Study of the nematocidal properties of the culture filtrate of the nematophagous fungus *Paecilomyces lilacinus*

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SUMMARY

The culture filtrates of *Paecilomyces lilacinus* (nematophagous fungus) growing on different liquid media and under various conditions showed a very inconstant toxicity towards nematodes. The best nematocidal activity was obtained on malt medium with motionless aerated cultures. Light had no effect on toxin production. Although it was only generated at pH 5, the toxin had an activity independant of the pH. The activity of the toxic metabolite was very specific. When tested on seventeen species of nematodes it was only efficient against the Heteroderidae family *(Meloidogyne, Heterodera).* The mechanism involved in the toxin activity appeared as a neurotropic one because it had a reversible effect when the treatment period was less than 48 h.

RÉSUMÉ

Étude des propriétés nématicides du filtrat de culture du champignon nématophage Paecilomyces lilacinus

Les filtrats de culture du champignon nématophage *Paecilomyces lilacinus* présentent une toxicité très variable vis-à-vis des nématodes selon les milieux et les conditions de cultures utilisés. La plus grande activité nématicide a été obtenue sur milieu au malt en cultures stables aérées sans influence de l'éclairement. Bien que n'étant produite qu'au dessous de pH 5, la substance toxique a une activité indépendante du pH. Cette substance toxique paraît très spécifique. Testée envers dix-sept espèces de nématodes, elle ne se révèle réellement active qu'envers les représentants de la famille des Heteroderidae *(Meloidogyne, Heterodera).* Le mode d'action de cette substance semble de type neurotrope car son effet est réversible si la durée du traitement ne dépasse pas 48 heures.

The toxic effect of culture filtrates of different fungi on *Meloidogyne* larvae has been studied previously (Sakhuja, Singh & Sharma, 1978; Mani & Sethi, 1984; Dahiya & Singh, 1985). Recently, Dechechi Gomes Carneiro (1986) has demonstrated that a toxic substance killing the eggs of *M. arenaria* was present in the culture filtrate of the nematophagous fungus *Paecilomyces lilacinus*. The objective of the present work was to observe the activity of the culture filtrate of *P. lilacinus* on adults and larvae of different species of nematodes (phytophagous, mycophagous, saprophagous, entomophagous) and to study the conditions necessary to obtain the maximum nematocidal effect of the filtrate.

Material and methods

ORGANISM

Fungus

The fungal culture *P. lilacinus* used in the present investigation originated from the Amazonian area of Brazil (Dechechi Gomes Carneiro, 1986).

Nematodes

Seventeen species of nematodes were tested for *P. lilacinus* activity. The origins of these species and the stages used for the biological tests are indicated in Table 1, col. 1 to 3.

BIOLOGICAL TESTS

After varied periods of incubation on different media, the culture filtrate was obtained by filtering it firstly through sterilized Whatman no. 2 filter paper which removes mycelium and secondly through 0.22 µm millipore filter avoiding spore contamination. The filtrate thus collected was tested pure or variously diluted in sterile distilled water (1/2; 1/4; 1/8; 1/10; 1/20; 1/30). The tests were done in 24 well Nunclon plates; each well received 1 ml of the test filtrate (pure or diluted) to which about 50 nematodes were added in 20 µl of water. Each treatment was replicated four times.

Toxicity was estimated according to the mean percentage of paralysed nematodes. Nematodes were considered paralysed if they did not move when probed with a fine needle. Two controls were used for comparison :

one in pure distilled water, the other in the uninoculated sterilized culture medium always corrected to the same pH value as the tested culture filtrate.

The reversible effect of the culture filtrate toxicity was also estimated according to the following method : the nematodes whose mobility was inhibited after various immersion periods in the filtrates were collected and dipped in fresh water. The proportion of paralysed nematodes was estimated after 24 h in water.

Table 1

Effect of the culture of *Paecilomyces lilacinus* on seventeen species of nematodes.

