TRICHODERMA MEDIATED CONTROL OF ROOT-KNOT NEMATODES ASSOCIATED WITH PEANUT PLANT

ABSTRACT

Peanut is one of the most world wide cultivated oil crop. Whenever it is cultivated, it is venerable to attack with nematodes. Biological control represents a safe environmentally, friendly and promising measure for limiting the loose caused by root-knot nematodes. Four isolates related to genus *Trichoderma* were randomly selected from about 50 isolates obtained from different soils. These isolates were able to reduce viability of nematodes in laboratory experiment. Their metabolites were toxic to free living and pathogenic nematodes. *Trichoderma viride* parasitized nematodes and finally killed them. When this isolate was applied to the infected field, it dramatically reduced the number and size of galls on peanut roots comparing to non treated soil. Total count of nematodes (free living and plant pathogenic) was minimized greatly due to application of *T. viride* to the soil. On the other hand, peanut canopy and yield increased significantly in *Trichoderma* treated soil. In all the peanut vigor increased due to the use of *Trichoderma.*

Key words: *Trichoderma,* root knot nematodes, biological control, protease, peanut, parasitism

INTRODUCTION

Peanut (groundnut; *Arachis hypogaea* L.) has been cultivated for more than 3500 years, (Singh and Simpson, 1994). It is one of the most important legume crops for several millions of people in the world and is a valuable cash crop for small-scale farmers in developing countries. It is one of the world's most popular and universal crops, cultivated in more than 100 countries all over the world .China, India, Nigeria and USA are the largest producers **(Weiss 2000)**. Peanut seeds are characterized by high contents of oil (40-50%), protein (20-40%), and a low percentage of carbohydrates (10-20%) **(Ahmed and Young, 1982),** making peanuts an important component in the daily diet of small holder subsistence farmers around the world. Peanuts have a variety of uses, including human food (roasted, boiled, cooking oil), animal feed (pressings, straw, seeds), and industrial raw materials (Soap, detergent, cosmetics) **(Maiti, 2002).** A large percentage of the world production of peanuts is used for edible oil. Also used for human food **(Ahmed and Young, 1982),** the principal uses being peanut butter, peanut candy, in-shell, and shelled nuts.

Many factors that limit production of peanut have been identified. The most prominent are root and foliar pathogens. Nematodes especially root knot, represent a destructive pest of peanut. There are numerous important plant parasitic nematodes reported worldwide, but root knot nematodes are the most economically-significant group of these sedentary endoparasites of roots and tubers **(Hussey and Janssen, 2001)**, causing serious problems on many cultivated plants. Within the root-knot group, *Meloidogyne* species (*M. incognita, M. javanica, M. arenaria, and M. hapla*) are responsible for yield loss in many crops **(Kokalis-Burelle *et al.*, 1997 and Hussey and Janssen, 2001)**. Nematodes were first identified on peanuts **in South Africa in 1926(Arant, 1951). Martin, (1958)** observed *Meloidogyne arenaria and M javanica* on pegs, pods and roots of peanut damaged by root-knot nematodes. Failure

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to respond normally to fertilizers and slow recovery from wilting are signs of root-knot nematode infestation. Peanut producers rely on crop rotation and nematocides for controlling of root-knot nematode in peanuts. However, chemical control has many disadvantages that lead to the search for other most reliable and globally accepted alternatives. Biological and physical means could be used safely to control phytopathogenic nematodes. The advantages of biological pest management are the safety handling, high degree of host specificity and the self perpetuation subsequently a less frequent need of reapplication.

Fungi can protect plant against nematodes through production of nematocide-antagonistic metabolites. The production of these metabolites is well documented phenomenon in different filtrate of fungi such as various rhizosphere fungi **(Alam *et al.*, 1973), *Trichoderma virens* Miller, Giddens & Foster (Meyer *et al.*, 2000), *Trichoderma harzianum* Rifai (Sharon *et al.*, 2001)**.The genus *Trichoderma* is cosmopolitan soil born fungus found on decaying wood and other forms of plant material. It exists in climates ranging from the tundra to the tropics. This may be attributable to their diverse metabolic capability and aggressive competitive nature **(Samuels 1996; Klein and Eveleigh 1998)**. The potential of *Trichoderma* species as biocontrol agents for plant diseases was early recognized **(Weindling, 1934).**

The aim of this study was to examine potentiality of *Trichoderma* to control root-knot nematodes associated with peanut root. Also to clarify the mechanism of action exhibited by this fungus.

MATERIALS AND METHODS

Isolation and identification of biocontrol agents

Soil and compost samples were collected from different sites. Plate dilution method as described by **Waksman, (1927) and Johnson *et al* (1960)** was adopted for isolation of *Trichoerma* spp. Specific *Trichoderma* modified E medium (TME) for *Trichoderma* spp. **(Papavizas and Lumsden 1982)** was used for cultivation of *Trichoerma*. Collected isolates were identified according to **Rifai, (1969).**

Preparation of fungal metabolites, spore suspension and extraction of nematodes.

Flasks containing potato dextrose broth (PDB) were inoculated with 1 ml of *Trichoderma* spore suspension and incubated on shaker for 7 days at 25°C. Liquid fungal cultures were filtrated and centrifuged at 5000 rpm for 10 min **(Meyer *et al.*, 2000)**. Supernatant was sterilized through 0.22 μm millipore filter, aseptically transferred to sterile 20 ml screw cap glass bottles and kept at 4°C until use.

Spore suspension was prepared from 7 days old Potato dextrose agar (PDA) and adjusted to 7×10^5spores ml^{-1} using haemacytometer.

Nematodes were extracted from composted soil sample (250 g) using sieving and Baermann pan technique (Barker *et al.*, 1985).

