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Screening culture filtrates of fungi for activity against *Tylenchulus semipenetrans*

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Abstract

Culture filtrates of 20 fungi isolated from citrus soils were screened for their activity against *Tylenchulus semipenetrans* in both *in vitro* and greenhouse tests. The filtrates of *Talaromyces cyanescens* (isolates 2-4 and 2-5), *Paecilomyces lilacinus, Chaetomium robustum, Acremonium strictum, Engyodontium album, Myrothecium verrucaria, Emericella rugulosa*, and *Tarracomyces gigaspora* consistently inhibited the motility of second-stage juveniles at various concentrations of the filtrate. Dose-response models were used to determine the filtrate concentration required to inhibit the motility of 50% of the juveniles (CI50). The culture filtrate of *P. lilacinus* showed the highest activity with a CI50 value of 58% that differed from that of *C. robustum* (CI50 = 68%), and *A. strictum* CI50 = 82%. The culture filtrates of *P. lilacinus*, *E. album*, and *T. cyanescens* 2-5 maintained their activity when autoclaved at 120ºC for 20 min. The autoclaved filtrate of *T. cyanescens* 2-4 was more effective at inhibiting juvenile motility (CI50 = 28%) than that of *T. cyanescens* 2-5 (CI50 = 80%), *C. robustum* (CI50 = 72 %) and *P. lilacinus* (CI50 = 72%). The culture filtrate of *T. cyanescens* 2-4 also inhibited egg hatching. Nematode reproduction on Cleopatra mandarin and Carrizo citrange were respectively reduced by the culture filtrate of *P. lilacinus* and the autoclaved filtrate of *T. cyanescens* 2-4. These results support the hypothesis that soil fungi may contribute to regulate nematode densities by the production of secondary metabolites with nematicidal activity.

Additional key words: Carrizo citrange, citrus nematode, Cleopatra mandarin, nematicidal activity, soil-borne fungi.

Resumen

Evaluación de filtrados de cultivos fúngicos de hongos por su actividad frente a *Tylenchulus semipenetrans*

Se evaluó la actividad de filtrados de cultivos de 20 hongos aislados del suelo en parcelas de cítricos frente a *Tylenchulus semipenetrans* mediante ensayos *in vitro* y en invernadero. Los filtrados de *Talaromyces cyanescens* (aislados 2-4 y 2-5), *Paecilomyces lilacinus, Chaetomium robustum, Acremonium strictum, Engyodontium album, Myrothecium verrucaria, Emericella rugulosa*, y *Tarracomyces gigaspora* inhibieron consistentemente la movilidad de los juveniles de segundo estadio del nematodo a varias concentraciones del filtrado. Se utilizaron modelos de dosis-respuesta para determinar la concentración del filtrado que inhibía la movilidad del 50% de los juveniles (CI50). El filtrado del cultivo de *P. lilacinus* mostró la máxima actividad entre todos los ensayados con un valor de CI50 del 58%, la cual era superior a la de los filtrados de *C. robustum* (CI50 = 68%) y *A. strictum* (CI50 = 82%). Los filtrados de *P. lilacinus*, *E. album*, y *T. cyanescens* 2-5 retenían su actividad después de autoclavarlos a 120ºC durante 20 min. La inhibición de la movilidad de los juveniles obtenida con el filtrado autoclavado de *T. cyanescens* 2-4 (CI50 = 28%) fue superior a la de los filtrados de *T. cyanescens* 2-5 (CI50 = 80%), *C. robustum* (CI50 = 72%), ó *P. lilacinus* (CI50 = 72%). El filtrado de *T. cyanescens* 2-4 también inhibió la eclosión de los huevos del nematodo. La reproducción del nematodo en mandarino Cleopatra y citrange Carrizo fue menor cuando los juveniles se incubaron en el filtrado de *P. lilacinus* y en el filtrado autoclavado *T. cyanescens* 2-4, respectivamente. Estos resultados apoyan la hipótesis de que los hongos del suelo pueden contribuir a la regulación de las poblacionales de los nematodos mediante la producción de metabolitos secundarios con acción nematicida.

Palabras clave adicionales: actividad nematicida, citrange Carrizo, hongos del suelo, mandarino Cleopatra, nematodo de los cítricos.

