Effect of fungal filtrates of *Fusarium oxyosporium* and *Aspergillus flavus* on *Meloidogyne javanica* in Okra plant under greenhouse conditions.

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Abstract. The aim of this study was to test the efficiency of fungal filtrates from *Fusarium oxyosporium* and *Aspergillus flavus* in the control of root-knot nematode in laboratory and field. The fungus had been grown on PDA medium and the nematode had been isolated from infection okra plant to prepare nematode suspension and test four concentration of fungal filtrates (25%,50%,75%,100%) on both the nematode and the plant. The effect of concentrations of fungal filtrates showed that, the concentration 25% was the least affected on the nematodes while concentration 100% was the best in reducing the number of nematodes and supporting plant growth. We concluded from this study that the time required to kill all nematode was reduced by increasing the concentration of filtrate and the fungal filtrates have had the capability to support plant growth and reduce number of knot nematode count.

1. Introduction

Plant-parasitic nematodes are important pests of the crops. It infest large numbers of vegetables and fruits of all types [1]. Despite the diversity of these pests, the most common is the root knot nematode *Meloidogyne* spp, which is common, widespread and one of the most harmful parasite [2]. The infection usually starts when the second juvenile stage of the parasite penetrates the root epidermis, then migrates to the cortex. After that it reach the vascular cylinder, reside their and begins to insert its excretions and complete its life cycle within the plant tissue. As a result of infection the plant's ability to resist adverse environmental conditions such as drought and pathogens will decrease. Because of the plants injury at an early age, this leads to plant death [9]. To reduce the damage caused by these pests, researchers have been turned to biological control methods which is often viewed as non-progressive and environmentally friendly way to control pest organisms. These methods leave behind no chemical residues that might have harmful impacts on humans or other organisms. These ways of control may provide widespread criterion of pest destruction in addition to cost effective and safe ways [3].

Okra (*Abelmoschus esculentus* Moench.) is one of the warm season crops that grown in the tropical and sub-tropical regions of the world [4]. It belongs to the Malvaceae family and is an important vegetable crop. The plant is grown for its green fruits in different parts of Iraq. Fruits of okra have basic useful properties in providing excellent health and nutritional qualities as these are rich in vitamins and enzymes necessary for body function and prevention of chronic diseases [5].
The fungus F. oxyosporium is widely distributed in nature and, some studies have indicated the ability of some of the isolates of F. oxyosporium to reduce the number of nematodes that hold roots in the soil as as well as their ability to parasite on their eggs. Some studies have indicated the ability of this fungus to inhibit the full hatch of eggs in the laboratory [6] Aspergillus flavus known for its ability to produce highly effective toxins against many microorganisms [10].

2. Materials and Method

2.1 Preparation of fungal filtrates
The fungal filtrates were prepared according to method of Musa Nima Mezher [11]. Briefly, 200 g of potatoes juice with 20 g of dextrose sugar were added into 500 ml of distilled water and filtrated with a piece of gauze. Then, the volume of filtrate was complete to a liter with distilled water. After that the medium was distributed in 250 ml volumetric flasks and sterilized in the Autoclave at 121 ° C for 20 min. After sterilization, the medium was cooled and chloramphenicol was added at a concentration of 250 mg / L. Then a growth from edge of colony (7day old) on PDA medium was taken and inoculated into potato dextrose broth. Incubation was carried out at 25 ° C for 28 days with shaking every three days . After that a the fungal filtrates was taken, filtered using the filter paper (Watman No. 1). Then the filtrate was sterilized employing Milipore type Nylon 0.22 diameter and kept at -20 C until use.