In vitro screening of nematicidal activity of *Trichoderma.*

Four *Trichoderma* isolates (viz, two *Trichoderma viride*, one *T. harzianum*, one *Trichoderma* sp) were *in vitro* screened for their activity against nematodes.

Fungal culture filtrate, fungal spore suspension or their combined mixture were tested in sterile perti dish (50mm). One ml of either preparation was added to10 ml of nematodes suspension (mixed stages). Three replicate for each treatment beside two controls of sterile distilled water and sterilized PDB were prepared. *Trichoderma* activity was assessed by recording the percentage of mobile and immobile nematode after different exposure time (2, 4 and 7 days). Per microscope field. The experiment was repeated twice.

Effect of different dilutions of *Trichoderma* culture filtrate

Culture filtrates of the most effective species (*T. viride* and *T. harzianum*) were tested at different dilutions. cultural filtrate were prepared as described before and diluted with sterile distilled water to give final dilution of (1: 3, 1: 1, 3: 1) and 100%. One ml of each single dilution was transferred to sterile petri dishes (50 mm) containing 10 ml of nematodes suspension (mixed stages) three replicates for each dilution beside two controls (sterile distilled water and sterilized PDB) were prepared. Percentage of mortality was recorded after 48 h.

Protease assay

Trichoderma spp.were grown in 100 ml flasks containing 25ml Czapex broth neither contains sucrose nor $NaNO_3$ but substituted with 1% gelatin. The broth was prepared in citrate phosphate buffer at pH 4.6, inoculated with 1 ml T.s.s and incubated at 25°C for 5 days on shaker. Medium was filtered through Whatman No. 1 filter paper followed by sterilization through 0.22 μm millipore filter. Filtrates of the four isolates were kept at 4°C until use.

Enzyme activity was measured by cup plate assay method (Dingle *et al,* 1953). Diameter of the clear zone (mm) was taken as a measure of activity. Five replicates for each isolate beside boiled enzyme extract (as control) were made.

Proteolytic activity of *Trichoderma* was measured Spectrophotometery according to **Monreal, and Reese, (1969).** Briefly one milliliter of 2% casein solution in phosphate buffer (*M* /20, pH 7.0) was incubated at 50 °C with 1 ml of crud enzyme, appropriately diluted with buffer. After one hour, 2 ml of protein precipitant solution (0.1 *M* trichloroacetic acid + 0.2 *M* sodium acetate), were added, shacked well and the tube was then place at 40 °C for 20 minutes. The mixture was centrifuged and filtered through Whatman No.1 paper. To 1 ml filtrate 5 ml of Na_2Co_3 (0.4 *M*) were added and left for 20 minutes at room temperature. One ml of Folin –Ciocalteau reagent (1*N*) was added to mixture and left for 30 minutes. Read at 550 mμ. Hydrolysis activity was estimated from standard curve of L-tyrosin **(Lowery *et al*, 1951).** One unit of proteolytic activity produces the equivalent of 1 μmol L-tyrosin under above conditions.

Field application of *Trichoderma viride*

The field experiment was conducted at the Botanical Garden of Botany Department, Faculty of Science. Ismalia, using seeds of peanut cultivar "Caltro" Apparently healthy seeds were sown in farm sandy soil, 5 seeds per each row with 30 cm intervals. The field was divided into two plots contain the same number of seeds and rows, one plot was drenched with 500 ml spore suspensions (6×10^6spores ml^{-1}) of *T. viride* after 10 days of cultivation, while the other plot was irrigated with normal water as control. Although *T. harzianum* showed higher potentiality the *T. viride* the

later was used in field experiment because it was the highest frequency during isolation. The experiment was observed daily to record any changes in growth pattern of treated and control peanut plants. After 110 days, ten plants from each plot were randomly selected to measure the plant vigor parameters such as canopy radius (cm), root lengths (cm), number of branches, leaves, secondary roots, pods, and plant fresh &dry weigh (g) as well as pods fresh weight (g) per each plant representative by weight of five random pods. The experiment was designed as plot – plot design with 20 replicas.

Yield was calculated by total fresh weight of pods / total fresh weight of plants. Few random leaves were taken to determine the concentration of pigments (chlorophyll a, chlorophyll b and carotenoids) as mg/g plant fresh weight according to **Metzner *et al.*, (1965).**

Impact of *Trichoderma viride* on root knot nematodes disease severity was measured by recording percentage of infection of peanut roots, and pods harvested from each plant, galls size (mm) by using vernier caliper and galls count per each plant and nematodes population count per gram soil compost sample from each plot. Data were analyzed by analysis of variance (**Cochran and Cox, 1957**) and significant differences among treatment were tested by the least significant difference test (LSD) at probability levels of 5% ($LSD_{0.05}$)

RESULTS

In vitro screening of nematicidal activity of *Trichoderma*

Culture filtrate of four *Trichoerma* sp (containing metabolites and spores) showed deleterious effect on nematodes represented by immobilization of different stages of nematodes. *T. harzianum* showed the highest immobility effect after 7 days of exposing nematodes to filtrate (76.5%) followed by *T. viride 1* (66.7 %) and *T. viride 2* (56.1%) However the culture filtrate was significantly effective even after 2 days of exposure comparing to control (Fig. 1).When the two components of culture filtrate (spore suspension and metabolites) were separated, each component alone showed great effect on nematodes. *T.harzianum* spore suspension. Inhibited mobility by (46.7 %) followed by *T. viride 1* spore suspension (42.8 %) (Fig.2.).Similarly the metabolite alone, suppressed mobility of nematodes *T.h*arzianum was more effective than *T. viride1* (58.8%, 53.3 % respectively) (Fig. 3). Comparing the three preparations (mixture, spore suspension and metabolite alone) to each other revealed that the mixture was more effective than the two other preparations (Fig.4). While spore suspension gave about 21.7 % immobilization metabolite alone gave 30.8 % and mixture gave the highest effect (36.9 %).