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Introduction

The citrus nematode *Tylenchulus semipenetrans* Cobb causes the disease known as slow decline, whose name refers to the gradual development of symptoms in the host, and the slow rate of nematode population growth in newly replanted orchards. Most studies estimate yield losses due to *T. semipenetrans* to be in the range of 10 to 30%, depending on the level of infection, the susceptibility of the rootstock, the climatic conditions, the soil characteristics, the presence of other pathogens or natural enemies, and orchard management practices (Duncan and Cohn, 1989; Duncan, 1999). In Spain, the tolerance limit of Clementine mandarin to *T. semipenetrans* has been estimated at 287 females per g of root in spring samples (Sorribas *et al.,* 2008). Management of the citrus nematode remains difficult as no single tactic provides adequate control of the nematode (Verdejo and McKenry, 2004).

Fumigant and non-fumigant nematicides have been used for nematode control for the last 50 years. However, increasing concerns over risks to the environment and human health have led to the withdrawal or restriction of some chemicals. For instance, methyl bromide, the most effective and widely used soil fumigant, has already been banned in developed countries in accordance with the Montreal Protocol. The use of some non-fumigant nematicides based on organophosphates and carbamates has been restricted depending on the region, crop, and production system, such as integrated production and organic farming. Consequently, the search for naturally occurring compounds with nematocidal activity has been stimulated as an alternative to using existing compounds (Chitwood, 2002). Soil fungi that inhabit the rhizosphere may serve as a source for such compounds since they share the same environment as the nematodes and produce metabolites as a strategy for protecting their habitat from plant-parasitic nematodes or other organisms (Anke and Sterner, 1997). These fungi may release compounds that directly kill nematodes, suppress nematode motility, reduce egg hatching and/or interfere with metabolic processes. Through such mechanisms, they could regulate nematode populations.

The objective of this study was to screen culture filtrates of selected fungi isolated from citrus soils to determine their activity against *T. semipenetrans* juveniles. The effects of filtrates of *Paecilomyces lilacinus* and *Talaromyces cyanescens* on nematode reproduction in greenhouse tests are also reported.

Material and methods

Fungi and preparation of the culture filtrates

Fungi were isolated from soil collected around citrus roots in orchards in Catalonia, Spain, except for *P. lilacinus* that was isolated from a female of *T. semipenetrans*. To recover the fungi, aqueous solutions (5 mL) of phenol (2% w/v), acetic acid (5% w/v), ethanol (60% w/v) or 2- furfuraldehide (10-3 M) (Warcup and Barker, 1963; Udagawa and Uchiyama, 1998; Stchigel *et al.,* 2001) were added to test tubes containing approximately 1 g soil. They were shaken vigorously for 1 min and left to settle for 9 min. The upper aqueous fraction was discarded, and the sediment was then resuspended in sterilized water (12 mL) and aliquots of the suspensions were plated in six 9 cm-diam Petri dishes. Potato carrot agar (PCA; potatoes 20 g, carrot 20 g, agar 20 g, tap water 1 L) with the addition of chloramphenicol (150 mg L^{-1}) and diehdrin (0.2 g L^{-1}) melted at 50-55°C was poured over the suspension and mixed. Plates were incubated at 15, 25 and 35°C, alternating periods of 12 h of darkness with 12 h of exposure to cool white fluorescent light. They were then examined every week, with the aid of a stereoscopic microscope, over a period of up to 2 months. Fungal colonies were transferred to 5 cm-diam Petri dishes containing either oatmeal agar (OMA; oatmeal 30 g, agar 15 g, water 1 L) or PCA to establish pure cultures. The fungal isolates were characterized both culturally and morphologically, and identified based on the bibliography. The taxonomic position of the fungi used to obtain culture filtrates is indicated in Table 1.

The isolates were grown on OMA and 5 mL fungal suspensions obtained from these cultures were added to 500 mL borosilicate glass flasks with chloramphenicol (100 mg L^{-1}) and 200 mL Saboureaud broth (meat peptone 10 g, sucrose 40 g, water 1 L). The flasks were maintained motionless and at a constant temperature under 12 h of darkness alternated with 12 h of cool white fluorescent light for 15-30 days, with the period depending on the growth rate of each fungal species.

Abbreviations used: CI50 (concentration that inhibit the motility of 50% of the juveniles), J2 (second-stage juveniles), OMA (oatmeal agar), PCA (potato carrot agar), SDW (sterile deionized water).