2.2 Preparation of the nematode suspension
The second stage juveniles (juveniles 2) was obtained according to method of Al abed Al kader [9]. The roots of the infected okra were washed with running water. The egg blocks were then picked up with fine forceps and then sterilized with 0.5% sodium hypochlorite solution for 2 minutes. After that, the eggs were washed three times with sterile distilled water. Then 1m of sterile distilled water containing penicillin, streptomycin and tetracycline at 0.1% concentration was added into test tubes and incubated at 25 ° C for two weeks. The water was changed twice during the incubation time. The adolescents were collected in one container. The contents of each test tube were poured into a Berman funnel covered With a layer of tissue paper and after 24 hours the second stage juveniles were collected. The size of the solution was calculated and then 0.1 mL was withdrawn from the solution and placed on the counting slide and was exposed to a light flame source for stabilizing of the worm movement. The number was calculated and then multiplied by the total volume of the solution . Then 100 juveniles 2 was added to the Petri dishes with three replicates per treatment. Then the concentration of each fungal filtrates was added to the suspension and the result was compared with the control (distilled water).

2.3 Preparation of concentrations
The concentrations used in the experiment were prepared from fungal filtrates. A stock was made at 100% concentration. Then a concentrations of 25%, 50%, 75%, and 100% were prepared by employing the mitigation law :

\[ N_1 \times V_1 = N_2 \times V_2 \]

\( N_1 \) = concentration of the fungal filtrates before dilution

\( V_1 \) = Volume of the fungal filtrates before dilution

\( N_2 \) = concentration of fungal filtrates after dilution

\( V_2 \) = Volume of the fungal filtrates after dilution

2.4 Detection of effective groups of fungal filtrations

2.4.1 Fungal filtration
Four flasks each with 250 ml liquid medium PDB (prepared as above) have had inoculated with three pieces of 5 mL diameter of 5 days old colony that has been grown on PDA medium. The flasks were incubated at 25°C for 14 days, with daily stirring. Then the fungal filtrates was sterilized using milipore filter (0.045 um) and the filtrate collected in a sterile flask under aseptic condition. After that,
the fungus filtrate was placed in the separation funnel, and chloroform was added at 1:1 (v:v). Then the mixture has been stirred up well to obtain two layers. After that the flask left and the residual residue has been collected and dried at 40°C then reconstituted to 5 ml of the original volume and placed in the refrigerator until use.

2.4.2 Detection of Alkaloids by using Mayer reagent
The reagent was prepared by adding 1.36 g of mercuric chloride HgCl₂ to 60 ml of distilled water. Then five grams of potassium iodide were dissolved in 10 ml of distilled water. The solvents were mixed and the volume was completed to 100 ml with distilled water. A few drops of the reagent were added to the fungus extracts. The appearance of a white deposit indicates the presence of alkaloids [17].

2.4.3 Detection of Tannins by using Lead acetate test
The solution was prepared by dissolving 1 g of lead acetate in 100 ml distilled water then several drops were dd to a test tube containing 0.50 mL of fungal extract. A white deposit of gelatin is a sign of the presence of tannins [21].

2.4.4 Detection of Glycosides by employing Molish reagent
The presence of the Glycosides was detected using a molash reagent. The test was carried out by taking 2 ml of the fungal extract and then few drops of α-naphthol solution has been added followed by stirring. Then the tube was held in an oblique position and 2 ml of concentrated sulfuric acid was added onto the wall of the tube until two layers appeared. The appearance of violet color separates the two layers is a sign of the presence of the Glycosides [22].

2.4.5 Detection of Flavonoids
Flavonoids were detected by adding 1 ml of 7.5% natural ferric chloride solution with concentration 7.5% to 1 ml of fungal extract. The appearance of black colour indicates the presence of flavonoids [23].

2.4.6 Detection of Phenolics
The preparation of phenolics reagent was carried out by dissolving 1 g of ferric chloride in 100 ml of distilled water. Then the filter paper was moistened with the fungus extract, followed by addition of a few drops of ferric chloride solution. After that, the paper exposed to ammonia vapor. The appearance of blue color is evidence of phenolic presences [Ref].

