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Rhizospheric Fungal Associations of Root Knot Nematode Infested Cucurbits: *In vitro* Assessment of Their Nematicidal Potential

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ABSTRACT

The root knot nematode (*Meloidogyne* spp.) is a polyphagous plant pest causing losses in a wide range of host crops. Due to environmental health concerns, the search for new microbial strains as nematode control agents is relevant. Soil samples were collected from the rhizosphere of nematode infested and non infested cucurbits plants and fungi isolated and identified using standard methods. Extraction of nematodes was done using Cobb's sieving and decanting method and modified Baerman's funnel technique. Cell free Fungal Culture Filtrates (FCF) were prepared from the most frequently occurring isolates and their nematicidal potentials assessed as the mean percentage mortality of nematodes at an interval of 24, 48 and 72 h. Sterile distilled water served as control. In all 18 fungal species were isolated from the rhizosphere soil of *Momordica charantia* control and 08 species from that of *Momordica charantia* infected and 09 species from *Lagenaria siceraria* infected. Among the nine fungi tested for nematicidal properties, *Aspergillus flavus* showed maximum nematode mortality (45%) after 24 h. After 48 h maximum nematode mortality was shown by *Aspergillus* species 2 and *Trichoderma viride* (68.75%). After 72 h, maximum nematode mortality was observed due to *Trichoderma viride* and *Aspergillus flavus* (93.75%). The results suggest that the various fungi associated with the rhizosphere soil of different vegetable crops could be used as a biocontrol agent of nematodes and the culture filtrates can serve as a source of novel nematicidal compound of fungal origin which is more environmental friendly.

Key words: Rhizospheric fungal associations, root knot nematode, *in vitro* assessment, nematicidal potential

INTRODUCTION

The rhizosphere is an important biotic zone of interaction and is the residence of a large variety of microbes such as, bacteria, actinomycetes, fungi, protozoa and micro algae, besides presence of higher densities of nematodes and mites (Bazin *et al.*, 1990). The root knot nematode (*Meloidogyne* spp.) is a cosmopolitan, polyphagous plant pest causing losses in a wide range of host crops. Since, indiscriminate use of nematicides is responsible for environmental and human health concerns; the search for new microbial strains as nematode control agents is relevant (Kerry, 2000; Regaieg *et al.*, 2010). Since, fungi cohabit together with nematodes in the rhizosphere, their toxic metabolites may be responsible for keeping a low level of nematode populations (Siddiqui *et al.*, 2001; Le *et al.*, 2009; Zarina and Shahina, 2010). Thus, fungi may be considered

as important in nematode control due to their ability for production of nematotoxic (Hallmann and Sikora, 1996; Kerry, 2000; Regaieg *et al.*, 2010; Lopez-Llorca and Jansson, 2006) and nematicidal compounds (Hallmann and Sikora, 1996; Anke and Sterner, 1997; Chen *et al.*, 2000; Meyer *et al.*, 2000, 2004).

Specific secondary metabolites or enzymes showing nematode antagonistic or toxic properties are a promising research field (Shinya *et al.*, 2008; Regaieg *et al.*, 2010). In recent years, the search for nematotoxic or antagonistic compounds in culture filtrates has been greatly intensified (Liu *et al.*, 2008; Lopez-Llorca *et al.*, 2008). Assays with culture filtrates show toxic and inhibitory effects towards plant parasitic nematodes (Nitao *et al.*, 1999; Chen and Chen, 2002; Olivares-Bernabeu and Lopez-Llorca, 2002; Mukhtar and Pervaz, 2003; Khan *et al.*, 2004). Toxic effects of fungal culture filtrates on *M. incognita* have been studied by several workers (Hallmann and Sikora, 1996; Anke and Sterner, 1997; Sharma, 1999; Wang *et al.*, 1999; Costa *et al.*, 2000; Randhawa *et al.*, 2001; Meyer *et al.*, 2004; Zareen *et al.*, 2001; El-Shanshoury *et al.*, 2005), although a broad range of fungi still remain unexplored. Hence, this work was undertaken with the objectives to isolate rhizospheric fungal associations of root knot nematode infected and uninfected Cucurbit plants and invitro assessment of their nematicidal potential against the *Meloidogyne* spp.