Nematodes tested			*Mean percentages of paralysed larvae after 20 h of dipping in pure culture filtrate***
Biological group	*Species*	*Origins*	
PHYTOPHAGOUS SPECIES	*Aphelenchoides besseyi**	*In vitro* cultures	90
	*A. fragariae**		87
	*A. ritzemabosi**		61
	*Meloidogyne arenaria***	From roots	100 *a*
	*M. incognita***		100 *a*
	*M. javanica***		100 *a*
	*Heterodera rostochiensis***		100 *a*
MYCOPHAGOUS SPECIES	*Aphelenchus avenae**	Fungi cultures on agar medium	18 *b*
	*Aphelenchoides subtenuis**		21 *b*
	*Ditylenchus myceliophagus**		2
SAPROPHAGOUS SPECIES	*Cephalobus parvus**	Cultures on agar medium (Nigon, 1949)	0 *c*
	*Panagrolaimus mycophilus**		1 *c*
	*Rhabditella axei**		82
	*Pristionchus lheritieri**		50
ENTOMOPHAGOUS SPECIES	*Neoplectana bibionis**	Cultures on *Galleria* caterpillars (Douenel, 1988)	30
	*N. carpocapsae**		20 *b*
	*N. glaseri**		25 *b*

* Adults and larvae.
** Second-stage larvae.
*** Ten day-old filtrate collected from montionless aerated cultures constantly exposed to light. Means followed by the same letter in the column are not significantly different (P = 0.05) according to the Student's test. Number of replicates : 4.

CULTURE MEDIA TESTED

The growth of *P. lilacinus* is dependent from the nutritious aptitude of the culture medium (Villanueva & Davide, 1984). As a general rule, the production of mycotoxins is also greatly influenced by the culture medium (Smith & Moss, 1985).

Consequently, the activity of the culture filtrate of *P. lilacinus* was tested after culturing the fungus on the following liquid media, all autoclaved at 115° during 20 min before fungus inoculation :

— media prepared with malt (15, 20, 25 and 30 g/l of water). pH after steam sterilization respectively : 5.5; 5.47; 5.44; 5.40.

— Czapek-Dox medium (Duncan, 1973). pH after steam sterilization : 6.4.

— Czapek-Dox modified medium (Naguib, 1960). pH after steam sterilization : 7.3.

— Arconteil modified medium (Vey, Quiot & Vago, 1987). pH after steam sterilization : 6.5.

— Mac Coy medium (Mac Coy, Hill & Cavanel, 1972). pH after steam sterilization : 6.9.

The culture media prepared with malt were undefined media containing many unknown components present in malt. Nevertheless, these malt media have consistently shown improved toxin production in a wide range of fungi (Smith & Moss, 1985) and so were included in the tests. The four other media are synthetic. They differ only in the mineral salts, the nature of the sugar (glucose or saccharose) and the presence or absence of iron salts. These four media are commonly employed for culturing

the toxin-producing fungi and it seems that their light differences between them are often sufficient to induce important variations in their toxic properties. All the tested media were inoculated with *P. lilacinus* according to the following standard method : spores were used from two month-old cultures of *P. lilacinus* in Petri dishes (100 mm diameter) on a standard agar medium (agar 15 g, malt 20 g/l of water). Each Petri dish was filled with 20 ml of sterile distilled water giving a spore suspension of about $3,2 \times 10^7$ spores per ml. This spore suspension of about 3.2×10^7 spores per ml. This spore 10^5 spores were inoculated per ml of liquid medium. The different liquid media thus inoculated were kept in laboratory temperature conditions (22-27°).

Culture conditions tested

All the cultures of *P. lilacinus* were prepared in 500 ml erlenmeyer flasks filled with 300 ml of liquid medium (32 flasks for each tested medium). These 32 flasks were subdivided into four replicates of the following eight factorial treatments : 1) constant exposure to light or darkness, 2) motionless or shaked cultures, 3) aerated or non-aerated cultures (sterile air blown into the liquid medium by means of an aquarium pump for five minutes a day).

Effects of the pH

Using the most performant culture medium and culture conditions, we have regularly measured the pH values of the culture filtrate in order to observe a possible correlation between pH and toxicity.

Results

Influence of the culture medium

The nematodes used to compare the toxicity between the eight different liquid media were second-stage juveniles of *M. arenaria* which were the most sensitive to the toxic activity of *P. lilacinus*. The results are given for 15 day-old filtrates collected from motionless non-aerated cultures constantly exposed to light.

Table 2

Influence of the culture medium on the activity of *Paecilomyces lilacinus* culture filtrates on *Meloidogyne arenaria* larvae (15 day-old filtrates collected from motionless non-aerated cultures constantly exposed to light)*.