Table 1. Identity of the fungal species isolated from citrus soils and used to obtain the culture filtrates and their respective isolate codes

Fungal species	Code
Acremonium strictum W. Gams	2-1
Acrophialophora fusispora (S.B. Saksena) Samson	3-3
Antrhacobia melaloma (Alb. & Schwein.) Arnould	1-7
Chaetomium robustum L. M. Ames	2-2
Emericella nidulans (Eidam) Vuillemin	1-6
Emericella rugulosa (Thom & Raper) C. R. Benj	V-2
Engyodontium album (Limber) de Hoog	1-1
Gliocladium roseum Bainier	4-15
Hapsidospora irregularis Malloch & Cain	1-9
Leptotrichopyxis sphaerospora Stchigel & Guarro	4-9
Monascus purpureus Went	1-3
Metarhizium anisopliae (Metschinikoff) Sorokin	IV-3
Myrothecium verrucaria (Alb. & Schwein.) Ditmar	V-4
Neosartorya glabra (Fennell & Raper) Kozakiewicz	1-11
Paecilomyces lilacinus (Thom) Samson	8260
Talaromyces cyanescens Stchigel & Guarro	2-4
T. cyanescens	2-5
T. cyanescens	1-4
Tarracomyces gigaspora Stchitgel & Guarro	1-8
Xylariaceae	2-8

The fungal mass was then removed through gauze, and the liquid phase was sterilized by filtration through a cellulose acetate membrane (0.45 µm diam., Whatman International Ltd.). The solutions collected after filtration constituted the culture filtrates that were then stored at –80°C until use. Preliminary tests showed that the Saboureaud broth did not affect the motility of the citrus nematode juveniles (data not shown).

Nematode source

Citrus nematode infected roots were used to obtain second-stage juveniles (J2) for the motility tests. The roots were dug up, washed free of soil, and cut into 1 cm sections. They were then blender macerated in a 0.5% NaOCl solution in a food blender, at approximately 1000 rpm for two successive 15 s intervals (McSorley *et al*., 1984). The egg suspension was passed through a 74 µm sieve to remove root debris, and the dispersed eggs were concentrated on a 20 µm sieve. The egg suspension was then subjected to centrifugation, using 50 mL-round bottom tubes combined with sugar flotation (Jenkins, 1964) followed by magnesium sulphate ($MgSO_4 \cdot 7H_2O$) differential centrifugation (Hendrickx *et al*., 1976) to further remove root debris. The $MgSO_4$ solution (225.9 g L^{-1}) was pipetted underneath the egg suspension, to form a density gradient and was then centrifuged at 1,500 rpm for 3 min. The upper aqueous fraction containing the eggs was then drawn off with a pipette and placed over a 20 µm sieve and rinsed repeatedly with tap water to remove any residual $MgSO_4$. The egg suspension was then placed on modified Baermann trays (Whitehead and Hemming, 1965), and hatched J2 were collected at 48-72 hour-intervals, concentrated into a small volume, and used for the motility test.

Motility tests

The tests were conducted in sterile, 24-microwell, flat-bottom, sterile, polystyrene plates (Iwaki, Scitech Div, Asahi Techno Glass, Japan). Three concentrations were tested for each culture filtrate, containing 100%, 50% and 25% of the original filtrate. The frozen filtrates were thawed at room temperature and dilutions were prepared by adding the appropriate volumes of sterile deionized water (SDW) to obtain a total volume of 1,000 µL per well after the addition of the J2 suspension. Wells with SDW served as controls (0% of the filtrate). Aliquots of the J2 suspension containing 30 to 40 individuals in 50-100 µL of water were added to the wells containing the culture filtrates. Five replicated wells were prepared per concentration, and each experiment (1 filtrate × 4 concentrations × 5 replications) was repeated at least once. The plates were incubated at 24°C in darkness for 24 h. Before reading the plates, they were gently agitated to allow aeration of the suspension and recovery of J2 motility, if they were deprived of oxygen. The number of moving and immobilized J2 was recorded separately with the aid of a stereomicroscope. The results were expressed as a percentage of immobilized J2. These J2 had adopted a straight shape.