Effect of different dilutions of *Trichoderma* culture filtrate

Metabolites of the most effective isolates (*T.harzianum* and *T.viride1)* were tested to evaluate their efficacy. Both exhibited significant effect on nematode mobilization. They were highly effective in suppression of nematodes even at low dilution (1: 3) where immobility reach about 22 % and 18.5 % with *T.harzianum* and *T.viride1* respectively (Fig. 5).

Direct parasitism

When the *Trichoerma* isolates were incubated with different stages of nematode, they were able to parasitize nematode and sporulate on them (Fig. 6.a.) Different degree of parasitism were detected, *T.h.* exhibited the highest percentage of parasitism (22 %) followed by *T.viride 1* and *T.* sp. (12 %, 11 % respectively), While *T.viride 2* showed the least percentage of parasites (7 %) (Fig. 6.b.)

Protease assay

Protease activity differed among the four tested *Trichoerma* isolates (Fig.7.a.). The highest protease activity was determined by *T.harzianum.* which gave the widest clear zone (2.08 mm) followed by *T.viride 1* (1.58 mm). Proteolytic activity of *T.harzianum* was (1.2 U/ml) while that *T.viride1* and *T.viride 2* were (0.9 U/ml, 0.7 U/ml respectively (Fig. 7.b).

Effect on population dynamic and galling

It was clearly evident that *Trichoderma* has a significant effect on population of pathogenic and free living nematode (Fig. 8). The population decreased dramatically from 44 to 14 nematodes /10 g soil at the same time percentage of pods infection was reduced down to 9 % (Fig. 9). Gall size of treated soils was 2.34 mm while, that of non- treated was 4.05 mm (Fig. 10). Reduction by 43% of galls number was determined (Fig. 11) *T.viride 1* treated plant showed very low number of gall (3.1) but non treated plant showed very high number (Fig. 12).

Effect on production

Trichoerma viride greatly affect peanut production. The treated peanut produced number of pods significantly greater than that of untreated peanut, (Fig. 13). Also the weight of pods produced from *Trichoerma* treated peanut was higher than untreated peanut, (Fig. 14.)With regard to biological production (relative production), there was no significant difference, (Fig. 15.a). It was calculated as percentage of produced pods to each plant (Fig. 15.b).

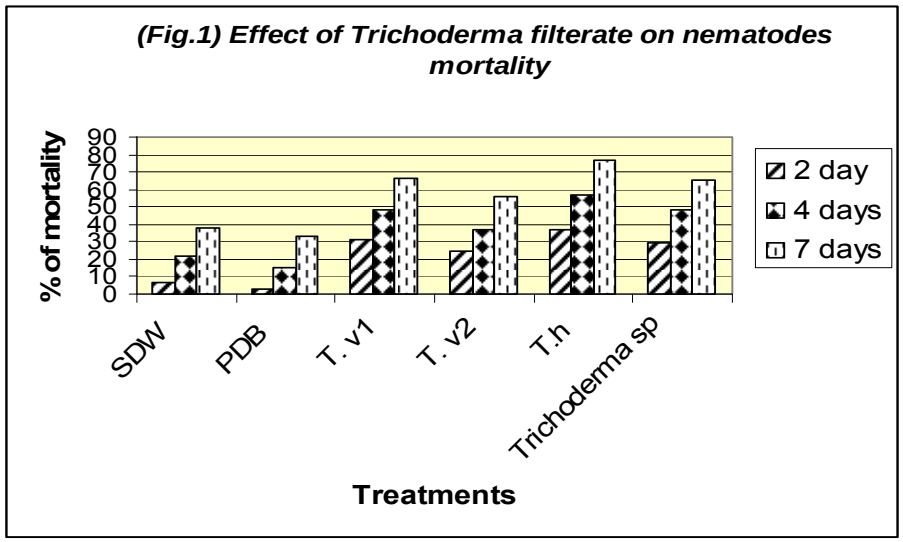
Effect on plant vigor

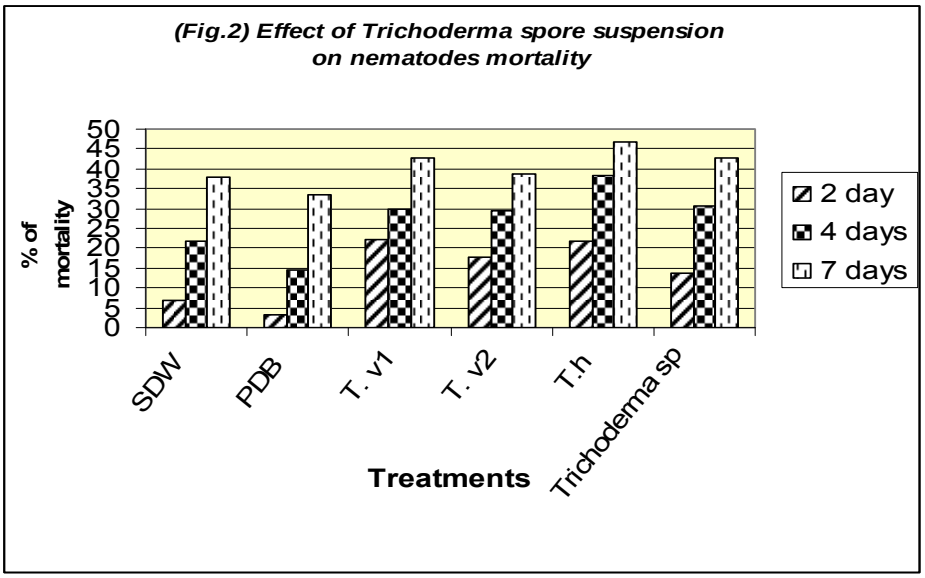
The canopies of treated plant as well as dry and fresh weight were significantly greater than that of untreated plant (Fig. 16). However number of leaves, branches, and root length were non- significant different in treated and non- treated plants (Fig. 17). It is worth to mention that the content of photosynthetic pigment in treated peanut was higher than that of untreated peanut (Fig. 18).