	Mean percentages of paralysed larvae after two hours of dipping					
	Tested media					
Culture filtrate dilutions	*Malt (15.20 g/l)*	*Malt (25.30 g/l)*	*Czapek-Dox modified medium*	*Arconteil modified medium*	*Czapek-Dox medium*	*Mac. Coy medium*
0	100 *a*	100 *a*	0 *ax*	54 *a*	100 *a*	100 *a*
1/2	100 *a*	100 *a*	1 *ax*	5 *bx*	78 *b*	90 *b*
1/4	97 *a*	100 *a*	1 *ax*	1 *bx*	51 *c*	13 *c*
1/8	37 *b*	52 *b*	0 *ax*	0 *bx*	0 *dx*	0 *dx*
1/10	2 *cx*	0 *cx*	0 *ax*	1 *bx*	1 *dx*	0 *dx*

* Means followed by the same letter in the columns and means underlined by the same line are not significantly different ($P < 0.05$) according to the Student's test. Number of replicates : 4.

As confirmation of the general rule, the present results showed that the toxic properties of *P. lilacinus* are greatly influenced by the liquid culture medium (Tab. 2). In Czapek-Dox modified medium the toxic metabolites were not produced at any dilution. In Arconteil modified medium the toxicity was weak in pure culture filtrate (about 50 % of paralyzed larvae) and decreased very rapidly when the filtrate was diluted. In the two other synthetic media (Czapek-Dox and Mac Coy media) the toxicity was greater. The origin of a so marked difference was possibly related to the larger amounts of nitrogen and potassium in these two last media (respectively 3 and 5 g of ammonium nitrate but only 0.7 g in the two first media — 1 and 1.5 g of potassium but only 0.01 and 0.36 g respectively in Czapek-Dox and Arconteil modified media).

The highest toxicity was obtained in the malt composed media irrespective of the malt concentration. The main point concerning these malt media was to avoid filtering the liquid medium after autoclaving in order not to eliminate the brown flocculent always present at the bottom of the flasks. The existence of this flocculent was absolutely necessary for the toxin production by the fungus and we assume that essential nutrients required for toxin production were present in this precipitate.

INFLUENCE OF THE CULTURE CONDITIONS

The culture medium used for these tests was malt medium (15 g/l). The nematodes used were *M. arenaria* larvae. Toxin production by the fungus was similar in light or dark conditions.

In contrast, the results in Figure 1 show that the rate of toxicity (expressed after the maximum filtrate dilution in which all larvae were paralysed after two hours of dipping) was greatly influenced by the four other culture conditions (motionless or shaken cultures; aerated or non-aerated). As a general rule, the toxic activity of *P. lilacinus* was better in motionless cultures than in shaked ones. It also appears that aerated cultures were more active than the non-aerated ones. Finally, the best toxin production was obtained with motionless aerated cultures where the *Meloidogyne* larvae were all paralysed when dipped two hours in the greatest tested dilution (1/30) in a 15 day-old culture filtrate.

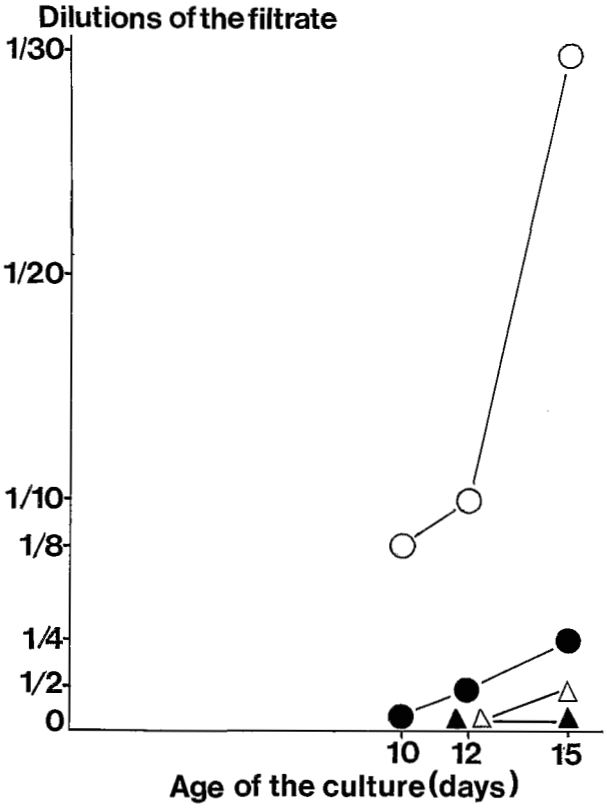

Fig. 1. Influence of the culture conditions on the nematocidal activity of *Paecilomyces lilacinus* culture filtrates on *Meloidogyne arenaria* larvae. (The graph shows the filtrate dilutions where the larvae are totally paralysed according to the different culture conditions : ○ = aerated motionless cultures; ● = non aerated motionless cultures; △ = aerated shaked cultures; ▲ = non aerated shaked cultures).

