IN VITRO SCREENING OF FUNGI FOR PARASITISM AGAINST SUGAR BEET CYST NEMATODE *HETERODERA SCHACHTII*

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Summary. *Heterodera schachtii* is one of the major limiting factors in sugar beet growing areas in Iran. Thirty fungal strains, isolated from the beet cyst nematode, were studied on water agar for their pathogenicity to the nematode. The rate of colonization of eggs by different fungi and hatch of second stage juveniles were measured after a month on water agar at 20 °C. The largest and smallest colony growth on potato dextrose agar medium, recorded after 5 days at 25 °C, were 67 and 12 mm for *Chrysosporium keratinophilum* and *Rhizoctonia* sp., respectively. Species of *Fusarium* grew from 26 to 68 mm, *Pochonia chlamydosporia* var. *chlamydosporia* and *Phoma* 17 mm, *Gliocladium* 27 to 37 mm, *Cylindrocarpon* 22 mm and *Paecilomyces lilacinus* 30 mm. The most virulent isolates were *Gliocladium cf. roseum, F. oxysporum, Ph. pomorum*, *P. c.* var. *chlamydsoporia* and *F. equiseti*, which colonized 72, 68, 67, 63 and 58% of the eggs respectively; 13 to 20% of juveniles hatched on these plates. *Fusarium graminiarum*, *E. chlamydospora*, *F. acuminatum armeniacum*, *C. destructans*, *P. lilacinus* and *Rhizonctonia* sp., with 51 to 41% pathogenicity, were moderately virulent. *Fusarium polyphialidicum*, *F. acuminatum acuminatum*, *G. roseum*, *F. oxysporum*, *F. solani*, *F. equiseti*, *C. obtusisporum*, *C. keratinophilum* and *F. camptoceras* were less virulent strains to *H. schachtii* and parasitized 38 to 12% of the immature eggs. Non-spore-producing strains colonized 18 to 50% of the eggs on agar.

Sugar beet culture in Iran began around 1896 and sugar beet cyst nematode (BCN), *Heterodera schachtii* Schmidt was first reported by Omidvar in 1968 (Omidvar, 1968) from Fars, and then from Khorasan, West Azarbaijan and Kohkiloieh (Schafer and Esmailpour, 1969; Talachian *et al*., 1976; Kalali and Farivar-Mahin, 1979; Sharafeh and Timoori, 1980; Noori *et al*., 1980; Akhiyani *et al*., 1993).

The average level of infestation of beet cyst nematode in some of these fields with a long history of sugar beet culture can reach 60 eggs/g soil and lack of proper management strategy and legislative measures has made *H. schachtii* one of the major limiting factors in beet production, both for subsistence farmers and the sugar beet industry (Kalali and Farivar-Mahin, 1979; Fatemy *et al*., 1999; Ahmadi and Damadzadeh, 2000; Akhiyani *et al*., 2001).

Biological agents, including antagonistic fungi, can play a role in the integrated management of *H. schachtii.* The species constituting the mycoflora of heteroderid nematodes are a relatively diverse group and some of them are reported as both animal and plant pathogens (Samson, 1974; Webster, 1985; Hoog *et al*., 2000).

Species of *Pochonia*, *Paecilomyces*, *Cylindrocarpon*, *Gliocladium*, *Fusarium* and many others have frequently been reported from cyst and root-knot nematodes from many countries around the world and some of them have been isolated from soils that are suppressive to cyst nematodes (Kerry and Crump, 1977; Rodriguez-Kabana and Morgan-Jones, 1988; Crump, 1989; Qadri and Saleh, 1990; Roccuzo *et al*., 1993; Crump and Flynn, 1995; Verdejo *et al.*, 2002).

The study of endemic mycoflora associated with BCN cysts in Iran was initiated when *Paecilomyces fumosoroseus* was isolated from eggs of *H. schachtii* (Fatemy, 1993) and further work has reported several strains of fungi associated with BCN (Fatemy, 1998, 2000; Fatemy *et al*., 1999; Saeidi Naeini and Fatemy, 2000). Further research is being undertaken to screen these fungi for their pathogenicity towards *H. schachtii*.

The objective of this paper is to report on the identification and *in vitro* screening of 30 strains of fungi isolated from eggs of *H. schachtii* taken from sugar beet fields in different regions of the country.