Thermostability of the culture filtrates

Filtrates demonstrating activity against *T. semipenetrans* were autoclaved at 120°C for 20 min to determine their heat stability. Tests were performed as described in the previous section using the autoclaved filtrates at concentrations of 100%, 50% and 25%. The wells containing SDW served as controls (0% of the filtrate). Five replicated wells were prepared per concentration, and each experiment was repeated at least once.

Hatching tests

Hatching chambers were prepared using 1.5 mL Eppendorf tubes by cutting their conical ends and fitting pieces of tissue paper placed over nylon cloth to the flattened bottoms using a ring obtained by cutting the upper parts of other tubes. Nematode eggs were obtained from *T. semipenetrans* infected citrus roots, as described in the nematode source section. Aliquots (200-300 μL) of the egg suspensions containing 5,000 eggs were pipetted onto the hatching chambers, which had previously been placed in a sterile micro-well plate (with one hatching chamber per well) containing 1 mL of SDW. The plate was then left for 24 h to collect any J2 present in the egg suspension. The chambers were then transferred to a new micro-well plate containing the original culture filtrate of *T. cyanescens* 2-4 at concentrations of 100%, 50%, and 25%. The wells containing SDW (0% of the filtrate) served as controls. Five replications were prepared per concentration. The plates were incubated at 24°C in darkness, and the number of hatched J2 was recorded after 3, 7, 10, and 14 days. At each time, the hatching chambers were removed from the wells and placed in new ones containing fresh culture filtrate. Two additional tests were done, following a similar procedure but using 1000 eggs per hatching chamber, and the number of hatched J2 were counted after 3 and 7 days. At the end of the experiments, the hatching chambers were transferred to wells containing only SDW and incubated for 3 (test 1) or 6 (tests 2 and 3) additional days to determine whether the effect of the filtrate on egg hatch could be reversed.

Greenhouse tests

Previously autoclaved culture filtrates of *P. lilacinus* and *T. cyanescens* 2-4 were used to determine their effects on nematode reproduction in Cleopatra mandarin (*Citrus reshni* Hort) and Carrizo citrange (*Citroncirus webberi* Ingram & Moore × *Poncirus trifoliata)* seedlings. Seedlings from pre-germinated seeds were transferred to root trainers (300 mL capacity) containing a potting mixture (Verdejo-Lucas *et al.,* 1997), and grown for five months before nematode infestation. The treatments consisted of J2 incubated for 24, 48 or 72 h at 24°C in darkness, in culture filtrates placed in sterile 35-mm diam plates. Juveniles incubated in SDW served as controls. Juveniles were washed over a 25 μm sieve, then concentrated and counted. Cleopatra mandarin seedlings were inoculated with 400 juveniles per plant, and eight replications were prepared per treatment and culture filtrate. Seedlings were maintained in a glasshouse, irrigated as required, and fertilized with a slow release fertilizer (15% N + 10% P_2O_5 + 12% K_2O + 2% MgO_2 + microelements). Plants were harvested 90 days after nematode infestation.

In a second test, Carrizo citrange seedlings were inoculated with 1200 juveniles per plant, having been previously incubated in the autoclaved culture filtrate of *T. cyanescens* 2-4 for 24 or 48 h. Five replicated plants were prepared per treatment and they were harvested 78 days after nematode infestation.

In a third test, seedlings of Carrizo citrange were inoculated with 4,600 juveniles per plant having previously been incubated for 48 h in the autoclaved culture filtrate of *T. cyanescens* 2-4. Five replicated plants were prepared per treatment. Plants were harvested 67 days after nematode infestation. At harvest, the tops were cut off at soil level and the roots were carefully washed free of soil. The fresh weights of the roots and shoots were determined. The roots were stained according to Bridge *et al.* (1982). Nematodes were then extracted by macerating the stained roots in a 0.5% NaOCl solution, as previously described. Eggs were counted and expressed per gram of fresh root.