2.5 Field experience
50 ml of stock filtrate of each extract were placed in plastic pot contains 500g of sandy loam soil. After 10 days, okra seed sterilized with 5% sodium hypochlorite solution were implanted. Five seeds were ingrained in each container with regular watering with sterilized water as needed. Ten days later, the growing plants were reduced to 2 in each pot. After that, a hole was made to a depth of 3 cm. Then 2,000 J2 (with 2000 J2 of M. javanica) was added to each pot, with 3 replicates per treatment and left to grow for 8 weeks. The roots were carefully pull out. The number of nodes per replicate with counting of nematodes were estimated, both in the fungal filtrate’s treated and control soil. Plant lengths and weights were also measured and compared with control plan.

2.6 Calculate the number of nematodes in the soil
The Assembly the Baermann funnel equipment was carried by placing a clamped rubber tube below the funnel. Then a piece of window screen is placed in the mouth of the funnel. After that, the funnel was placed to holder. Then a tissue-paper wrapped soil sample placed on to the screen material. Then water was added into the funnel setup until the window screen and soil sample are immersed and left overnight. After that the first couple of drops of water was gathered from the bottom of the tube by slowly releasing the clamp on the tubing and examined under the microscope, looking for actively mobile, living nematodes [12]. The suspension was placed into a glass container and the volume of the
suspension was measured. Then 0.1 ml of suspension was taken and placed on Hematocytometer. The number of nematodes found in 100 g of soil was calculated from the following equation:

Number of nematodes present in 0.1 X total size of suspension

Statistical analysis:
The results were statistically analyzed by applying the T test using the Minitab statistical program and comparing the arithmetic mean of the various treatment by the multivariate Duncan test at a probability level of $P \leq 0.05$

3. Results

| Table 1: Effect of exposure time for different concentrations of fungal filtrates of F. oxysporium on juveniles 2. |
|---|---|---|---|
| Concentration% | 6 hour | 12 hour | 24 hour |
| 0 | 0 | 0 | 0 |
| 25 | 21.3 ± 3.05 c | 44.0 ± 4.58 b' | 72.33 ±10.69a |
| 50 | 51.6 ± 3.05 c | 80.6 ±5.50 b | 91.33 ± 1.52a |
| 75 | 62.67 ± 2.08 c | 86.67 ± 1.5 b | 100.0 ± 0.00a |
| 100 | 97.33 ± 0.57 b | 100.0 ± 0.0a | 100.0 ± 0.00a |

* Numbers with similar letters in the same row have no significant differences according to the Duncan Multiplicity test at a probability level of $P \leq 0.05$

| Table 2: Effect of exposure time for different concentrations of fungal filtrates of A. flavus on juveniles 2 |
|---|---|---|---|
| Concentration% | 6 hour | 12 hour | 24 hour |
| 0 | 0 | 0 | 0 |
| 25 | 45.3c ± 13.1 | 83.3b ± 4.9 | 100. a ± 0.0 |
| 50 | 56.3c ± 4.9 | 92.3b ± 1.1 | 100. a ± 0.0 |
| 75 | 76.3c ± 2.3 | 95.3b ± 2.5 | 100. a ± 0.0 |
| 100 | 93.6c ± 2.3 | 100. b ± 0.0 | 100. a ± 0.0 |

* the numbers with similar letters in the same row have no significant differences according to the Duncan Multiplicity test at a probability level of $P \leq 0.05$

| Table 3: Effect of Different Concentrations of fungal filtration of F. oxysporium on Plant |
|---|---|---|---|---|---|
| Plant length | Number of nodes | Nematode count | Root weight | Stem weight |
| Concentration% | 19.83 ± 2.3B | 45.0 ± 2.0A | 1185.0 ± 11.5A | 0.73 ± 0.05D | 0.63 ± 0.15D |
| 25 | 20.06 ± 0.2B | 42.0 ± 2.0B | 1169.0 ± 9.8A | 0.73 ± 0.15D | 0.73 ± 0.05 D |
| 50 | 20.33 ± 0.2B | 36.66 ± 1.52C | 894.3 ± 58.5B | 0.86 ± 0.11C | 0.86 ± 0.05 C |
| 75 | 22.70 ± 0.4A | 30.0 ± 1.73D | 793.0 ± 42.7C | 1.23 ± 0.15B | 1.00 ± 0.10B |
| 100 | 23.76 ± 0.2A | 25.33 ± 1.5E | 731.7 ± 15.5D | 1.36 ± 0.05A | 1.20 ± 0.10 A |

The numbers with the same large letters in the same column have no significant differences according to the Duncan Multiplicity test at a probability level of $P \leq 0.05$. 