MATERIALS AND METHODS

Production of *H. schachtii*. To provide enough inoculum of *H. schachtii* for the experiments, sugar beet seedlings of cultivar IC1 were planted in soil infested with the nematode. Two months later, 200-g sub-samples of moist soil were processed through 850- and 250-µm sieves (Kim and Riggs, 1995) and females and cysts were hand picked with the help of a stereomicroscope.

To obtain uninfected, healthy eggs for the tests, females and young cysts of *H. schachtii* were surface sterilized with 0.1% NaOCl for 30 seconds, rinsed with sterile distilled water and transferred aseptically to 9-cm diameter Petri dishes containing potato dextrose agar (PDA) medium (17 g/l, Difco, USA), for 48 h. Only healthy uninfected individuals that did not develop fungal colonies were used for the three separate tests of pathogenicity that were done. An egg suspension was

prepared by crushing the selected females and young cysts between a microscope slide and cover slip and washing them into a beaker. The suspension was stirrered for 30 seconds at low speed with an electric stirrer. To separate the eggs from the cyst debris, the egg suspension was passed through a 250-µm sieve and collected on 10-µm sintered glass filters, all under sterile conditions and with the aid of a vacuum pump. The eggs were rinsed on the filters five times with sterile distilled water (Kerry and Crump, 1977; Fatemy, 1998). The eggs were then resuspended in a known volume of water to give about 200 eggs per ml.

Pathogenicity tests. For the three pathogenicity tests, 8 g of agar (Difco, USA) were put in a conical flask, made up to 1 litre with distilled water, mixed, and autoclaved at 121 °C for 20 min. One hundred ppm each of streptomycin sulphate, chlorotetracycline hydrochloride and chloramphenicol were added when the temperature of the medium had cooled to about 45 °C. Plates were poured by adding 11 ml of the medium to 5-mm diameter sterile Petri dishes. Under sterile conditions, the centre of each Petri dish was inoculated with a 5-mm disk of the fungus growing actively on PDA. Uninfected PDA disks were added to control dishes and all dishes were kept at 20 °C for a month in the dark. One ml of the egg suspension was spread on the surface of each dish, and there were four replicates per treatment arranged randomly in an incubator at 20 °C (Kerry and Crump, 1977; Fatemy, 1998). After a month, the content of each dish was transferred to a blender (Waring), mixed in 200 ml of distilled water for 30 seconds, and passed through a 0.2-mm sieve to remove agar pieces followed by a 20-µm sieve to collect eggs and juveniles (Fatemy, 1998). The contents of this sieve were washed into a beaker with water and the whole suspension was observed, 1 ml at a time, at 20× and 40× magnification on an inverted microscope to estimate the number of infected and uninfected immature and mature eggs and emerged juveniles.

Fungal cultures. Fungal strains originally isolated from eggs and juveniles of *H. schachtii* (Fatemy *et al.*, 1999) were kept on PDA at +4 °C and re-cultured on PDA for further identification and pathogenicity tests. Slant and slide culture identification was carried out on two types of media, based on macroscopic and microscopic characters such as colony diameter, colour, topography, presence or absence of pigments, morphology of conidia, conidiophores, chlamydospores, etc. Strains were grown on 25 ml PDA slants in 20×2-cm tubes kept at 25 °C in the dark for 8 days, or longer in the case of slow growing strains, and re-examined after 2 months for sexual reproduction.

Slide cultures were prepared for microscopic measurements. Under sterile conditions, a PDA cube measuring 3×12×12 mm (height × width × length) was transferred to a slide, infected with a fungal isolate, covered with an 18×18 mm cover slip and placed on a sterile V-shaped plastic drinking straw, 12 cm long, in a 9-cm Petri dish containing 5 ml of sterile distilled water and kept at 25 °C in an incubator. Two such preparations were made per isolate and, on day 3 and 6 (9 and 12 for some strains), the PDA cube was removed and two slides were made of the infected cover slips and slides. The slides were then stained with a drop of 0.05% cotton blue lactophenol, sealed, and growth measurements were made at 20× and 40× magnification under a light microscope.

To identify *Fusarium* to species level, carnation leaf agar medium was used (Burgess *et al.*, 1994). Pieces of carnation leaves of 5×5 mm, untreated with any pesticides, were oven-dried at 70 °C for 4 hr and five pieces placed on the surface of 15 ml of 2% sterile water agar in a 7-cm Petri dish. A single microconidium was put near the leaf pieces and, after 6-10 days, measurements were taken of the sporodochium, and vegetative and reproductive organs. Identification of some very similar strains was made after 3 days at 25 and 30 °C or after 13 days at 12 hour intervals of light and dark at 25 and 20 °C respectively.