Statistical analyses

Version 8 of the SAS General Linear Model procedure (SAS Institute Inc., Cary, NC) was used for statistical analyses. The activity of a particular culture filtrate was established by comparing the percentage of immobilized J2 at each concentration of the filtrate with that in SDW by Dunnett's test (Dunnet, 1955). Percentages were transformed to arcsin (square root) before analyses. The SAS Probit procedure was used to determine the relationship between the logarithm of the dose and the percentage of immobilized J2. Goodness of fit to the probabilistic, logistic or Gompertz functions was determined, and the concentration that inhibited the motility of 50% of the J2 (CI50) was estimated. For the greenhouse tests, data on the number of eggs per gram root were transformed to log $(x + 1)$ and the effect of the culture filtrate on nematode reproduction was analyzed by Dunnett's test ($P < 0.05$). The effect of the incubation time in the culture filtrate on nematode reproduction was analyzed by Tukey´s test ($P < 0.05$).

Results

Nine out of 20 culture filtrates consistently immobilized J2 of *T. semipenetrans* at various concentrations compared to SDW (Table 2). The remaining filtrates did not display activity against the citrus nematode (data not shown). Significant results ranged from 17% to 94% of J2 being immobilized by the filtrates, depending on their respective concentrations and the fungal isolates from which they originated.

The culture filtrate from *T. cyanescens* 2-4 inhibited J2 motility at concentrations of 50% and 25% but was inactive at 100%, whereas that of *T. cyanescens* 2-5 inhibited motility at 100 and 50% (Table 2). The filtrate of *Engyodontium album* showed similar levels of activity irrespective of the concentration tested. The activity of the filtrate of *Myrothecium verrucaria* was not affected by the dilution of the original filtrate. Culture filtrates of *Tarracomyces gigaspora* and *Emericella rugulosa* inhibited J2 motility, although the results obtained were inconsistent (Table 2). The activity of the filtrates of *P. lilacinus*, *Chaetomium robustum* and *Acremonium strictum* was respectively related to the probit, logit, and the Gompit dose-response models (Table 3). The culture filtrate of *P. lilacinus* showed the highest activity with a CI50 value of 58%; this differed significantly from that of the filtrates of *C. robustum* (CI50 = 68%), and *A. strictum* (CI50 = 82%).

Thermostability of the culture filtrates

The autoclaved and non-autoclaved culture filtrates of *P. lilacinus*, *E. album*, and *T. cyanescens* 2-5 showed similar inhibitory effects on J2 motility (Table 2). The

Table 2. Percentage of second-stage juveniles of *Tylenchulus semipenetrans* immobilized by culture filtrates of fungi isolated from citrus soils after 24 h incubation at 24°C in darkness

Fungus and isolate code	Exp	Original filtrate[1]				Autoclaved filtrate[1]			
		100	50	25	0[2]	100	50	25	0[2]
Paecilomyces lilacinus	1	87 ± 4*	53 ± 6*	26 ± 4*	14 ± 5	70 ± 8*	44 ± 12*	19 ± 5	12 ± 4
	2	72 ± 9*	42 ± 13*	17 ± 3	12 ± 7	72 ± 9*	39 ± 7*	16 ± 5	12 ± 3
Chaetomium robustum 2-2	1	80 ± 11*	42 ± 11*	22 ± 9*	11 ± 5	71 ± 8*	40 ± 8*	28 ± 10*	12 ± 4
	2	73 ± 3*	20 ± 7*	8 ± 5	9 ± 3	76 ± 8*	27 ± 11*	19 ± 6*	10 ± 4
Acremonium strictum 2-1	1	71 ± 6*	37 ± 12*	14 ± 7	9 ± 5	81 ± 3*	56 ± 9*	30 ± 9*	12 ± 3
	2	75 ± 11*	56 ± 5*	19 ± 7*	9 ± 5	32 ± 6*	18 ± 4	14 ± 11	10 ± 4
	3	na[3]	na	na	na	23 ± 5*	10 ± 4	11 ± 4	13 ± 3
Engyodontium album 1-1	1	65 ± 7*	54 ± 3*	57 ± 12*	11 ± 5	44 ± 6*	31 ± 5*	20 ± 6*	10 ± 3
	2	57 ± 12*	48 ± 5*	31 ± 6*	14 ± 5	46 ± 6*	52 ± 5*	69 ± 1*	10 ± 4
Tarracomyces gigaspora	1	29 ± 7*	27 ± 1*	24 ± 8*	12 ± 6	18 ± 6*	12 ± 3	15 ± 5	8 ± 3
	2	89 ± 5*	38 ± 9	28 ± 3	25 ± 9	18 ± 5*	12 ± 3	13 ± 4	9 ± 3
Talaromyces cyanescens 2-4	1	18 ± 9	94 ± 4*	51 ± 8*	11 ± 6	85 ± 5*	81 ± 6*	38 ± 26*	12 ± 5
	2	10 ± 10	82 ± 13*	78 ± 4*	11 ± 15	91 ± 11*	81 ± 4*	44 ± 15*	6 ± 5
T. cyanescens 2-5	1	36 ± 6*	30 ± 7*	21 ± 4	15 ± 6	56 ± 17*	30 ± 18*	11 ± 4	7 ± 5
	2	29 ± 5*	18 ± 7*	17 ± 5*	10 ± 4	63 ± 10*	47 ± 12*	28 ± 8*	8 ± 4
T. cyanescens 1-4	1	11 ± 4	4 ± 3	7 ± 4	8 ± 3	12 ± 5	15 ± 6	11 ± 2	8 ± 4
	2	14 ± 3	17 ± 7	9 ± 3	12 ± 4	41 ± 4*	24 ± 6*	18 ± 7*	10 ± 4
	3	17 ± 4*	13 ± 6	16 ± 5*	9 ± 3	38 ± 23*	26 ± 9*	11 ± 3	4 ± 6
Myrothecium verrucaria	1	25 ± 10*	30 ± 6*	29 ± 13*	8 ± 5	26 ± 13*	21 ± 8 *	42 ± 7*	9 ± 3
	2	44 ± 10*	19 ± 8	44 ± 12*	23 ± 11	30 ± 9*	20 ± 7*	21 ± 10*	3 ± 3
Emericella rugulosa	1	12 ± 5	67 ± 17*	73 ± 13*	7 ± 5	19 ± 3	14 ± 7	20 ± 14	12 ± 4
	2	18 ± 6*	15 ± 3	32 ± 11*	9 ± 5	11 ± 3	19 ± 5	20 ± 14	19 ± 8

