culture filtrate (1.2 ml) was placed in Petri dishes (90-mm diameter) and approximately 12 ml of PDA was added and mixed with the filtrate (10% v/v). The filtrate-amended PDA plates were then centrally inoculated with 7-mm mycelial plugs of *S. rolfii*. Plates were incubated at 15, 20, 25°C with four replicates; unamended PDA served as control. The linear growth rate of *S. rolfii* was measured after 48 hour and calculated as cm<sup>2</sup> /day and percent inhibition was



calculated. The experimental design was completely randomized with four replicates (plates) for each treatment.

## B) Hyphal interaction on thin films of agar:

Hyphal interaction was made on sterile glass cover slips coated with 2% water agar (20g Difco agar / 1L distilled water). Each cover slip was immersed for 1-2 sec in autoclaved melted water agar at about 45 °C, allowed to drain and then placed on the surface of 2% solidified water agar in a 90mm diameter Petri dishes, so that a thin film of agar set on the upper surface. Five mm disk of one week old growing colonies cut from the margin of each of *S. rolfsii* and *Trichoderma* isolates were placed 3 cm apart on the agar surface and then incubated at 25 °C. Cultures were inspected daily for mycoparasitism; host and mycoparasite colony margins should meet across the coated coverslips in less than three days. Each cover slip was removed carefully without damaging the mycelial contact and then it was inverted on a sterile microscopic slide (25.4 x 76.2). Microscopic examination was carried out through the coated cover slip using fresh direct mounts in Lactophenol cotton blue under medium and high magnifications x 20, x40, respectively. Specimens were always sealed by nail varnish to prevent drying (Laing & Deacon , 1991). Mycoparasitism was indicated by hyphal coiling and internal colonization of the host hyphae (*S. rolfsii*) by the mycoparasite *Trichoderma*.

## Induce growth response (IGR):

The ability to induce and increase the emergence of seedling, increase plant height, and dry weight were tested. Most effective fifteen *Trichoderma* isolates (H2, H3, H4, J8, J9, Jn14, Jn18, Jn21, Q26, Q27, T36, T37, N38, R42, and B47) were used. The isolates were grown on plate's (90-mm in diameter) containing PDA for 10 days at 25 °C under illumination. Conidia were harvested from the cultures, washed several times in water, and suspended in 0.001% Tween 20 (Polyoxyethylene-sorbitan, Sigma- aldrich. Com.) (Chang et al., 1986). These suspensions were added to the peat moss growth substrate at a concentration of  $5 \times 10^6$  conidia per gram of soil and incubated for 14 days at 25 °C. After 14 days, six bean (*Phaseolus vulgaris*) seeds were sown per pot and five replicates were employed. Plants were grown in a growth chamber at 25 °C for 4 weeks. The various measurements of plant growth responses were made including number and time of emergence of seedlings. Plant heights were measured from soil surface to apical buds. Concerning fresh and dry weights, plants were washed under running tap water to remove soil from roots; plants were then dried at 80 °C in drying oven after recording fresh weights. After 72hr, plant dry weights were determined (Shenker et al., 1992).

## Statistical analysis:

The results of all experiments were analyzed statistically using One-Way Analysis of Variance (ANOVA) to test for significance, and the Fisher Test was used for mean separations by SigmaStat Software (1999).