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Table 4: Effect of Different Concentrations of fungal filtration of *A. flavus* on Plant

| Concentration% | Plant length | Number of nodes | Nematode count | Root weight | Stem weight |
|----------------|-------------|-----------------|----------------|-------------|-------------|
| 25             | 21.3 ± 1.5E | 41. ± 3.0A      | 1162.0 ±        | 0.5 ± 0.1D  | 0.7 ± 0.05D |
| 50             | 22.6 ± 2.6C | 36.3 ± 2.5B     | 906.7 ± 84.7B   | 0.6 ± 0.05C | 0.8 ± 0.1D  |
| 75             | 25.1 ± 0.7D | 30.0C ± 2.6C    | 672.7 ± 69.0C   | 0.8 ± 0.1B  | 1.17 ± 0.3C |
| 100            | 27.2 ± 0.5C | 24.6 ± 4.5D     | 634.0 ± 6.6D    | 0.9A ± 0.1B | 1.12 ± 0.12B|
|                | 29.1 ± 0.8B | 20.3 ± 2.5E     | 585.0 ± 41.6E   | 0.9 ± 0.2A  | 1.3 ± 0.1A  |

The numbers with the same large letters in the same column have no significant differences according to the Duncan Multiplicity test at a a probability level of P≤0.05.

Table 5: Results of qualitative components estimation of data of active fungal extracts.

| Fungi            | Alkaloids | Tannins | Glycosides | Flavonoids | Carbohydrates | Phenols |
|------------------|-----------|---------|------------|------------|---------------|---------|
| *A. flavus*      | +++       | +++     | +++        | +++        | +             | +       |
| *F. oxyosporium* |           |         |            |            |               |         |

The results of effect of exposure time for different concentrations of fungal filtrates of *F. oxyosporium* and *A. flavus* are presented in Table 1 and 2. The study showed that, the numbers of the second stage juveniles killed, are proportional with the length of time exposure. As for fungal filtrate of *F. oxyosporium*, at a concentration of 25%, the percentage of killed juveniles 2 after 6 hours and 24 hours were, 21.3 and 72.3 percent respectively, while for *A. flavus*, the percentage of killed juveniles 2 at same time and concentration were, 45.3 and 100 percent respectively. However, at concentration of 50% of fungal filtrate of *F. oxyosporium*, the percentage of juveniles 2 killed after 6 and 24 hours were, 51.6 and 91.3 respectively. While for *A. flavus*, the percentage of killed juveniles 2 at same time and concentration were, 56.3 and 100 percent respectively. At concentration of 75%, fungal filtrate of *F. oxyosporium*, the rate of the juveniles 2 killed after 6 hours and 24 hours of incubation was 62.6 and 100 percent respectively. while for *A. flavus*, the percentage of killed juveniles 2 at same time and concentration were, 76.3 and 100 percent respectively. Nevertheless the use of 100% concentration is the most efficient in the elimination of juveniles 2, where the rate of killing were, 97.3% and 100% after 6 hours and 12 hours of incubation with fungal filtrate of *F. oxyosporium* while for *A. flavus*, the percentage of killed juveniles 2 at same time and concentration were, 93.3 and 100 percent respectively [Table 1 and 2]. From comparing of both results that we obtained, it would appeare that both fungi gave an approximate close results. The effect of Different Concentrations of fungal filtrate of *F. oxyosporium* on Plant is presented in Table 3. When comparing the treatment of fungal filtrates with control, the low concentration did not show any significant differences in the effect on plant length. We found that at 25% and 50% concentration, plant length was 20.06 cm and 20.3 cm respectively. When using concentrations of 75% and 100% the length was, 22.7 cm and 23.7 cm respectively, compared with the control, where the length of the plant was 19.83 cm. The number of nematodes knode in the plant, were influenced by treatment with various concentration of fungal filtrate. The average number of knots was decreased from 42 to 30 knots at concentration of 25%, to 100% respectively in comparison to 45 knots in the control. As for the number of nodes in the plant, the fungal filtrate treatment contributed to a significant reduction in the number of knots in comparison to control. The average
number of the knots was; 42 knots at 25%, 36.6 at 50%, 30 at 75% and 25.3 at concentration of 100% in comparison to number of knots in the control which it was 45 knots.