Values are means ± standard deviations of five replications per treatment. [1] Percentage concentration of the culture filtrate in solution. [2] Sterile deionized water. [3] Data not available. * indicates statistical differences compared with the sterile deionized water according to Dunnett's test ($P < 0.05$).

Table 3. Goodness of fit of the dose-response models and concentrations of the culture filtrates that immobilized 50% of the second-stage juveniles (CI50) of *Tylenchulus semipenetrans*

Culture filtrate	Model	Goodness of fit	CI50	Confidence limits
Non-autoclaved				
Paecilomyces lilacinus	Probit	0.683	58	53.3-62.1
Chaetomium robustum 2-2	Logit	0.760	68	63.3-71.8
Acremonium strictum. 2-1	Gompit	0.645	82	76.2-87.7
Autoclaved				
Paecilomyces lilacinus	Probit	0.530	72	66.5-77.3
Chaetomium robustum 2-2	Gompit	0.196	72	63.9-81.2
Talaromyces cyanescens 2-4	Probit	0.112	28	21.8-33.6
T. cyanescens 2-5	Probit	0.669	80	69.8-93.6

activity of the remaining active culture filtrates was modified by autoclaving, and the most striking result was that the culture filtrate of *T. cyanescens* 2-4 showed activity at a concentration of 100%, whereas the non-autoclaved one did not. The percentage of immobile J2 at concentrations of 50% and 25% was similar whether the filtrate had been autoclaved or not. Furthermore, the culture filtrate of *T. cyanescens* 1-4 showed activity at concentrations of 100% and 50% when autoclaved but the results obtained were inconsistent (Table 2). The autoclaved culture filtrate of *E. rugulosa* became inactive after autoclaving, and that of *A. strictum* only demonstrated activity at a concentration of 100%, although the percentage of immobilized J2 greatly varied from test to test. As regards to the dose-response relationships, the autoclaved culture filtrate of *T. cyanescens* 2-4 was more effective at inhibiting J2 motility (CI50 = 28%) than those of *T. cyanescens* 2-5 (CI50 = 80%), *C. robustum* (CI50 = 72%), and *P. lilacinus* (CI50 = 72%) (Table 3).