MATERIALS AND METHODS

Collection of samples: Soil samples (nematode infested and non infested) were collected from the rhizosphere of four species of cucurbits plants, namely *Lagenaria siceraria*, *Cucumis sativus*, *Cucurbita pepo* and *Momordica charantia* from a depth of 6 inches, at the rate of one unit sample per acre crop area. Each unit sample was a composite of 20 cores obtained from four corners and centre of the field. Two hundred gram of soil sub-samples prepared from the unit samples were stored in polythene bags and stored at 4°C in a refrigerator for not more than one week (Goodey, 1957).

Fungal isolation and identification: Rhizospheric fungi of the various vegetable crops were isolated by using soil plate method (Warcup, 1950) and Serial dilution method (Aneja, 2003). Materials from the pure culture were mounted in Lactophenol, stained with Cotton blue and the morphological observations of hyphae, sporangiophore/conidiophore and conidia were done with the help of a Compound light microscope, Labomed CXL Mono, at 100X, 400X and 1000X magnifications.

Preparation of cell free Fungal Culture Filtrate (FCF): To test the nematicidal potential of the cell free Fungal Culture Filtrate (FCF) the most frequently occurring isolates belonging to the genera of *Aspergillus*, *Trichoderma* and *Fusarium* were selected. These strains were inoculated on to petri plates containing Potato Dextrose Agar medium and incubated for 7 to 10 days at 27°C, which served as inocula. From these actively growing cultures, one disc each of 0.5 cm diameter was transferred to 250 mL Erlenmeyer flask containing 50 mL Potato Dextrose broth. These flasks were incubated in a rotary incubator at 27±1°C and 150 rpm for 15 days. After 15 days of incubation, the cell free culture filtrate was obtained by filtering the metabolized growth medium through pre weighed Whatmann filter paper. The filtrates were used in their original concentrations to test their toxicity against nematodes.

Extraction of root knot nematode from rhizosphere: Soil samples from the rhizosphere of nematode infected cucurbits were collected and processed for nematode extraction using Cobb's

sieving and decanting method and modified Baerman's funnel technique (Southey, 1985). Populations of nematodes were counted microscopically in specially designed counting dishes.

Evaluation of nematicidal potential of fungal culture filtrates: The nematode suspension and fungal culture filtrate were pipetted into sterile multichambered counting dishes at the ratio of 1: 3 and number of nematodes counted at 0 h. The experiment was set in triplicates. The counting chambers were kept for incubation at room temperature in a moist chamber. The mortality was assessed as the mean percentage of dead (immobilized) nematodes at an interval of 24, 48 and 72 h. Sterile distilled water served as control (Radwan, 2007).

RESULTS

Fungal isolation and identification: The distribution of fungal species in the rhizosphere of Cucurbits uninfected and infected with root knot nematodes is shown in Table 1.

Twenty seven different fungal isolates belonging to various genera were isolated from six rhizosphere soil samples of four cucurbit plant species by using Serial dilution method and Warcup

Table 1: Distribution of fungal species in the rhizosphere of cucurbits uninfected and infected with Root Knot Nematodes (RKN)