## Results:

### *Trichoderma* spp. Isolates:

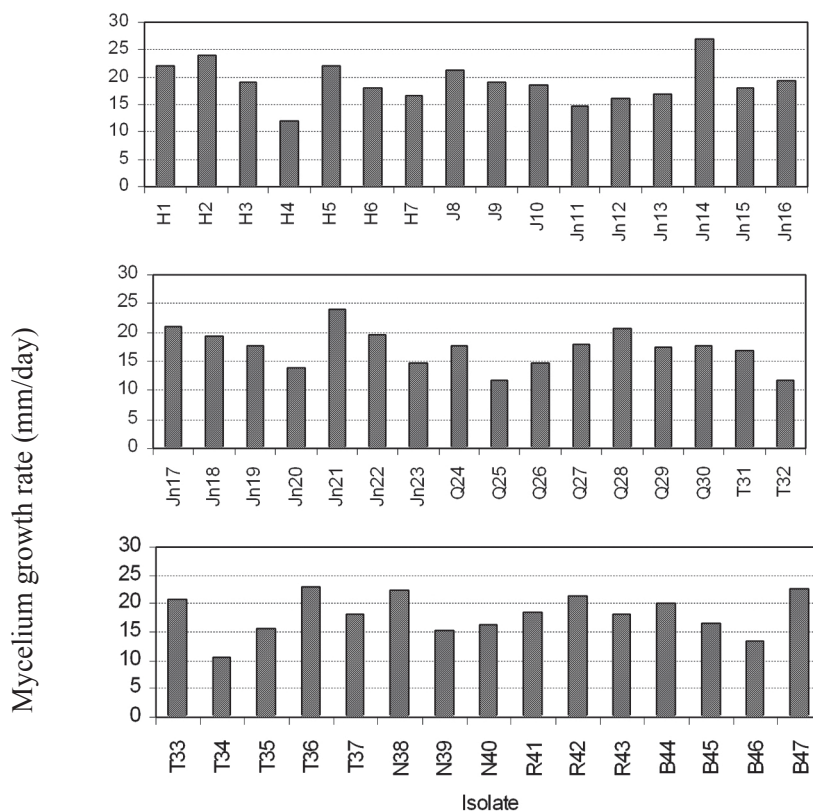
Forty-seven isolates of *Trichoderma* species were recovered from 169 soil samples collected from different locations in the Palestinian areas of the West Bank during the period between April and September 2000. The numbers of *Trichoderma* isolates recovered were 7, 3, 13, 7, 7, 3, 3, and 4 *Trichoderma* from Hebron, Jericho, Jenin, Qalqilya, Tulkarem, Nablus, Ramallah, and Bethlehem, respectively, obtained from the rhizosphere of different soil types. The average percentage of *Trichoderma* isolates recovered to soil samples collected was 27.8%. The percentage of isolates recovered to soil samples in respect to location were 28%, 12.5%, 40.6%, 35%, 38.9%, 20%, 15%, and 26.7% in Hebron, Jericho, Jenin, Qalqilya, Tulkarem, Nablus, Ramallah, and Bethlehem, respectively. Of the 47 isolates recovered, Forty-four isolates were recovered from irrigated vegetable fields fertilized with manure and solarized in current or previous season; two isolates were recovered from fields planted with grape and banana in Jericho, and one isolate from field planted with thyme in Ramallah. In addition, fourteen isolates out of the forty four

isolates were collected from soil fumigated with methyl bromide and/or drenched with fungicides Dynone and Benlate in present or previous agricultural seasons (H2, H3, J9, Jn12, Jn17, Jn18, Q27, Q28, Q29, Q30, T31, N38, B46, and B47). Eleven isolates were recovered from greenhouses (H2, H3, H7, Jn12, Q27, Q28, Q29, Q30, N38, B46, and B47). The *Trichoderma* isolates were named as (X B). Where X: the first letter of the name of district area where the isolate was collected and B: the serial number of the isolate.

### Mycelial Growth Rate (Average Linear Growth):

The mean of average linear growth rate for isolates ranged from 10.67 mm/day for isolate T34 and 27.0 mm/day for isolate J14. Results of radial mycelial growth rate of the local *Trichoderma* isolates growing on PDA are presented (Fig. 1) The radial mycelial growth rate between local isolates varied according to the tested isolate. The highest radial mycelium growth rate was 27.0 mm/day for the isolate Jn14 later identified as *Trichoderma harzianum*. The effect of temperatures on mycelial growth rate (MGR) and sporulation of the most effective *Trichoderma* isolates was tested. The results indicate that reached a peak in MGR at 30 °C and was completely inhibited at 40 °C (Fig. 3). The variability between the isolates was significantly observed at 30 °C. The isolate Jn14 (*Trichoderma harzianum*) recorded a growth of 16.75 cm<sup>2</sup>/day at 30 °C and was clearly the highest while