Other culture media used for the identification of some strains of *Fusarium*, *Cylindrocarpon*, *Paecilomyces*, *Pochonia* (formerly *Verticillium*), *Phoma*, *Chrysosporium* and non-spore-producing isolates were soil agar medium, malt agar, Sabouraud's dextrose agar, potato-carrot agar and corn meal agar (Booth, 1966; Gams, 1971; Samson, 1974; Ellis, 1976; Domsch *et al.*, 1980).

RESULTS

One strain of *Pochonia chlamydosporia* var. *chlamydosporia*, *Paecilomyces lilacinus*, *Chrysosporium keratinophilum*, *Embellisia chlamydospora*, *Cylindrocarpon destructans*, *Rhizoctonia* sp., *Phoma pomorum*, *Fusarium solani*, *F. polyphialidicum*, *F. graminiarum*, *F. camptoceras*, *F. acuminatum acuminatum*, and *F. acuminatum armeniacum*, two strains of *Gliocladium roseum*, *G. cf. roseum*, *C. obtusisporum*, and *F. equiseti*, four isolates of *F. oxysporum*, and seven non-spore-producing isolates were among the fungi identified.

There were large differences in the diameter of growth of colonies between the various species when data for morphometric measurements were compared after 5 days on PDA at 25 °C (Fig. 1). The largest and smallest colony diameters (67 and 12 mm) were for *C. keratinophilum* and *Rhizoctonia* sp., respectively; species of *Cylindrocarpon* grew 22 mm, *Gliocladium* between 27 and 37 mm, *Pochonia* and *Phoma* nearly 17 mm, *Embellisia* 46 mm, *P. lilacinus* 30 mm and species of *Fusarium* from 26 to 68 mm diameter during this period.

In the first pathogenicity test, the isolates showed various pathogenicity effects, with the maximum recorded kill of just 51% (Fig. 2). *Cylindrocarpon obtusisporum* and three sterile strains infected 51, 50, 48 and

47% of the eggs, respectively; a strain of *C. obtusisporum*, two isolates of *F. oxysporum* and *F. solani* penetrated 25 to 36% of the eggs on water agar ($P < 0.05$).

Of ten strains evaluated in the second pathogenicity test, *F. graminiarum* infected nearly 51% of the eggs, followed by *P. lilacinus*, a sterile isolate and *Rhizoctonia* sp., which each killed 41% of the immature eggs (Fig. 3) ($P < 0.05$). Mycelium of different species of *Fusarium*, including *F. polyphialidicum, F. oxysporum, F. equiseti*, and *F. camptoceras*, was found in 38 to 12% and two sterile strains in less than 20% of the young eggs.

In the third pathogenicity test, between 50 and 72% of the unembryonated eggs of *H. schachtii* were penetrated by mycelium of *E. chlamydospora*, *F. equiseti*, *P. c. var. chlamydosporia*, *Ph. pomorum*, *F. oxysporum* and *G. cf. roseum* (Fig. 4) ($P < 0.05$). The percentages of eggs infected by other fungi were 49% for *C. destructans* and *F. a. armeniacum*, 32% for *G. roseum* and 14% for *C. keratinophilum*.

The fungi that colonized more eggs allowed less egg hatch, although correlation was not consistent among different isolates (Fig. 5).

DISCUSSION

The spore-producing fungi in this study belonged to nine genera and 18 species, and seven isolates could not be identified. *Fusarium* species comprised 40% of the total isolates and were the most commonly occurring fungi at all sites (Hay and Skipp, 1993; Fatemy *et al.*, 1999).

Most of these fungi have frequently been isolated from heteroderid nematodes from North and South America, Europe, Asia and Australia, supporting the view that a certain group of fungi occur regularly in association with Heteroderidae in widely scattered geographical locations (Willcox and Tribe, 1974; Kerry, 1975; Oshima and Akazawa, 1980; Lal *et al.*, 1982; Freirie and Bridge, 1985; Rodriguez-Kabana and Morgan-Jones, 1988; Qadri and Saleh, 1990; Stirling, 1991; Hay and Skipp, 1993).