Fungi	<i>L. sicereria</i>		<i>M. charantia</i>		<i>C. pepo</i>	<i>C. sativus</i>
	RKNU	RKNI	RKNU	RKNI	RKNU	RKNU
<i>Aspergillus sydowii</i>	-	-	+	+	+	-
<i>Aspergillus flavus</i>	-	+	+	-	+	-
<i>Aspergillus terreus</i>	+	+	-	+	+	+
<i>Aspergillus niger</i>	+	+	+	+	+	+
<i>Aspergillus albus</i>	-	+	+	+	+	-
<i>Aspergillus nidulance</i>	+	+	+	+	+	+
<i>Aspergillus versicolor</i>	+	+	+	+	+	+
<i>Aspergillus</i> species 1	+	-	-	-	+	+
<i>Aspergillus</i> species 2	+	-	+	-	-	+
<i>Aspergillus</i> species 3	-	-	+	-	-	-
<i>Aspergillus</i> species 4	+	-	-	-	-	+
<i>Aspergillus</i> species 5	-	-	+	-	+	-
<i>Cladosporium</i> species	+	-	-	-	+	-
<i>Humicola</i> species	-	-	-	-	+	-
<i>Penicillium</i> species	-	-	+	-	-	-
<i>Rhizopus</i> species	-	-	+	-	-	+
<i>Fusarium oxysporium</i>	-	+	+	+	+	+
<i>Tricothecium roseum</i>	-	-	+	-	-	+
<i>Alternaria alternate</i>	-	+	-	-	-	+
<i>Trichoderma viride</i>	-	+	+	+	+	+
<i>Mucor</i> species 1	-	-	-	-	-	+
<i>Mucor</i> species 2	-	-	-	-	+	-
Unidentified 1	-	-	-	-	+	-
Unidentified 2	-	-	+	-	-	+
Unidentified 3	-	-	+	-	-	-
Unidentified 4	-	-	+	-	-	-
Unidentified 5	-	-	+	-	+	-

RKNU: Uninfected with root knot nematode, RKNI: Infected with root knot nematode, Present (+), Absent (-), *L. sicereria* (*Lagenaria sicereria*), *M. charantia* (*Momordica charantia*), *C. pepo* (*Cucurbita pepo*), *C. sativus* (*Cucumis sativus*)

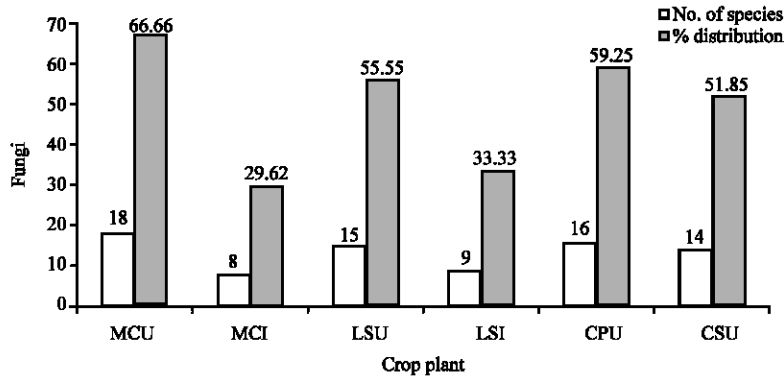


Fig. 1: No. and % distribution of fungal species associated with rhizosphere of root knot nematode infected and uninfected Cucurbits, MC: *M. charantia*, LS: *Lagenaria siceraria*, CP: *Cucurbita pepo*, CS: *Cucumis sativus*, Infected with Root Knot Nematode (I), Uninfected with Root Knot Nematode (U)

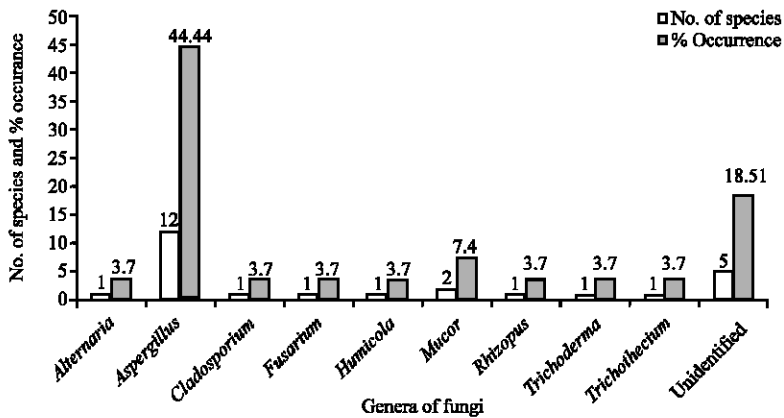


Fig. 2: Percent occurrence of various fungal genera in the rhizosphere of cucurbits