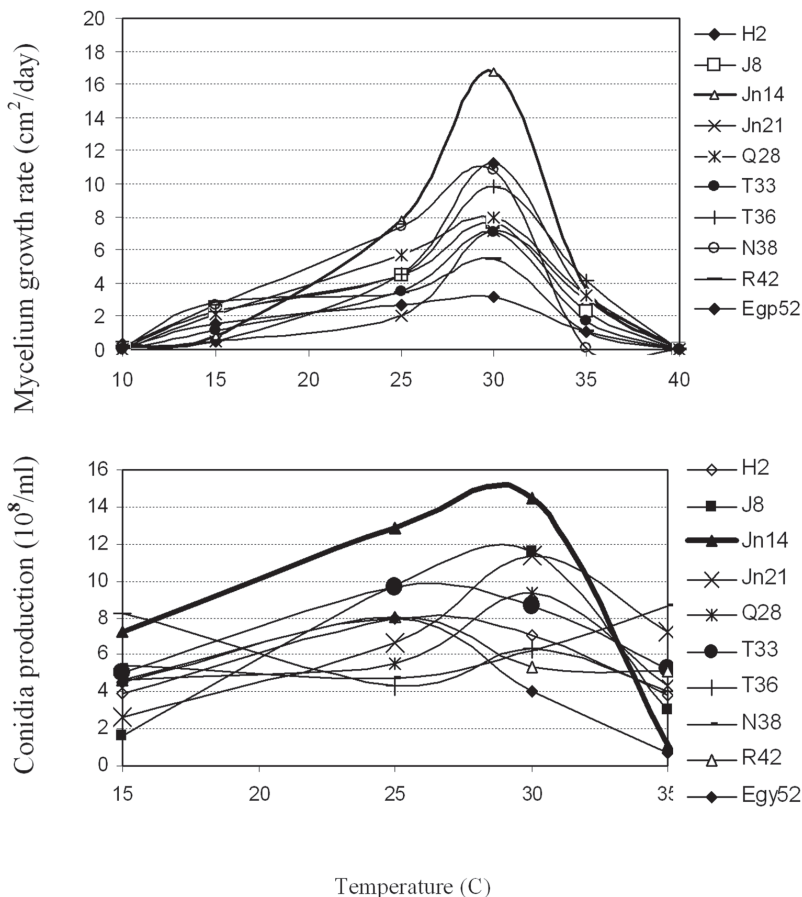




**Fig.1 :** Mycelium growth rate of *Trichoderma* isolates (mm/day) growing on PDA medium incubated at 25 C.

the lowest measurements were recorded by the isolate Q28 (*Trichoderma hamatum*) at 10 °C (Fig 3). Conidia production occurred at temperatures between 15 and 35 °C. Conidia production was significantly affected by temperatures. Sporulation of the isolates reached a

peak at 30 °C and declined at 35 °C. The highest conidia production rate ( $1.44 \times 10^8$ ) conidia / ml was recorded by the isolate Jn14 (*Trichoderma harzianum*) at 30 °C and the lowest value was recorded by the isolate Egy52 at 35°C ( $0.74 \times 10^8$ )conidia / ml (Fig 3)



**Fig.3 :** . Effect of temperature on mycelium growth rate (cm<sup>2</sup>/day) (A) and conidia production (B) of *Trichoderma* isolates (H2, J8, Jn14, Jn21, Q28, T33, T36, N38, R42, and Egy52) growing on PDA medium and incubated at different temperatures (10, 15, 25, 30, 35 and 40°C).

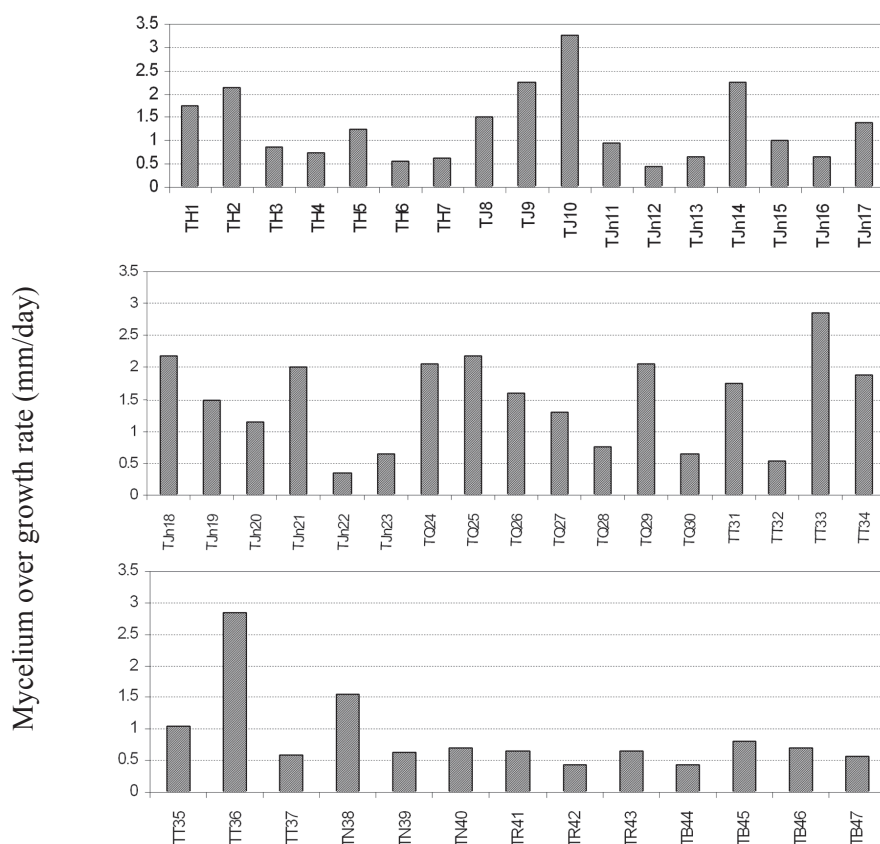
### Mycoparasitism in dual culture:

The ability of local *Trichoderma* isolates to inhibit the mycelial growth of *S. rolfii* in dual culture was determined on PDA medium. Results of mycoparasitism in dual culture for all *Trichoderma* isolates are presented in (Fig. 2). By 24 hr after interaction between mycelia of the bioagent *Trichoderma* isolates and

the pathogen mycelia, a clear zone of interaction was formed on PDA media. The mean overgrowth rate of *Trichoderma* isolates on *S. rolfii* ranged from 3.25 for isolate J10 and 0.35 for isolate Jn22 and the most effective isolates were (J10, T36, and T33). The results of interactions showed that there is a statistically significant difference in the overgrowth rate between the different isolates on the *S. rolfii* (Fig. 2).

Results of the effect of temperatures on the interaction between *Trichoderma* isolates mycelia and the pathogen mycelium showed that there was a statistically significant difference between groups (LSD= 0.279) (Table 1). The interaction between *S. rolf sii* and the *Trichoderma* isolates studied were highly dependent on temperature. The mean overgrowth rate of *Trichoderma*

isolates on *S. rolf sii* ranged from 0.17 mm/ day for isolates N38 and Egy52 at 35 °C and 30 °C, respectively, and 2.4 mm/day for the isolate Jn14 at 25 °C (Table 1). A clear zone of inhibition was formed between the two fungal colonies at 25 and 30 °C after 48 hours of contact in the isolate N38 when used against *S. rolf sii*.



**Fig.2 :** . Mycelium overgrowth rate (mm/day) of *Trichoderma* isolates on phytopathogen *S. rolf sii* in dual culture both growing on PDA medium and incubated at 25°C.

**Table 1:** An overgrowth rate (mm/ day) of *Trichoderma* isolates (H2, J8, Jn14, Jn21, Q28, T33, T36, N38, R42, and Egy52) on *S. rolfii* growing on PDA medium in dual culture at different temperatures (15, 25, 30, and 35 °C).

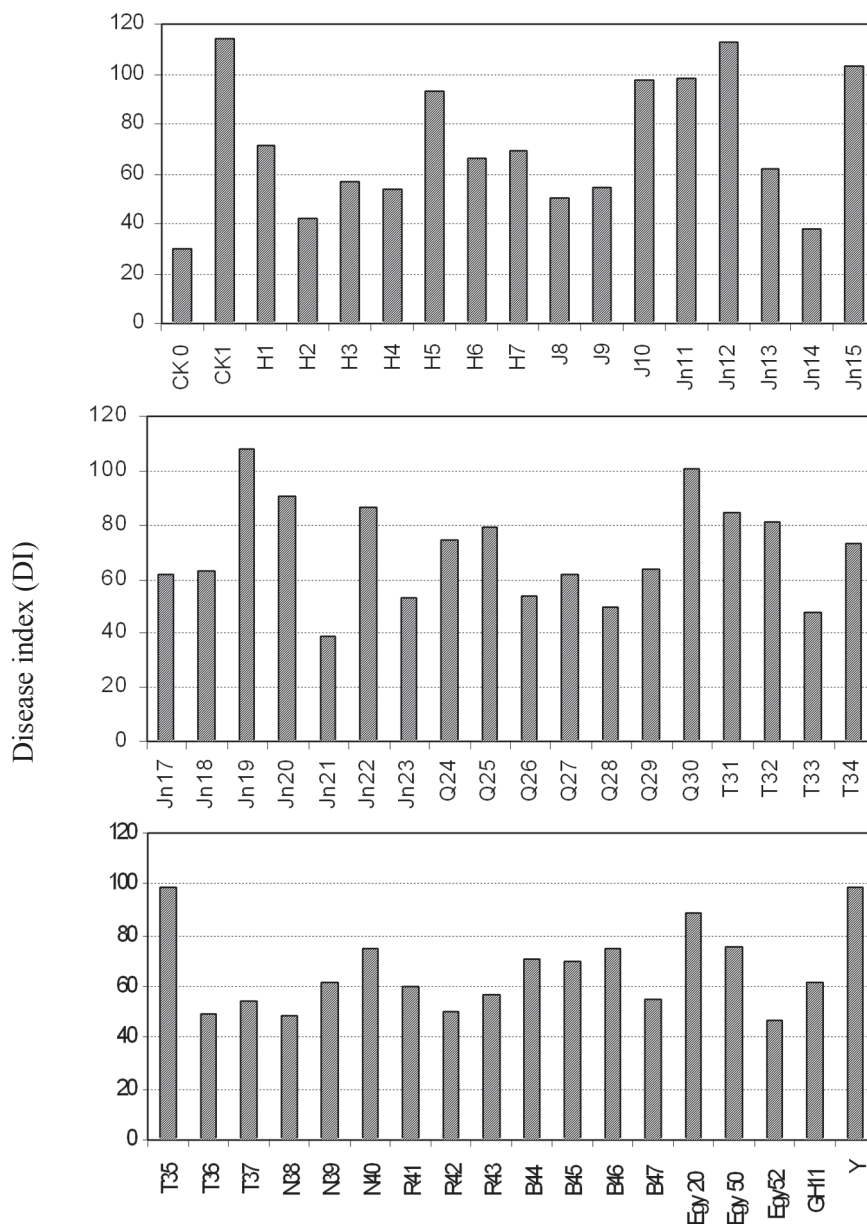
Isolate	15 °C	25 °C	30 °C	35 °C
H2	0.63* f	1.08 d	0.63 f	0.25 g
J8	0.71 f	1.08 d	0.96 e	0.37 g
Jn14	0.38 g	2.42 a	1.88 b	1.25 d
Jn21	0.54 f	0.38 g	0.50 f	0.29 g
Q28	0.67 f	0.37 g	0.33 f	0.21 g
T33	1.08 d	0.67 f	0.63 f	0.21 g
T36	0.71 f	1.63 c	0.83 e	0.29 g
N38	1.21 d	0.71 f	0.25 g	0.17 h
R42	0.21 g	0.17 h	0.21 g	0.38 g
Egy52	0.42 g	0.54 f	0.17 h	0.21 g