In the calculation of nematode number, the treatment showed no significant differences at a concentration of 25% of fungal filtrate. While at; 50%, 75% and 100% the number of nematodes was 894.3, 793 and 731.7 respectively, in comparison to the rate of nematode number in the control which it was 1185 (Table). In regards to plant weight, no significant result was obtained at a concentration of 25%, while the weight of the plant increased by 50%, 75% and 100%. The root weights were 0.86 g, 1.23 g and 1.36 g respectively. The weight of the stem showed no significant difference at concentration of 25%, while differences were found in the concentrations of; 50%, 75% and 100% , where the stem weight was 0.86 g, 1 g and 1.2 g, respectively. The control showed a stem weight of 0.63 g (Tale). The effect of different concentrations of fungal filtrate of A. flavus on Plant is presented in Table 4. The results of the study showed, that the effect of filtrate of A. flavus on plant length showed a significant difference in comparison with the control. The plant length rate was 22.6, 25.1, 27.2 and 29.1 cm at a the concentration of; 25%, 50%, 75% and 100% respectively compared to the control which it was 21.3. The number of nematodes knode in the plant, were influenced by treatment with various concentration of fungal filtrate. The average number of knots was decreased from 36.3 to 20.3 knots at concentration of 25%, and 100% respectively in comparison to 41 knots in the control. The calculation of nematode number at a concentration of 25% 50%, 75% and 100% was, 906.7, 762.7, 634 and 585, respectively. This result is the differences in the number of nematodes in each treatment was significant in comparison to the rate of nematode number in the control which it was 1162 [Table]. The root weight showed a difference In the concentrations of 25%, 50%, 75% and 100, where it was 0.6, 0.8, 0.9 and 0.9 gm, respectively, compared with control which was 0.5 g, while the weight of the stem did not show a significant difference at 25% concentration, where it was 0.8 g. The differences were significant at concentrations 50%, 75% and 100%, respectively, which was 1.17 g, 1.12 g and 1.3 , respectively, compared to control with a stem weight was 0.7 g.

5 Discussions
Successful biocontrol program usually significantly reduce the abundance of the pest but in some cases they simply prevent the damage caused by the pest with out reducing pest abundance [16]. There are a large number of research in the area of using fungi in the control of nematode and the fungal natural products are very promising potential source to manage plant parasitic nematode. In the present study, the number of root knot nematode has been reduced resulted in reduction of the number of knot and promoted plant growth. These results are in consistent with the results of a previous studies [15]. The fungal filtrates of several types of Aspergillus have been studied and the effect in controlling root knot formation in tomato root and nematode population in the soil were demonstrated. The workers found that treatment with the filtrate reduced the knot formation and nematode number in the rhizosphere and promotes the growth of plant. In another study conducted by Roccuzzo, et. al. [18], they found that, the number of knots reduced in presence of F. oxysporum in the soil of infected plant. The study of Shahda et.al. The ability of fungi to reduce the member of nematode in the soil and its knot may be due to secondary metabolic compounds produced by fungi that my have an effect on nematode such as flavonoid which has ability to dissolve the cytoplasmic membrane of nematode cell and their functional groups interfering with enzyme protein structures of nematode [19]. Also Aspergillus produces many toxins such as aflatoxin B1, B2, G and G2. Fumonisins, Trichothecens and Diacetoxyscirpenol which are produced by Fusarium, have been promoted plant growth. Also, the constituents of glycosides and carbohydrate are important sources of energy for growth of plants.

6 References
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