method. Twenty two species of moulds belonging to thirteen genera were identified. Five isolates could not be identified as they lacked sporulation in the conditions that were provided. Most were representatives of the class Deuteromycetes, although members of Zygomycetes and Ascomycetes were also found. The highest numbers of fungal species (18) were isolated from the rhizosphere soil of *Momordica charantia* uninfected with root knot nematodes, while 08 species were recorded from those of infected ones. Fifteen species were recorded from rhizosphere of uninfected *Lagenaria siceraria* as compared from 9 species from infected ones. Sixteen and fourteen species were respectively recorded from the rhizosphere of uninfected *Cucurbita pepo* and *Cucumis sativus*. Percent distribution of fungi was highest in *M. charantia* (66.66) followed by *C. pepo* (59.25), *L. siceraria* (55.55) and *C. sativus* (51.85) (Fig. 1). The most frequent genus was *Aspergillus* accounting 44.4% of the total mould isolates with *Aspergillus niger* being the most frequent and abundant species. The second most common genera were *Trichoderma* followed by *Rhizopus* and *Fusarium* (Fig. 2).

The various filamentous fungi isolated were identified, based on the following characteristics in accordance with the standard literature available.

Aspergillus sydowii: Colonies on Czapek's agar were blue-green, with the bluish effect prominent; velvety, growing well, attaining 3-4 cm diameter after ten days. Reverse and substratum had shades of orange to red, becoming almost black. Conidiophores mostly arose from submerged hyphae, up to 500×4-8 µm, colourless, smooth and thick-walled. Heads radiate or globose; vesicles 12-20 µm in diameter. Phialides radiated in two series; conidia globose 2.5-3.5 µm.

Aspergillus flavus: Colonies on Czapek's agar widely spreading, growing rapidly, 7.5-8.5 cm to slow growing, 3-4 cm in 10 days; conidial heads yellow when young, becoming dark yellow-green in age, in older cultures deep grey-green, reverse colourless to pale yellow brown. Conidiophores arose separately from the substratum, 400-700 or 1000 µm long×5-15 µm in diameter, broadening upward. Walls were pitted as to appear rough or spiny with low magnification, occasionally granular, gradually enlarging upward to form a vesicle 10-30 or 40 µm in diameter.

Aspergillus fumigatus: Colonies on Czapek's agar spread rapidly, white at first becoming dull blue-green, velvety to floccose; reverse colourless. Conidial heads columnar, compact often densely crowded, up to 400×50 µm. Conidiophores short, smooth, light green, up to 300 µm in length and 5-8 µm in breadth, septate, gradually enlarging into a flask shaped vesicle. Vesicles, fertile on the upper 1/2 to 2/3, 20-30 µm in diameter, bearing a single series of phialides. Phialides closely packed, 6-8×2-3 µm; conidia globose to sub globose, 2.5-3 µm, green in mass.

Aspergillus niger: Colonies on Czapek's agar rapidly growing with abundant submerged mycelium, attaining a diameter of 8-9 cm in 10 days. Conidial heads carbon black or sometimes deep brownish black. Reverse colourless to pale yellow. Conidial heads large and black, at first globose, then radiate or splitting, in well defined columns. Conidiophores arising directly from the substratum, smooth, non septate, thick walled, 1-2 mm×15-20 µm, vesicles globose, walls thick, commonly 45-75 µm in diameter, occasionally longer, bearing two series of fully packed phialides, conidia globose, at first smooth but later spinulose with colouring substance, black, 4-5 µm.

Aspergillus terreus: Colonies on Czapek's agar form tints of pinkish-cinnamon through cinnamon to deeper brown shades in age, velvety, spreading well, attaining 3.5-5 cm in 10 days. Reverse pale yellow or fairly deep brown; exudates amber coloured; conidial heads long columnar, compact with uniform diameter, cinnamon brown, 150-500×30-50 µm, conidiophores smooth, colourless, 100-250×4.5-6 µm, vesicles hemispherical, dome like, 10-16 µm in diameter, phialides biseriata; conidia globose to subglobose, 1.8-2.4 µm.