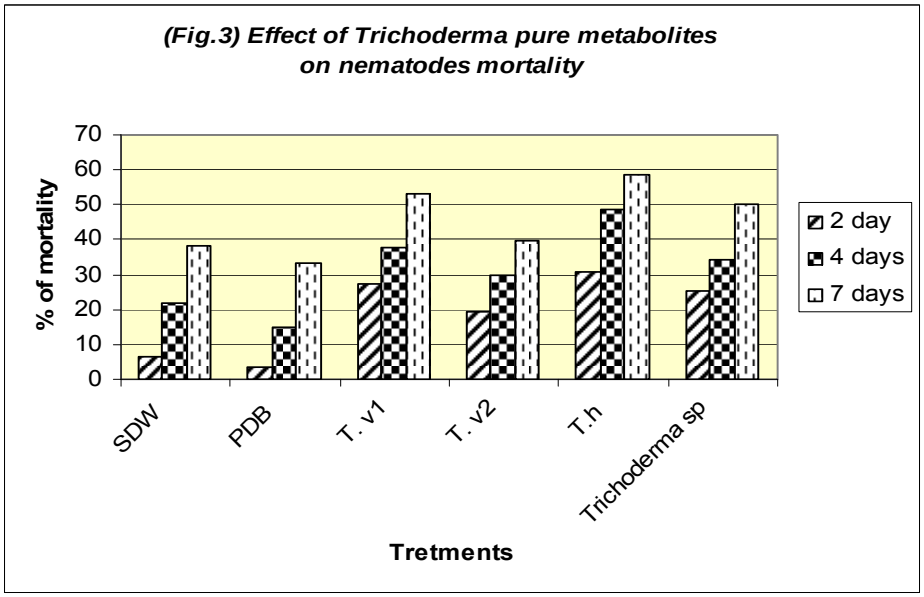
DISCUSSION

Trichoerma is widely used to control soil borne plant pathogenic fungi (Chet., 1987: Abdul Wahid., 2006).However it is less frequently used against nematodes. Evaluation of four *Trichoerma* isolates for their effective potential against root knot nematodes revealed significant suppression activity. Both two *Trichoderma viride* and *T. harzianum* were highly efficient for inhibiting nematodes comparing to the other two *Trichoderma* species as well as control. Different degree of inhibition were noticed with various preparations viz, metabolites alone, spore suspension and mixture of both metabolites and spore suspension. The later was prevalent on the others. This could be attributed to deleterious substances found in metabolites as well as to substances secreted during spore germination **(Khan and Saxena., 1997)** Metabolites contain very active anti-nematode compound (s) which was effective

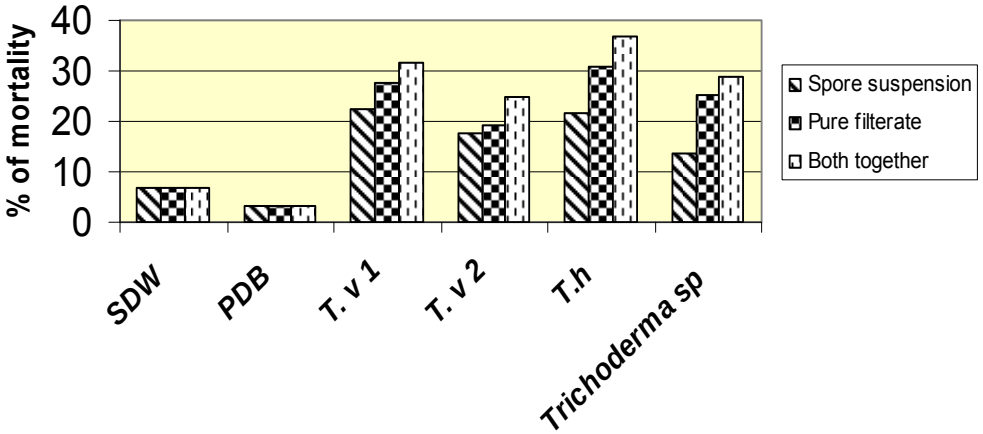
even at low concentration and play role in suppression nematodes reproduction **(Windham *et al.*, 1989**), inhibiting penetration and egg hatch (Sharon *et al.,* 2001) and decreasing the presence of galls **(Parveen *et al.*, 1993**).In addition *Trichoderma* was able to parasitize living nematodes and sporulate on it. These findings were reported by **(Sharon *et al.*, 2001, Meyer *et al.*, 2000**). The parasitism nature of *Trichoderma* is supported by the high proteolytic activity exhibited by the four *Trichoderma* isolates these isolates differ in their capability to secret protease enzymes, but showed considerable activity. When the isolate *T.viride1* was applied to naturally infested soil, it dramatically reduced number and size of galls of peanut roots comparing to non treated soil. Also the population of nematodes in soil decreased significantly due to application of *T.viride 1* to the soil as a drench. Data of laboratory and field experiment consolidate the suggesion that two mechanisms may be involved in biocontrol efficacy of *T.viride 1*. These mechanisms are production of anti-nematode substances and direct parasitism of nematodes. Increasing of peanut canopy, root length as well as photosynthetic pigment content in *T. viride 1*treated peanut strengthened the proposition of a third mechanism, which is the growth promotion of the plant. The ability of *Trichoderma* to promote plant growth is documented by other investigators **(Chet., *et al* 1997, Harman., *et al* 2004**). It's presumably that *T.viride 1* exhibited multiple mechanisms through which it mediates its action and prop up plant health. It is most likely that these mechanisms act in synergistic and synchronized way to protect and support plants. Using a biocontrol agent of various mode of action is a key factor in the successful biocontrol system.