\* Mean of four replicates  $\pm$  standard deviation; values followed by the same letter within columns or rows are not significantly different according to Fisher LSD test (LSD= 0.279,  $P \leq 0.05$ ). The *Trichoderma* isolates were named as (X B). Where X: the first letter of the name of district area where the isolate was collected and B: the serial number of the isolate.

## Evaluation of antagonistic potential in bioassay:

The tested *Trichoderma* isolates reduced disease index of bean plants in different rates; means of disease index ranged from 37.8 to 114 (Fig. 4). *Trichoderma* isolates significantly reduced disease index (LSD= 23.585). Forty three *Trichoderma* isolates significantly reduced disease index by 20.8%-66.8%. The most effective isolates Jn14 (*Trichoderma harzianum*), Jn21 (*Trichoderma pseudokoningii*), and H2 (*Trichoderma lactea*) reduced disease index by 66.8%, 65.8%, and 63% respectively. The other nine isolates (Jn12, Jn19, Jn15, Q30, T35, Y, Jn11, J10, and H5) reduced disease index by 1.3%-18.5%. Results in this study showed that most effective local *Trichoderma* isolate (Jn14) (*Trichode-*

*rma harzianum*) reduced disease index more than those *Trichoderma* isolates from Hebrew University (GH11) and Egypt (Egy52); isolate GH11 reduced disease index by 45.7%, and isolate Egy52 reduced disease index 58.9%.



**Fig.4 :** The effect of *Trichoderma* isolates on diseases index caused by *S. rolfisii* on bean plants.

# Taxonomy identification of *Trichoderma* Isolates:

Most effective Local *Trichoderma* fungal isolates (Table 2) were taxonomy identified according to their conidial morphology, color and texture, and

growth characteristics. Microscopic examination was carried out according to Bissett (1984, 1991 a, b, c) classification method (Table 2).

**Table 2:** Identification of the local *Trichoderma* isolates (H2, J8, Jn14, Jn17, Jn21, Q28, T33, T36, N38, R42, and B47).

Isolate	<i>Trichoderma</i> species
H2	<i>Trichoderma lacteal</i>
J8	<i>Trichoderma viride</i>
Jn14	<i>Trichoderma harzianum</i>
Jn17	<i>Trichoderma atroviride</i>
Jn21	<i>Trichoderma pseudokoningii</i>
Q28	<i>Trichoderma hamatum</i>
T33	<i>Trichoderma citrinoviride</i>
T36	<i>Trichoderma hamatum</i>
R42	<i>Trichoderma koningii</i>
B47	<i>Trichoderma citrinoviride</i>

The isolate (H2), *Trichoderma lacteal* was characterized by limited aerial mycelium, white in color and arachnoid; culture reverse was dull yellow in color. Conidiation effuse, and white; conidiophores hyaline, straight and stout, wide near the base, unbranched in the lower part, irregularly branched toward the apex. Phialides subulate, arising singly or in whorls. Conidia ellipsoid to ovoid, it belongs to *Trichoderma* section *Hypocreanum*. The isolates (J8, Jn17, Jn21, T33, R42 and B47) belonged to *Trichoderma* section *Longibrachiatum*. Species in this section were characterized by sparsely branched conidiophores having a high proportion of solitary phialides, in addition to the production of characteris-

tic bright yellow-green pigments visible in the colony reverse. The isolates (T33 and B47) were identified as *T. citrinoviride*; conidia were ellipsoid and smaller than in the other species, conidiation formed yellow-green shades or was dark olive in older cultures. The isolate (Jn17) identified as *T. atroviride*, has relatively large, ellipsoidal conidia and a very sparse branching system with curved or sinuous conidiophores branches and phialides. The isolate (J8) identified as *T. viride* has conidiophores with side branches relatively long and rebranched several times; branches often curved or sinuous. The isolate (Jn21) identified as *T. pseudokoningii* has conidiophores branching similar to *T. citrinoviride*; colonies with conidial