The mycelium of all isolates of fungi tested in our experiments penetrated eggs of *H. schachtii* on water agar, with *G. roseum* and *F. camptoceras*, parasitizing 72 and 12% of the eggs respectively, being the most and least virulent isolates evaluated (Figs. 2-4). *Phoma pomorum*, *P. c. var. chlamydosporia*, *C. obtusisporum*, *E. chlamydospora* and *F. a. acuminatum*, *F. graminiarum*, *F. equiseti* and *F. oxysporum* were the most pathogenic spore-producing fungi, destroying more than 49% of the eggs on agar.

Wide variations in virulence of fungal species and isolates of a species against nematodes have been reported by several researchers (Bursnall and Tribe, 1974; Rodriguez-Kabana *et al.*, 1984; Irving and Kerry, 1986). In our tests (Figs. 2-4), three isolates of *F. oxysporum* parasitized 68% and one isolate 32%, whilst two strains of *C. obtusisporum* and *G. roseum* infected 25% and 51%, and 72 and 32% of the eggs, respectively.

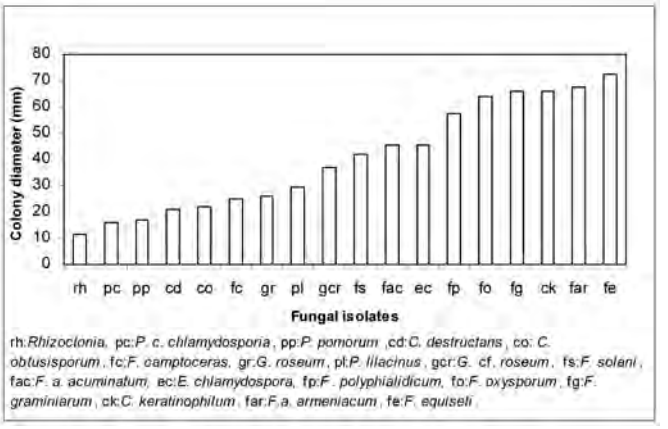

Fig. 1. Radial growth of fungi isolated from *Heterodera schachtii* on PDA after 5 days at 25 ºC.

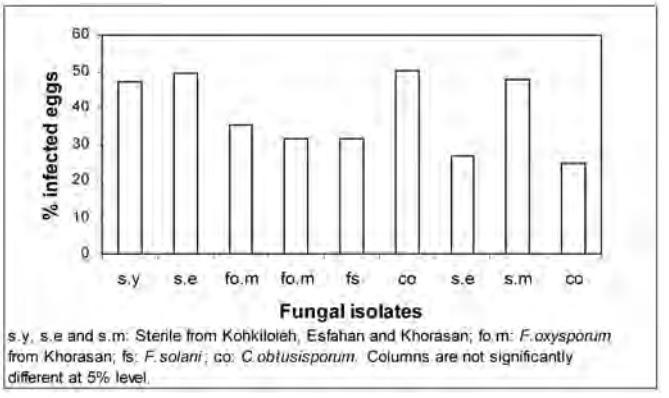

Fig. 2. Pathogenicity of fungi on eggs of *H. schachtii* at 20 ºC after 30 days on water agar, test 1.

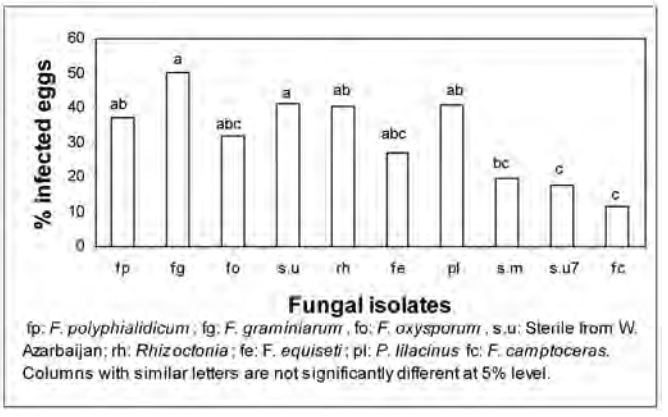

Fig. 3. Pathogenicity of fungi on eggs of *H. schachtii* at 20º C after 30 days on water agar, test 2.