Alternaria alternata: Colonies grew rapidly on PDA, reaching 7.5-8.0 in 10 days at 27°C, olive black without aerial mycelium. Conidiophores short, simple, straight, branched or unbranched up to 50 µm long. Conidia often in long branched chains of 2-10 or more, uniform with 3-8 transverse septa, longitudinal or oblique septa present.

Fusarium oxysporum: Colonies reaching 4.5 cm diameter in 4 days at 25°C on PDA; white or peach but usually with a purple or violet tinge; reverse colourless, blue to dark purple; conidiophores unbranched, monophialidic. Stroma white, plectenchymatous, smooth, effuse; microconidia usually abundant, mostly zero septate, oval, ellipsoidal, kidney shaped or straight,

produced on simple lateral phialides, solitary on free conidiophores, never formed chains. 5-12×2.5-3.5 µm; Macroconidia 2-5 septate, spindle to fusiform, curved or almost straight, pointed at both ends, 27-60×3-5 µm.

***Humicola* species:** Colonies effuse, cottony, at first white, later grey brown with age. Mycelia, superficial and immersed, becoming 4 cm diameter in 10 days. Hyphae, hyaline, 0.5-2.5 µm wide, bearing masses of chlamydospores and conidia. Conidia white at first, rough, thick walled, typically globose, one celled.

***Mucor* species:** Colonies fast growing, filling petridish in 3-5 days, often several cm high, white to yellow, becoming dark grey with the development of sporangia. Mycelia present in and on the substratum but without rhizoids or stolons. Sporangiphores forming a thick turf, springing from the mycelium, either unbranched with terminal sporangia or branched.

***Rhizopus* species:** Colonies very fast growing, 1-2 cm high, white at first, pale, grey, dark brown-grey, reddish brown, brownish black. Mycelium branched, non septate, stolons and rhizoids present; sporangiphores arise from these points, sporangia globose, hemispherical, zygospores round or oval.

***Trichoderma viride*:** Colonies grew rapidly up to 9 cm in 4 days, watery white, becoming hairy from the formation of loose scanty aerial mycelium, floccose to arachnoid; somewhat whitish; Conidiation effuse or in compact tufts; reverse uncolored; chlamydospores common, intercalary or terminal. Conidiophores much branched; arise in compact or loose tufts. Main conidiophores 4-5 µm, producing smaller side branches. Conidia globose or short obovoid, or broadly ellipsoidal, bluish green to dark green, 3.5-4.5 µm or 4-4.8×3.5-4 µm.

***Trichothecium roseum*:** Colonies growing fast, reaching 7-8 cm diameter in 10 days, pinkish, zonate in diurnal rhythm, powdery due to conidial formation; reverse colourless to light pink; exudates lacking; conidiophores erect, produced singly or in groups, mostly simple, hyaline and septate. Conidia ellipsoidal to pyriform, two celled, 12-23×8-10 µm, smooth, thick walled, hyaline.

***Cladosporium cladosporioides*:** Colonies reaching 3-5 cm diameter in 10 days at 27°C, velvety, light olive green to olive green; reverse olivaceous black; conidiophores macronematous, light olivaceous brown, smooth, without swellings and sympodial elongations, branching acropleurogenously, producing conidia in chains below septum, 35-340×2-6 µm. Conidia formed in long branched chains, aseptate, ellipsoidal, olive green, olivaceous brown, 3.5-10.2×2.6-3.8 µm.

***Aspergillus nidulance*:** Colonies fast growing, reaching 6-7 cm diameter in 7 days, smooth walled conidiophore, small echinulate conidia 3-4 µm in diameter. Quickly ripening ascospores, purple red in color and with equatorial binding and large thick walled, globose bodies, termed "hulle cells" forming an irregular layer about the perithecia.