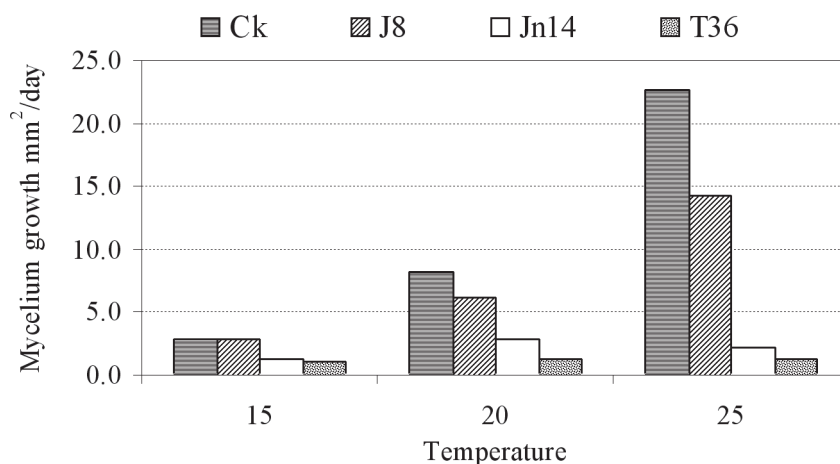


areas widely effused and not forming pustules; conidiation form bluish-green shades and not darkening appreciably in age. *T. koningii* (isolate R42) was characterized by phialides arising predominantly in false whorls and does not produce intercalary phialides. The isolates (Jn14, Q28 and T36) belonged to section *Pachybasium* which is characterized by broad or inflated conidiophores elements and phialides, which give the conidiophores a stout or rigid appearance. Phialides were ampulliform, divergent, and arranged in crowds on terminal branches of conidiophore that are repeatedly branched and rebranched at an indefinite number of levels. In addition, many species have conspicuous,

sterile elongation of the conidiophore main axis.

### Antibiosis:

The growth rate of *S. rolfsii* was reduced significantly at 20 °C and 25 °C; Growth rate inhibition percentages at 25 °C were 37.4%, 90.5% and 94.7% when it was grown on PDA amended medium with 10% of PDB containing metabolites produced by the isolates J8, Jn14, and T36, respectively. Looking at variation in inhibition of mycelial growth of *S. rolfsii*, T36 was the most effective isolate and reduced the pathogen mycelium growth at the temperatures 15 °C, 20 °C, and 25 °C by 64.5%, 84.1%, and 94%, respectively (Fig. 5).



**Fig.5 :** .Mycelium overgrowth rate (mm<sup>2</sup>/day) of *S. rolfsii* growing on PDA medium amended with metabolites produced by the *Trichoderma* isolates (J8, Jn14, T36) and incubated at different temperatures (15, 20, and 25 °C).

## Hyphal interaction on thin films of agar:

The *Trichoderma* isolates (H2, J8, Jn14, Jn17, Jn21, T33 and T36) showed identical mode of action during interacting with *S. rolf sii* on water agar films. After contact, the hyphae of *Trichoderma* grew along the pathogens hyphae; sometimes the main hyphae coiled around the host or produced short branches that tightly surrounded the host hyphae. Dense coiling around host hyphae and internal growth within the host mycelium had commonly been seen during interaction between hosts (*S. rolf sii*) and *Trichoderma*; disintegration of the host cell wall was observed as well. When *Trichoderma* hyphae reached the older portions of the pathogen colony, they were seen to be growing inside the host hyphae. At this stage most of the host cells were already lacking cytoplasm (Inbar et al., 1996). Microscopic examination had also revealed that the isolate Jn14 (*T. harzianum*) was found to be a very efficient mycoparasite during interaction on thin film of agar. Coiling of *Trichoderma harzianum* (Jn14), *T. hamatum* (T36), and *T. Pseudokoningii* (Jn21) hyphae around *S. rolf sii* hyphae was noticed on 40x microscopic magnification. Internal colonization by local *Trichoderma* isolates Jn14, T33, and J8 was abundant and well-developed throughout the *S. rolf sii* mycelium. Five types of hyphal interactions were observed in this study: Coiling around the host hyphae; extensive coiling around the host hyphae; penetration of the host hyphae; penetration of the host hyphae and lacking cytoplasm of the host cells; and subsequent lysis of the infected hyphae.