Nearly all of the eggs infected by the different fungi were at the embryonic stage and none of the isolates were found to parasitize mature eggs or juveniles, confirming the suggestion that immature eggs are more susceptible to invasion by some fungal strains (Irving and

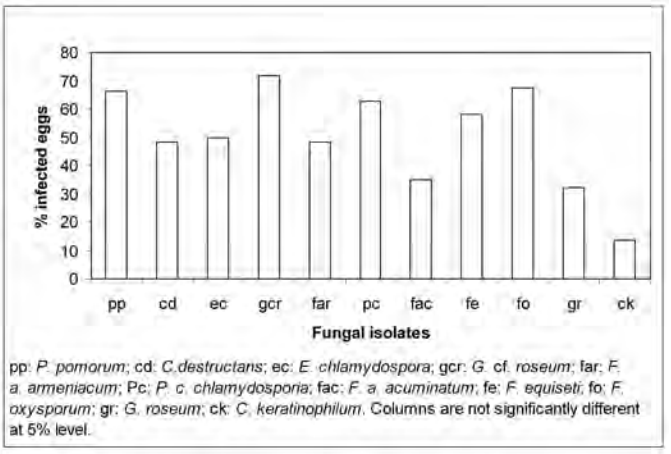

Fig. 4. Pathogenicity of fungi on eggs of *H. schachtii* at 20 ºC after 30 days on water agar, test 3.

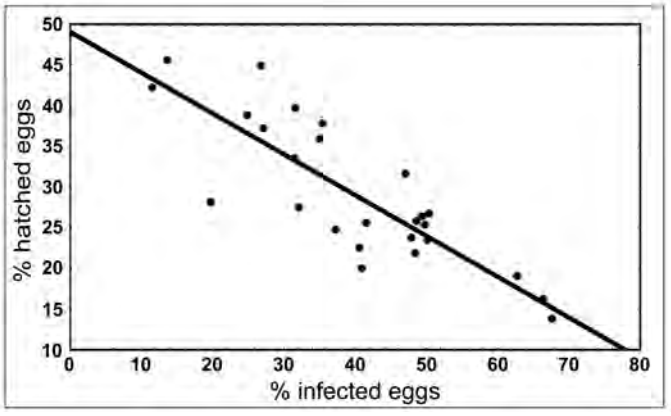

Fig. 5. Relationship between fungal infection and hatching of eggs of *H. schachtii*.

Kerry, 1986; Roccuzzo *et al.*, 1993). Furthermore, by the end of the experiments there were still a number of uninfected eggs, suggesting that the effects of other factors, such as metabolites, in killing or inhibiting hatch of juveniles could not be ruled out in our experiments (Caroppo *et al.*, 1990; Coosemans, 1991).

Fungi associated with heteroderid nematodes show different temperature requirements between and within genera and isolates (Dackman and Nordbring-Hertz, 1985; Kerry *et al.*, 1986; Rodriguez-Kabana and Morgan-Jones, 1988; Hojat-Jalali and Cooseman, 1997). Our data tend to confirm these findings in that mycelium development on potato dextrose agar at a constant temperature varied between different genera (Fig. 1). Some of the isolated fungi have a narrow and some a wide range of growth temperature optima. Isolates of *F. solani* and *F. oxysporum* grew well between 10 and 30º C, with maximum growth occurring at 25-30 ºC (Fatemy, unpublished). *Paecilomyces fumosoroseus* grew slowly at 15 and faster at 20 and 25 ºC on PDA (Fatemy, 1998). Kerry *et al.* (1986) found the optimal temperature for growth of some strains of *Pochonia chlamydosporia* to be 18 ºC and for others 20-25 ºC. Our isolates of *P. c.* var. *chlamydosporia* also grew between 15 and 30 ºC, with maximum growth at 25 ºC (Alizadeh and Fatemy, 2004).

The regions from where these fungi were isolated are located in temperate and cold parts in north-east, north-west and central parts of Iran, at altitudes of around 1000 m above sea level. The minimum average temperature in winter in these areas may fall to -7 to -14 °C. Sugar beet is planted in April and harvested in late autumn, during which period *H. schachtii* completes three generations but only begins the fourth, because plants are harvested by then (Kalali and Farivar-Mahain, 1979; Parvizi *et al.*, 1998; Sharafeh and Farsinejad, 1993; Damadzadeh *et al.*, 1995).

The variation in temperature demand for growth of the fungi isolated from these fields indicates that some are more active during cooler parts of the growing season, some during warmer parts and some are infective over the whole growing season. Therefore, when fungi virulent towards BCN occur, the overall effects of different fungi can limit (or even decrease) the development of the nematode population in the field.

The results of this study indicate that there are species of fungi that are potential biocontrol agents for nematodes in Iran and that additional research in natural conditions is needed to evaluate some of these fungal species.

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