(Fig.2) Effect of Trichoderma spore suspension on nematodes mortality
% of mortality
50
45
40
35
30
25
20
15
10
5
0
SDW
PDB
T. v1
T. v2
T.h
Trichoderma sp
Treatments
2 day
4 days
7 days


(Fig.3) Effect of Trichoderma pure metabolites on nematodes mortality
% of mortality
70
60
50
40
30
20
10
0
SDW
PDB
T. v1
T. v2
T.h
Trichoderma sp
Tretments
2 day
4 days
7 days

(Fig.4) Comparison betwwen three prepations of Trichoderma after 48 h



(Fig.5) Effect of different dilution of Trichoderma culture filterate on nematodes

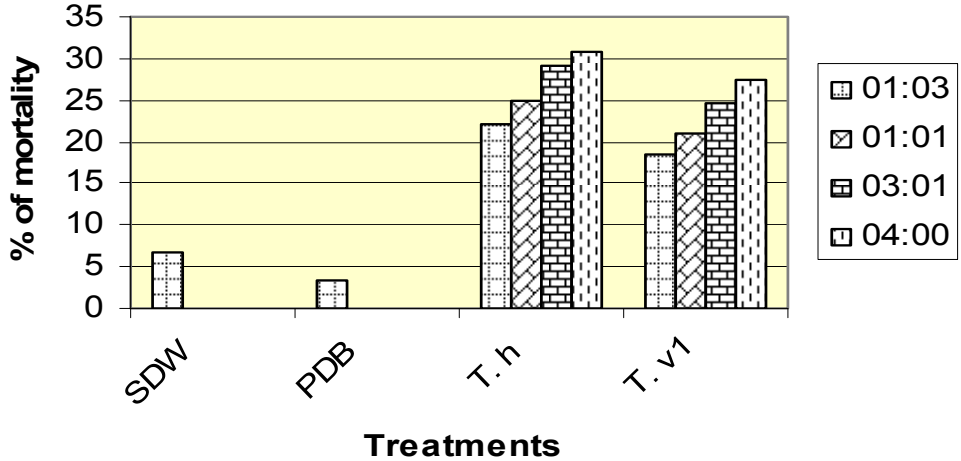


Fig.6.a

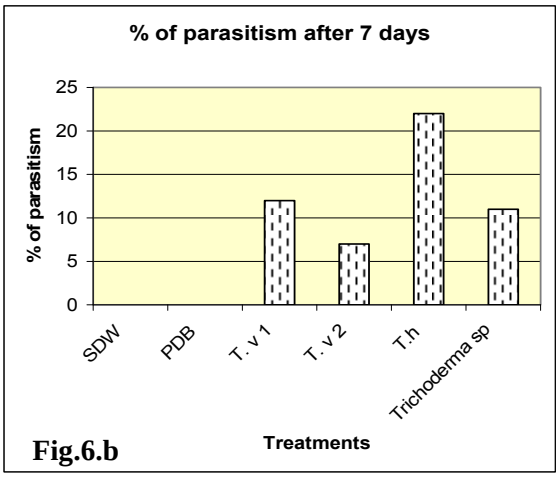
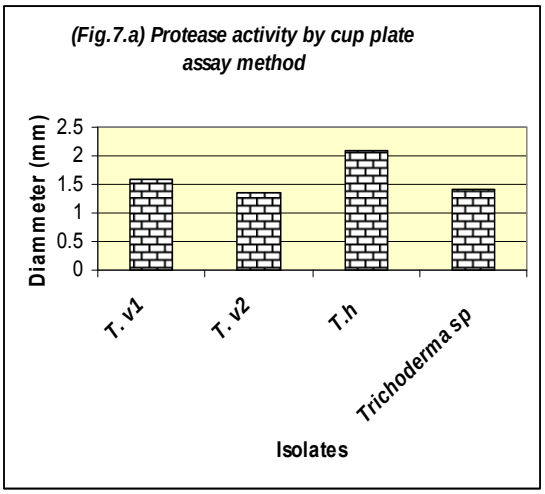
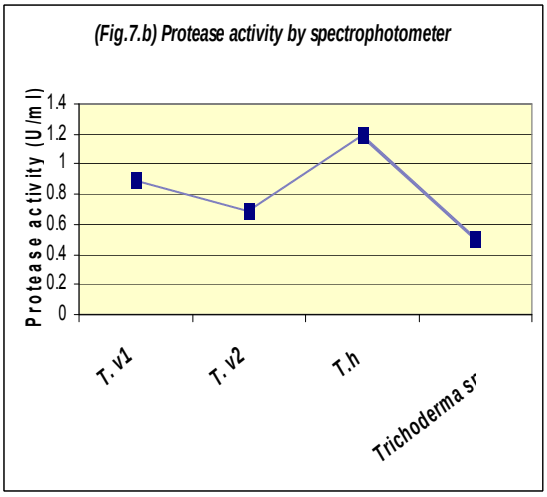
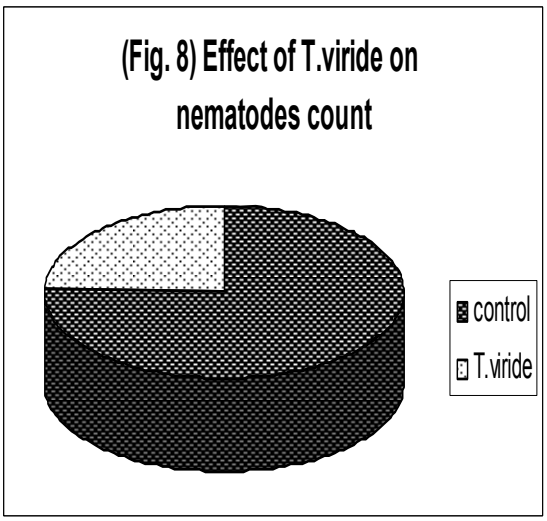
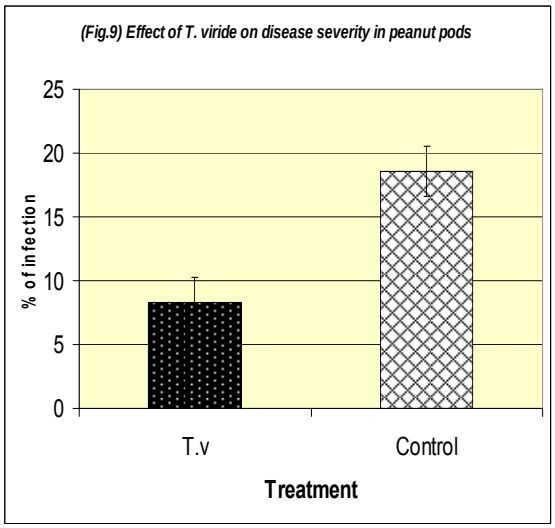