## Induce growth response (IGR):

*Trichoderma* isolates were applied to soil as conidial suspension. The results showed that there was a significant increase for each of the parameters measured (plants emergence, plant height, plant fresh, and plant dry weight) in bean seedlings, 4 weeks after sowing compared to the non-treated seedlings (Table 3). Bean seedlings treated with *Trichoderma* isolates J8 and T36 increased in height by 160 to 200%, respectively. In addition, seedlings treated with the isolates Jn14 and Jn21 increased in fresh weight in the range of 133 % to 217%, respectively. There was no significant difference, however, in respect to plant dry weights within treatments. Germination of bean seeds planted in soils treated with *Trichoderma* isolates mentioned above occurred about four days earlier than those planted in untreated soil. Germination of seeds planted in treated soil increased in the range of (16.7% - 55.6%) compared to the control. The enhancement was induced by the isolates Jn14 and T36 (55.6%) after the 7 days.

**Table 3:** Effect of local *Trichoderma* isolates on bean plant growth response (PGR).

Isolate	Seedlings emergence (Average of seedling no.)		Plant height (cm)	Plant weight	
	3 days	7 days		Fresh (g)	Dry (g)
Ck	0.00 <sup>a</sup> f	3.6 b	18.3 <sup>a</sup> c	5.9 d	0.59 d
H2	1.6 d e	4.6 a g	29.9 b	10.4 b	1.04 b
H3	1.5 e	4.75 a	31.0 b	10.6 b	1.07 a
H4	1.6 d e	4.4 b	34.7 a	11.1 a	1.11a
J8	1.75 d	4.25 b	29.8 b	8.9 b	0.89 b
J9	1.0 e	4.2 b	29.8 b	8.8 b	0.88 b
Jn14	3.4 c	5.6 a	36.7 a	12.9 a	1.28 a
Jn18	2.0 d	4.2 b	29.7 b	11.2 a	1.12 a
Jn21	1.8 d	5.2 a	32.1 b	8.4 c	0.84 c
Q27	1.6 e	4.8 a	32.5 b	11.8 a	1.18 a
Q28	1.6 e	4.8 a	33.4 a	11.9 a	1.19 a
T36	2.2 d	5.6 a	36.8 a	12.7 a	1.27 a
T37	0.6 f	4.6 a	29.7 b	9.5 b	0.95 b
N38	2.2 d	5.2 a	34.7 a	11.0 a	1.10 a
R42	2.4 d	5.2 a	34.7 a	9.8 b	0.98 b
B47	1.2 e	4.6 a	34.0 a	10.9 a	1.09 a

\* Mean of five replicates; values followed by the same letter within columns are not significantly different according to Fisher LSD test ( $P \leq 0.05$ ). # LSD of Seedling emergence = 0.998 , Plant height = 4.190, Plant weight (Fresh) = 2.232 and Plant weight (Dry) = 0.223. The *Trichoderma* isolates were named as (X B). Where X: the first letter of the name of district area where the isolate was collected and B: the serial number of the isolate.

## Discussion:

The bioagent *Trichoderma* spp. is presence in the Palestinian studied areas that are irrigated and cultivated with different crops. Forty-seven *Trichoderma* isolates were obtained from 169 soil samples; five isolates (H2, Q29, N38, B46 and B47) were isolated from soil fumigated with methyl bromide in the same or previous agricultural season. Similar results were observed by Munnecke *et al.*, 1981 who reported that *Trichoderma* spp. is the most com-

mon antagonist to appear after soil fumigation with methyl bromide and it can reproduce rapidly. Also, Strashnov *et al.* (1985) found that combination of *T. harzianum* and methyl bromide enhanced proliferation and establishment of the antagonist in soil. The isolates (H2, H3, J9, Jn12, Jn17, Jn18, Q27, Q28, Q29, Q30, and isolate T31) were recovered from soil irrigated by fungicide Dynone® in the same or previous agricultural season, while the isolates

Jn12 and Q28 recovered from soil irrigated by fungicides Dynone® and Benlate®. The rest isolates were recovered from non treated soils.