Hatching tests

The filtrate of *T. cyanescens* 2-4 reduced ($P < 0.05$) egg hatching at concentrations of 100% and 50% after egg incubation for 7 and 10 days in the culture filtrate, respectively (Fig. 1). Egg in SDW continued hatching for 14 days when the test was terminated. The inhibitory effect of the culture filtrate on egg hatching was confirmed in tests 2 and 3; inhibition occurred at all three concentrations tested and ranged from 85% to 92% in test 2, and from 81% to 93% in test 3 (data not shown). This effect was not reversed when eggs that had previously been incubated in the culture filtrate were placed in water (data not shown).

Greenhouse tests

The fresh shoot and root weights of the citrus seedlings were unaffected by the treatments. Nematode reproduction on Cleopatra mandarin was lower ($P < 0.05$) when the J2 inoculum was incubated in the culture filtrates of *P. lilacinus* for 72 h (Table 4). The autoclaved culture filtrate of *T. cyanescens* 2-4 reduced nematode

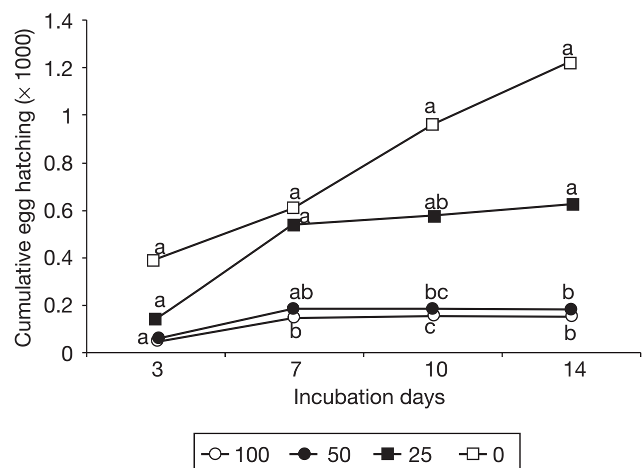

Figure 1. Cumulative hatching of *Tylenchulus semipenetrans* eggs incubated in a series of concentrations of a culture filtrate of *Talaromyces cyanescens* isolate 2-4 for 3, 7, 10, and 14 days. Values are means of five replications per treatment. Low case letters indicate statistical differences ($P < 0.05$) between concentrations of the culture filtrate at a given time.

Table 4. Number of eggs per gram of root of *Tylenchulus semipenetrans* on Cleopatra mandarin and Carrizo citrange infested with second-stage juveniles previously incubated for 24, 48 or 72 h in culture filtrates of *Paecilomyces lilacinus* and *Talaromyces cyanescens* isolate 2-4

Culture filtrate and citrus rootstock	Inoculum (J2/plant)	Incubation time (h)	Treatment	Eggs g^{-1} root	Percentage reduction
P. lilacinus					
Cleopatra mandarin	400	24	Filtrate	114 ± 114	57
			Water[1]	264 ± 297	
		48	Filtrate	143 ± 83	None
			Water	23 ± 56	
		72	Filtrate	11 ± 16*	82
			Water	62 ± 32	
T. cyanescens 2-4[2]					
Cleopatra mandarin	400	24			
			Filtrate	149 ± 144	60
			Water	370 ± 366	
		48	Filtrate	164 ± 172	60
			Water	410 ± 419	
		72	Filtrate	380 ± 608	None
			Water	123 ± 36	
Carrizo citrange	1,200	24	Filtrate	131 ± 93*	68
			Water	409 ± 311	
		48	Filtrate	84 ± 99*	84
			Water	508 ± 352	
Carrizo citrange	4,600	48	Filtrate	141 ± 59*	75
			Water	570 ± 589	

Values are means ± standard deviations of five to eight replicated plants. [1] Sterile deionized water. [2] Autoclaved culture filtrate at 120°C for 20 min. * indicates statistical differences between treatments according to Dunnett's test ($P < 0.05$)

reproduction ($P < 0.05$) on Carrizo citrange, but not on Cleopatra mandarin.