% of parasitism after 7 days
% of parasitism
25
20
15
10
5
0
SDW
PDB
T. v 1
T. v 2
T.h
Trichoderma sp
Treatments

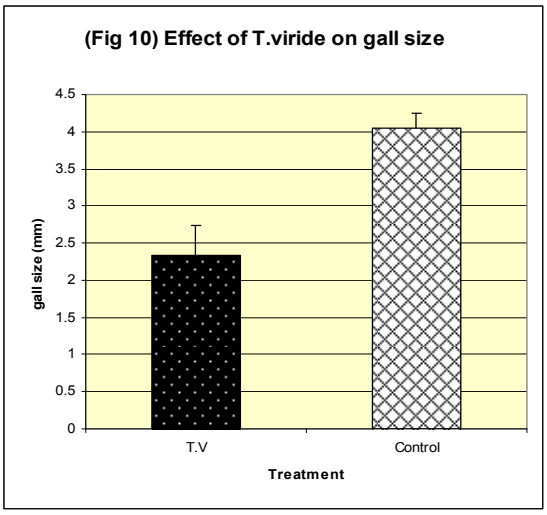
Fig.6.b


(Fig.7.a) Protease activity by cup plate assay method
Diammeter (mm)
2.5
2
1.5
1
0.5
0
T. v1
T. v2
T.h
Trichoderma sp
Isolates


(Fig.7.b) Protease activity by spectrophotometer
Protease activity (U/ml)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
T. v1
T. v2
T.h
Trichoderma sr


(Fig. 8) Effect of T.viride on nematodes count
control
T.viride


(Fig.9) Effect of T. viride on disease severity in peanut pods
% of infection
25
20
15
10
5
0
T.v
Control
Treatment