INFLUENCE OF THE pH OF THE MEDIUM

For these experiments, malt medium (15 g/l) in motionless aerated cultures exposed to light was used. The nematodes tested were *M. arenaria* larvae. The results in Figure 2 show that the increase of the toxic properties of the culture filtrate (expressed after the greatest filtrate dilution in which all larvae were paralysed after two hours of dipping) were related to the decrease of the pH. In 15 day-old filtrate, the *Meloido-*

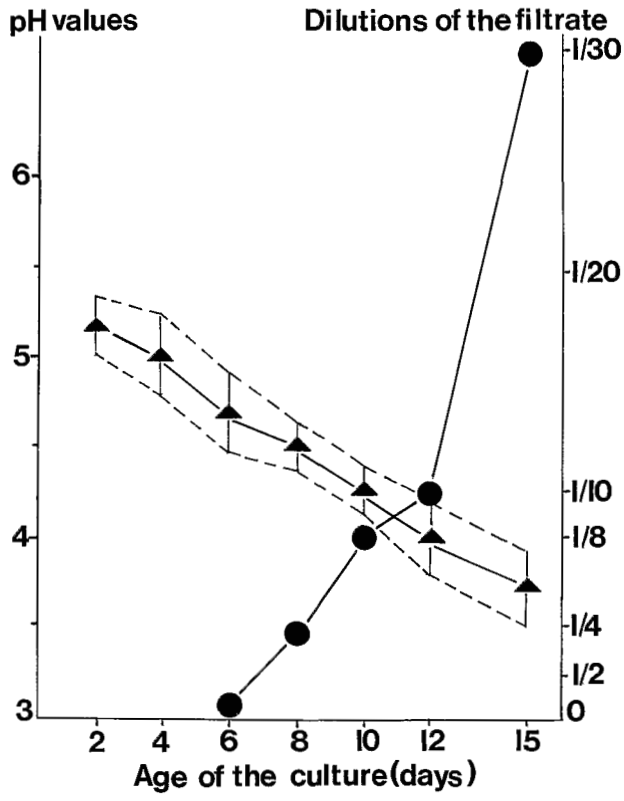

Fig. 2. Relationship between pH and the toxic activity of *Paecilomyces lilacinus* culture filtrates on *Meloidogyne arenaria* larvae. (The graph shows the filtrate dilutions where *Meloidogyne arenaria* juveniles are totally paralysed) (▲ = pH values; ● = filtrate dilutions paralysing the larvae).

gyne larvae were all paralysed in the greatest filtrate dilution (1/30) when the pH value was the lowest (pH 3.7). In order to observe if the activity of the culture filtrate was strictly limited to low pH, the pH of the culture filtrate was raised up to different pH values (5, 6, 7, 8, 9) by adding some µl of 0.01 N sodium hydroxyde (without toxic effect on *Meloidogyne* larvae). A 10 day-old culture filtrate obtained in the same medium and in the same conditions as previously described was used for this purpose. The different pH values ranging from 4.2 (natural filtrate) up to 9, were tested combined with various dilutions of the filtrate (0, 1/2, 1/4, 1/8) : no significative difference was observed between these various treatments.

Two major informations are proceeding from these results : *i)* the increase of the toxin production was in inverse ratio to the decrease of the pH of the culture medium; it seems consequently that the toxin production by *P. lilacinus* is more important when the fungus is in maximum growth period; *ii)* the so-produced toxin was able to act towards *Meloidogyne* larvae whatever was the pH of the culture filtrate. Consequently we conclude that the toxin production only occurs at low pH conditions but that the so-produced toxin acts in a wide range of pH values.