***Aspergillus albus*:** Colonies on Czapek Dox agar fast growing, velvety, felted or floccose, plane, attaining a diameter of 4.0-8.5 cm in 4 to 12 days at 25-35°C. Reverse colorless to bright yellow, green, dark red, brown or even black in age. Conidiophore short, sometimes faintly yellow, upto 575.0 µm to 2.8 to 10.0 µm, conidia globose to subglobose.

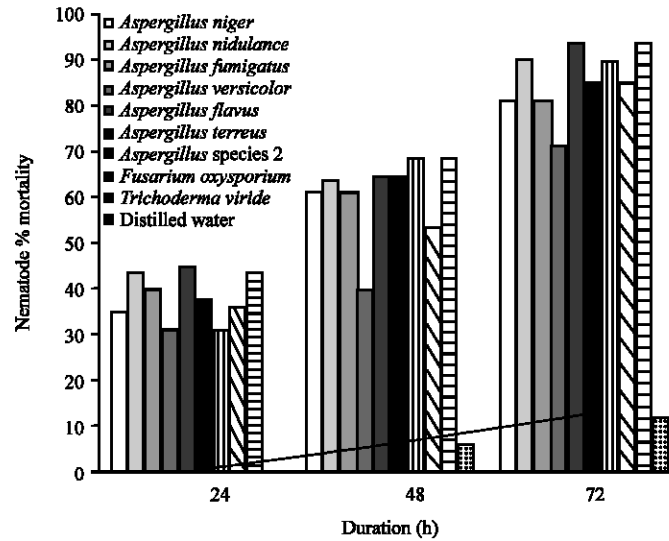


Fig. 3: Percent mortality of nematodes in response to culture filtrates of various fungal genera

***Aspergillus versicolor*:** Colonies on Potato Dextrose Agar (PDA) at 25°C grew rapidly, reaching 21-23 mm in diameter after 7 days and 47-49 mm after two weeks, with sometimes centrally rising, velvety to rather floccose. Colour blue-green, exudates absent and reverse pale yellowish. It was interesting to find that a large number of heads were fragmentary and like penicillium. Conidiophores monoverticillate or biverticillate, hyaline, smooth-walled, 7.0-9.0×2.0-3.0 μm. Metulae 7.0-10.0×2.0-3.0 μm long. Phialides, 7.0-8.0×2.0-3.0 μm long, flask-shaped.

***In vitro* assessment of nematicidal property of fungal filtrates (Fig. 3):** Among the nine fungi tested for nematicidal properties, the filtrate of *Aspergillus flavus* showed maximum mortality (45%) after 24 h. After 48 h maximum mortality was shown by *Aspergillus species 2* and *Trichoderma viride* (68.75%). After 72 h, maximum mortality was reported by filtrates of *Trichoderma viride* and *Aspergillus flavus* (93.75%). Minimum activity was exhibited by *Aspergillus species 2* and *Aspergillus versicolor* after 24 h (31.25%) and after 48 h minimum mortality in *Aspergillus versicolor* and *Fusarium oxysporium* (40 and 53.75%). After 72 h minimum mortality was reported by filtrate of *Aspergillus versicolor* (71.25%). The mortality rate of filtrate of *Fusarium oxysporium* was only 36.25% after 24 h but showed 85% mortality after 72 h. All the fungal filtrates tested showed some percentage of toxicity as compared to the control experiment using distilled water which showed zero mortality after 24 h and 6.25% mortality after 48 h and 12.5% mortality after 72 h.

DISCUSSION

Results show that the highest numbers of fungal species were isolated from the rhizosphere soil of *Momordica charantia* control (18 species). Lowest number was recorded from the rhizosphere of *Momordica charantia* infected (08 species) and *Lagenaria siceraria* infected (09 species). This result is in accordance with that of Altalhi (2004) and Abou-Zeid and El-Fattah (2007). Altalhi (2004) reported that the highest numbers of fungal colonies were recorded in the rhizosphere soil of *Artemisia judaica* and the lowest number in *Desmostachya bipinnata*. According to Abou-Zeid

and El-Fattah (2007) the highest number of fungal populations were found in the rhizosphere of *Asphodelus aestivus* followed by *Pulicaria crispa* and the lowest numbers were reported on the rhizosphere of *Argemone ochroleuca*.