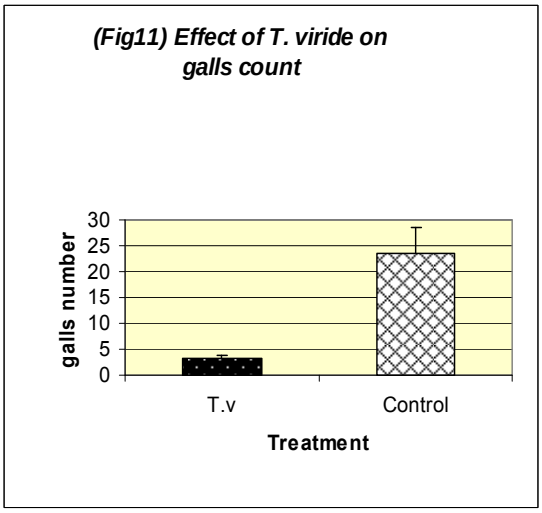
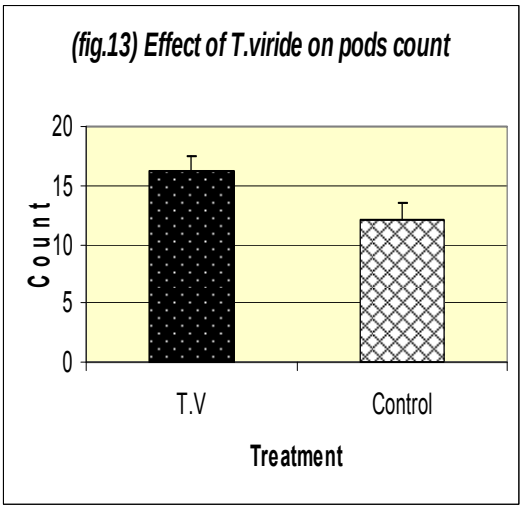
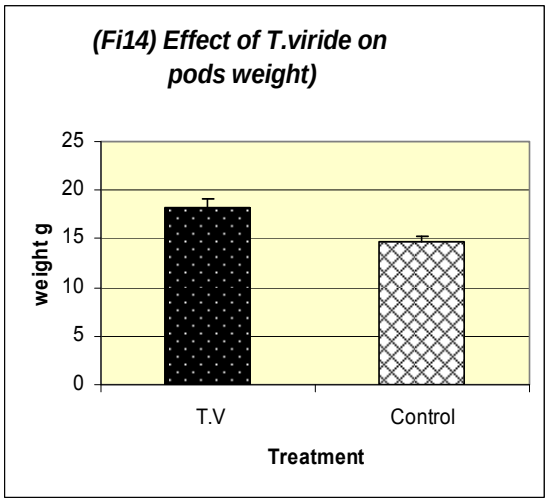
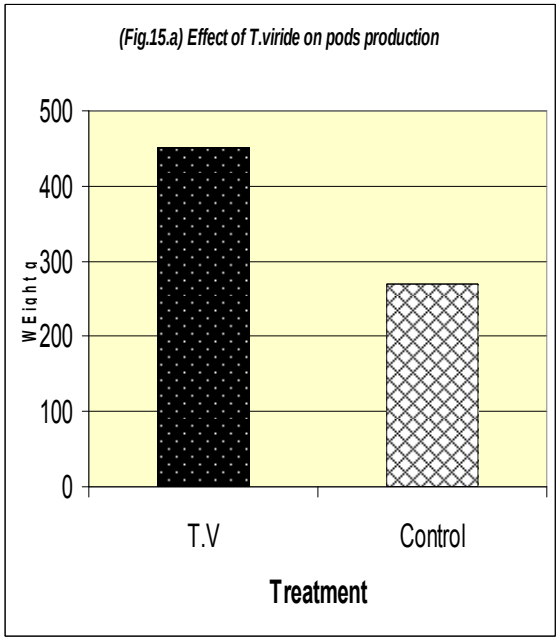
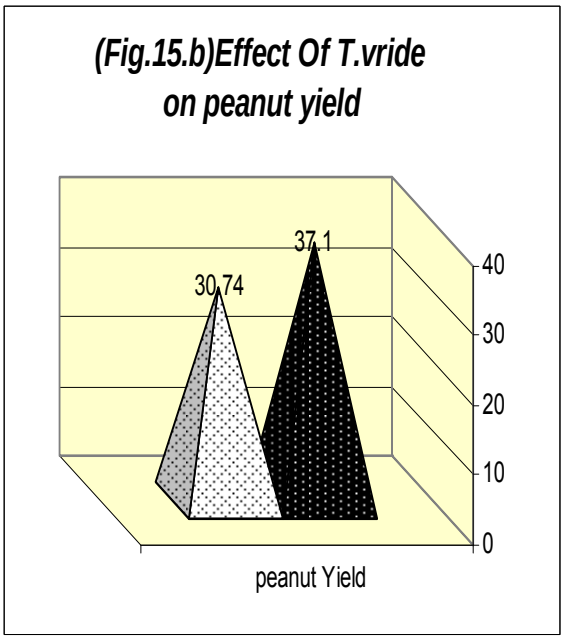


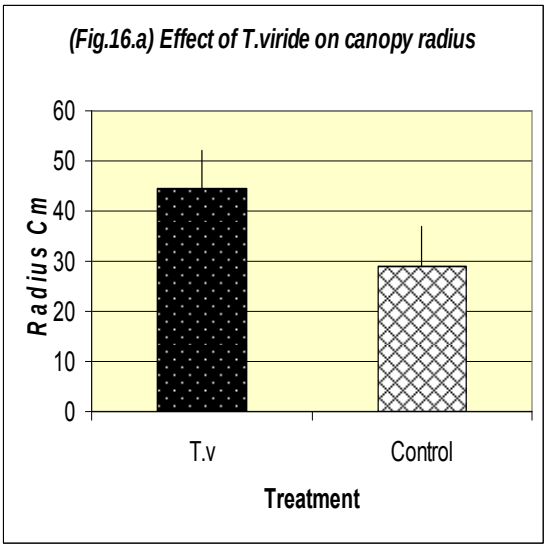
Fig. (12): Effect on population dynamic and galling