Results showed that the contact between *Trichoderma* isolates and *S. rolfsii* in dual culture after 3 days. Not all *Trichoderma* isolates grew on *S. rolfsii* colony except the isolates (H1, H2, J8, J9, Jn14, Jn21, T34, and N38). The isolates (Q24, Q25, Q26, Q29, and T31) grew toward *S. rolfsii* colony and stopped its growth before reaching it, forming a clear zone. The results revealed that *Trichoderma* isolates (H2, J8, Jn14, Jn17, Jn21, T33 and T36) parasitized the hyphae of *S. rolfsii*. The isolates hyphae of (Jn14, Jn21, and T36) grew over the pathogen and formed branches that coiled around them. The penetration and growth of isolates (Jn14, T33, and J8) inside the hyphae of *S. rolfsii* was observed. Similar observations have been reported for the *Trichoderma harzianum* and *Sclerotinia sclerotiorum* interaction by Inbar et al. (1996). The antagonistic ability of *Trichoderma* isolates is highly variable (Chet et al., 1979), as was shown in this study in which only 5.77% of the *Trichoderma* isolates tested were effective in controlling *S. rolfsii*. In the bioassay studies, the most effective isolates were (Jn14, Jn21, T33, T36, H2, and R42). Application of *Trichoderma* isolates as a conidial suspension greatly reduced disease index. The ability of *Trichoderma* to reduce diseases caused by soil borne pathogens is well known and it is related to the antagonistic properties of *Trichoderma*, which involve parasitism and lysis of pathogenic fungi and

/or competition for limiting factors in the rhizosphere mainly iron and carbon (Sivan & Chet, 1986). Another mechanism has been suggested by Kleifeld and Chet (1992) and related to *Trichoderma*-induced resistance in host plants to fungal attack. Increased growth response of several plants including vegetables, following the application of *Trichoderma* to pathogen-free soil has been documented (Baker, 1989; Chang et al., 1986; Kleifeld and Chet, 1992). In this study, bean seeds which were planted in *Trichoderma* treated soils germinated earlier by 4 days than those planted in nontreated soils in addition to better emergence rate. In relation to this, Yedidia et al. (2001) suggested that a 30% increase in cucumber seedling emergence was observed up to 8 days after sowing when soil was amended with *T. harzianum* propagules. This can be explained as expensed by (Kleifeld and Chet, 1992) by the ability of *Trichoderma* to inhibit minor pathogens in the rhizosphere which might induce seed rots and preemergence damping off. Furthermore, seedlings grown in *Trichoderma* treated soils recorded higher values of plant heights and weights. This agrees again with the work of (Kleifeld and Chet, 1992). Some investigators reported that the increased growth response caused by *Trichoderma* isolates resulted in large increase in root area and root lengths and may be related to the effect on root system. These results were similar to the results revealed by (Yedidia et al., 2001) who showed that treatment of cucumber plants in soil with *T. harzianum* (T-203) resulted in large in-

crease in root area and cumulative root lengths, and significant increase in dry weight, shoot length and leaf area over that of the untreated control. Yedidia *et al.* (2001) suggested a direct role for *T. harzianum* in mineral uptake by the plant at a very early stage of the fungal-plant association. In addition, Harman (2000) established that *Trichoderma* spp. are opportunistic plant colonizers that affect plant growth by promoting abundant and healthy plant roots, possibly via the production or control of plant hormones. Increased growth response has been demonstrated by several other investigators (Altomare *et al.*, 1999; Anusuya and Jayarajan, 1998). They demonstrated the ability of *T. viride* and *T. harzianum* to solubilize insoluble tricalcium phosphate in vitro. The optimum temperature for mycelium growth rate and conidia production of most effective isolates was 30 °C and MGR ranged from 0.02 cm<sup>2</sup> for the isolate (Q28) and 16.75 cm<sup>2</sup> for the isolate (Jn14). These results agree with the work of Prasun and Kanthdai (1997). They found that the optimum temperature for *Trichoderma* sp. was 25-30 °C. In addition, Chet (1990) reported that the optimal temperature for *Trichoderma* growth is around 28 °C and growth is very slow below 18 °C. The effect of temperature on the interaction between the bioagent and the pathogen was evaluated as well. *Trichoderma* overgrew *S. rolfii* at 25 °C and 30 °C in dual culture. Similar results were demonstrated by Prasun and Kanthdai (1997). In contrast to the interaction in dual culture, results revealed that the growth rate of *S. rolfii*

was reduced due to the production of fungitoxic metabolites produced by *Trichoderma* isolates at different temperatures. The growth rate of *S. rolfii* was reduced significantly at 20 °C and 25 °C by the isolate (Jn14). The isolate (T36) was the most effective isolate when used against *S. rolfii*; it reduced the pathogen mycelium growth at temperatures 15 °C, 20 °C, and 25 °C. Similar results were observed by Brasun and Kanthdai (1997) who reported that *Trichoderma* (isolate Td-1) produced higher concentration of fungitoxic metabolites at higher temperatures and it effectively suppressed the growth of *S. rolfii* at or below 33 °C.

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