Twenty seven different fungal isolates belonging to various genera were isolated from six rhizosphere soil samples of four cucurbit plant species by using Serial dilution method and Warcup method. Most frequent genera was *Aspergillus* accounting 44.4% of the total mold isolates and *Aspergillus niger*, the most frequent and abundant species. This finding supports the observation made by Abdel-Hafez (1982a). According to their study, *Aspergillus* contributed the broadest spectrum of species in the rhizosphere and *Aspergillus niger* the most frequent fungi. These results are also agreeable with Abdel-Hafez (1981, 1982b) who isolated different fungal genera and species from the desert soils in Saudi Arabia. The present results also agree with El-Abyad *et al.* (1982) and Abdel-Hafez and Shoreit (1985) who isolated 70 species and 31 genera from 20 dust samples of Taif, Saudi Arabia and the most common genera were *Aspergillus*, *Fusarium*, *Penicillium* and *Mucor*.

In the present study the second most common genera was *Mucor*. Hence, it can be concluded that the frequency and type of fungal species vary in different cucurbit plants, both control and infected.

The effect of culture filtrates on nematodes differed according to nematode and fungal biological groups as well as exposure time. The nematicidal activity of culture filtrate from the most frequently occurring isolate nine fungal species the filtrate of *Aspergillus flavus* showed maximum mortality (45%) after 24 h. After 48 h maximum mortality *Trichoderma viride* and *Aspergillus* species showed (68.75%). These result also agreed with Siddiqui *et al.* (2004) and Zareen *et al.* (2001). After 72 h maximum mortality was reported by filtrates of *Aspergillus flavus* and *Trichoderma viride* (93.75%). Although, *Trichoderma* spp. are known to suppress plant-parasitic nematodes (Sharon *et al.*, 2001; Jegathambigai *et al.*, 2011), in this study its fungal culture filtrate had a toxic effect on *M. incognita*. Minimum activity was exhibited by *Aspergillus* species and *Aspergillus versicolor* after 24 h (31.25%) and after 48 h minimum mortality was reported by *Fusarium oxysporium* (53.75%). After 72 h minimum mortality was reported by filtrate of *Aspergillus versicolor*. Hence, it may be expressed undoubtedly that the culture filtrates of *Aspergillus* species different have a toxic effect on *M. incognita* similar to the results of Siddiqui *et al.* (2004) and Amer-Zareen *et al.* (1999). In contrast to the finding by Radwan (2007), filtrates of *Aspergillus niger* showed only 35% mortality after 24 h and 81.25% after 72 h. According to his study, filtrate of *Aspergillus niger* exhibited 100% mortality after 12 h in original concentration and *Fusarium oxysporum* showed 52% mortality after 12 h and 78.8% after 48 h. According to present investigation, the mortality rate of filtrate of *Fusarium oxysporum* was only 36.25% after 24 h but showed 85% mortality after 72 h. However, all the fungal filtrates tested showed some percentage of toxicity as compared to the control experiment using distilled water which showed zero mortality after 24 h and 6.25% mortality after 48 h and 12.5% mortality after 72 h (Fig. 3). The nematicidal efficacy of each fungal filtrate increased with duration of exposure.

CONCLUSION

The variations in the nematicidal action of the tested fungal filtrates may be attributed to the differences in number and nature of metabolites produced by different fungi and would be worth investigating. The result of the present study suggests that the various fungi associated with the rhizosphere soil of different vegetable crops could be used as a biocontrol agent of nematodes and the culture filtrates can serve as a source of novel nematicidal compound of fungal origin which is

more environmental friendly. However, the effective components of the fungal metabolites, which are responsible for the nematicidal activity in the filtrate, need to be purified and characterized.

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