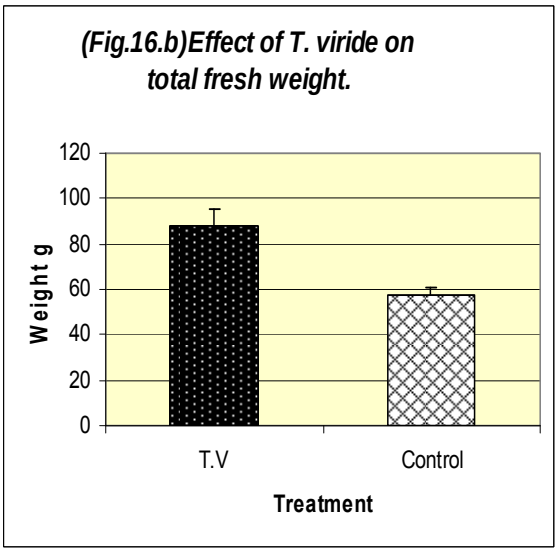


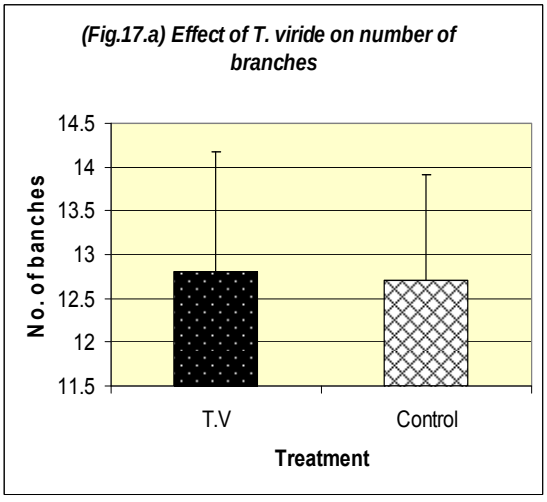


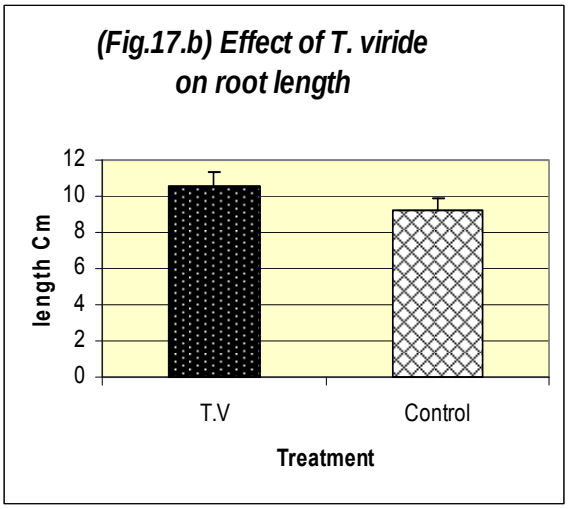

(Fig.15.a) Effect of T.viride on pods production
500
400
300
200
100
0
Weight g
T.V
Control
Treatment

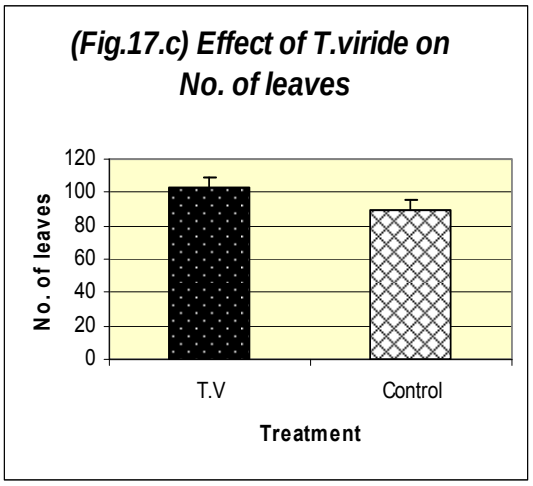

(Fig.15.b)Effect Of T.vride on peanut yield
37.1
30.74
40
30
20
10
0
peanut Yield

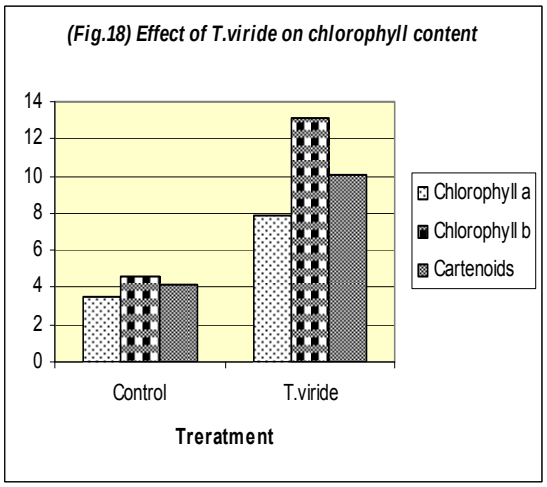

(Fig.16.a) Effect of T.viride on canopy radius
60
50
40
30
20
10
0
Radius Cm
T.v
Control
Treatment


(Fig.16.b)Effect of T. viride on total fresh weight.
120
100
80
60
40
20
0
Weight g
T.V
Control
Treatment


(Fig.17.a) Effect of T. viride on number of branches
14.5
14
13.5
13
12.5
12
11.5
No. of banches
T.V
Control
Treatment


(Fig.17.b) Effect of T. viride on root length
12
10
8
6
4
2
0
length Cm
T.V
Control
Treatment





دور فطر الترايكوديرما في المقاومة الحيوية لنيماتودا تعقد الجذور في الفول السودانى

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يعتبر الفول السوداني واحد من أهم المحاصيل الزيتية فى العالم وهو عرضة للإصابة ببعض الأمراض وأحد أهم هذه الإصابات بنيماتودا تعقد الجذور. تعتبر المقاومة الحيوية أحد الحلول الآمنة وصديقة للبيئة للحد من إصابة الجذور بواسطة نيماتودا تعقد الجذور. تم اختيار 4 عزلات عشوائيا من فطر تريكوديرما من إجمالي 50 عزلة تم الحصول عليها من مصادر تربة مختلفة و هذه العزلات لها القدرة على الحد من حيوية النيماتودا فى التجارب المعملية من خلال الافرازات الثانوية الناتجة من تنمية هذه العزلات فى بيئات سائلة هذة الإفرازات لها تأثير سمى على كل من النيماتودا الممرضة والغير ممرضة . فطرى *Trichoderma viride*& *T. harzianum* كانا لهما قدرة عالية في التطفل على النيماتودا وإفراز أنزيمات لتحليلها وأخيرا القضاء عليها .

عندما أضيف فطر *Trichoderma viride* إلى تربة مصابة طبيعيا واظهر هذا الفطر نتائج جيدة فى خفض عدد وحجم العقد الناتجة من إصابة الجذور بواسطة نيماتودا تعقد الجذور مقارنة بتربة غير معالجة وكذلك أنخفض عدد النيماتودا فى التربة التي تحتوى على الفطر على الجانب الأخر انعكس تأثير الفطر إيجابيا على نمو النبات من خلال زيادة معنوية فى المجموع الخضري ووزن النبات وكذلك زيادة فى الإنتاجية بشكل عام نتيجة تطبيق التريكوديرما على محصول الفول السودانى.