Discussion

The results of this study indicate that fungi native to citrus soils can be a good source of compounds that demonstrate activity against the citrus nematode and that the most promising candidates are those yielding filtrates with a strong inhibitory effect on J2 motility and reproducible results. The original and autoclaved filtrates generally showed similar activity, indicating that their active compounds were thermostable. The exception was the filtrate of *E. rugulosa*, which lost its activity, suggesting the presence of a protein-like compound in this filtrate. *Paecilomyces lilacinus* is, by far, the most abundant and frequent fungal species isolated from citrus nematode eggs, females, and juveniles in citrus orchards of Catalonia, Spain (Gené *et al.*, 2005). The isolation of the fungus from J2 suggested another mode-of-action besides direct parasitism (Morgan-Jones *et al.*, 1984) since the fungus do not produce traps or adhesive spores. The culture filtrate of *P. lilacinus* reduced the motility of a high percentage of J2 following a dose-response model, reduced nematode reproduction on Cleopatra mandarin, and retained its activity after autoclaving. Whether immobilisation of J2 is a prerequisite for fungal parasitism is, as yet, unknown, but the release of nematocidal compounds into the medium could be an additional mode-of-action of this fungus that is unrelated to direct parasitism. Conversely, the culture filtrate could affect nematode behaviour, but lack nematocidal activity. To the best of our knowledge, the effect of *P. lilacinus* culture filtrates on *T. semipenetrans* have not been

investigated, although the production in liquid cultures of compounds with activity against root-knot and cyst-forming nematodes has been reported (Molina and Davide, 1986; Cayrol *et al.,* 1989; Shabana and Khan, 1992; Chen *et al*., 2000; Meyer *et al.,* 2004). Nevertheless, the activity of a given culture filtrate can vary with the culture medium, culture conditions, and fungal isolate, and they can determine the type of secondary metabolites produced, and may even cause shifts in their biogenic pathways (Cayrol *et al.,* 1989; Stadler and Anke, 1995; Hallmann and Sikora, 1996; Chen *et al*., 2000). Culture filtrates can also differ in their activity according to the stage of the nematode life cycle, and from one nematode species to another (Cayrol *et al*., 1989; Hallmann and Sikora, 1996; Zareen *et al.,* 2001; Meyer *et al*., 2004). The culture filtrate of *T. cyanescens* 2-4 inhibited J2 motility and egg hatching and reduced nematode reproduction on citrus seedlings. However, the greatest activity was shown when the culture filtrate was diluted or autoclaved. This result was consistent, but unexpected, and cannot be explained at the moment. The culture filtrates of the three isolates of *T. cyanescens* showed different inhibitory effects on nematode motility. Although the production of secondary metabolites is a genetic trait that can vary between isolates, environmental factors can affect the expression of these genes, and therefore the activity of these cultures filtrates against nematodes. The isolates of *T. cyanescens* 2-4 and 2-5 came from the same geographical area and were isolated from loamy soils with high organic matter contents (3.9% and 3%, respectively), whereas isolate 1-4 was isolated from a sandy soil in a different area with only 1.4% of organic matter.

The culture filtrate of *C. robustum* showed a high inhibitory effect on nematode motility and heat stability and followed a dose-response model. A closely related species, *C. globosum*, produced compounds that inhibited egg hatching and J2 motility in *Meloidogyne incognita* and egg hatch in *Heterodera glycines* (Nitao *et al.,* 2002). The activity of the culture filtrate of *A. strictum* decreased after autoclaving. This fungus has been isolated from citrus nematode females in natural infestations (Gené *et al.,* 2005). Culture filtrates of an *A. strictum* isolate from *H. glycines* displayed a strong inhibitory effect on egg hatch in both *H. glycines* and *M. incognita* (Meyer *et al*., 2004). The culture filtrate of *E. album* ranked fourth in inhibitory activity and immobilized up to 69% of the J2. This fungus has not been isolated from root-knot nematode eggs in natural infestations (Verdejo-Lucas *et al.,* 2002). The isolate of *M. verrucaria* produced a filtrate with a low, but consistent level of activity. The commercial product DiTera™ is produced by submerged fermentation of an isolate of this fungal species and was developed through motility tests (Grau *et al*., 1996).

Under natural conditions, *T. semipenetrans* populations are regulated by biotic and abiotic factors (Sorribas *et al.,* 2000). A recent study (Gené *et al.,* 2005) has shown that a wide range of organisms with antagonistic, pathogenic or predatory activities are present in the citrus rizosphere. The results of this preliminary screening and previous investigations support the hypothesis that soil fungi could contribute to regulating nematode densities by parasitism and predation, or indirectly through the production of biologically active secondary metabolites. Further investigations are needed to determine the chemical nature of such metabolites, their precise mode of action, and their effects over a wider range of conditions.

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