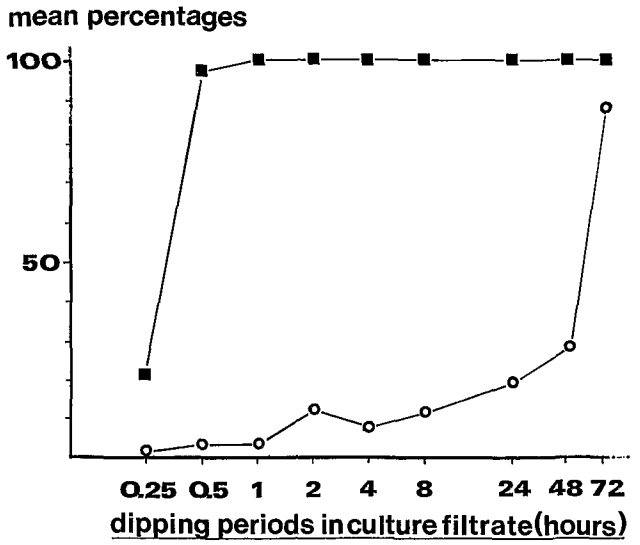

Fig. 3. Reversibility of the toxic effect of culture filtrate of *Paecilomyces lilacinus* on *Meloidogyne arenaria* larvae.[1] (Ten day-old filtrate, collected from motionless aerated cultures constantly exposed to light; ■ = paralysed larvae in filtrate; O = still paralysed larvae in water.)

STUDY OF THE REVERSIBILITY OF THE TOXIC EFFECT

A ten day-old culture filtrate collected from a motionless aerated culture on malt medium (15 g/l) exposed to light was used for this experiment. The nematodes tested were *M. arenaria* larvae. These larvae were dipped in pure culture filtrate during different periods (from 15 min to 72 h). The proportion of paralysed larvae present after 24 h in fresh water following different times in filtrate are presented in Figure 3. The toxic effect is clearly rapid since 97 % of larvae are already paralysed after only 30 min in the culture filtrate. Secondly, the majority of larvae are able to recover the mobility in fresh water following all treatment times up to 48 h. Only the 72 h treatment seems to kill the nematodes.

STUDY OF THE EFFECT OF THE CULTURE FILTRATE ON DIFFERENT SPECIES OF NEMATODES

A 10 day-old culture filtrate produced in the same conditions as those described in the preceding experiment was used for this screening. The nematodes of the different species were immerged for 20 h in the culture filtrate. The results in Table 1, col. 4 show the mean percentages of paralysed nematodes for the different species. There were marqued differences in the toxicity of the culture filtrate according to the different species of nematodes tested.

The four species belonging to the family of Heteroderidae *(Meloidogyne* and *Heterodera)* were totally paralysed. Some others species were weakly affected *(Aphelenchoides besseyi, A. fragariae, A. ritzemabosi* and *Rhabditella axei).* There was no significant activity against the nine other species.

Discussion

The influence of the culture medium on toxin production was very important as the filtrate from the Czapek-Dox modified medium was inactive whereas maximum toxicity was observed with malt medium. Likewise, the toxic properties of the fungus were greatly influenced by the culture conditions. So the toxic metabolite was almost absent in shaked cultures but very active in motionless aerated cultures. Very different results have been observed with other fungi. For example, the entomogenous fungus *Metarrhizium anisopliae* gives the maximum toxin production in shaked cultures (Roberts, 1966).

Concerning the age of the culture, the results show that the toxin production is more important when the fungus is in maximum growth period. That is also in contradiction with some others papers (Sakhuja, Singh & Sharma, 1978; Fargues & Robert, 1986) according to which most of fungal pathogens produced toxins during their initial growth period. Hence it appears that the conditions necessary for mycotoxin production (medium, culture conditions, age) are very variable from one fungus to the other. Consequently when the ability of fungi to produce any toxins are studied, it is absolutely necessary to test several parameters. Also to establish the

identity of the toxic metabolite in the near future, it is absolutely necessary to define a well known synthetic medium which gives as good results as the malt medium. Indeed, the composition of the latter is too ill-defined to accurately consider the chemical analysis of the culture filtrate.

The activity of the toxic metabolite of *P. lilacinus* suggests a neurotropic mechanism. Firstly, the cuticles of stylet bearing nematodes are very impermeable; in the experiments, the activity of the toxin was tested by dipping the nematodes in the culture filtrate. Secondly, the paralysis of the susceptible species appeared very quickly (after 30 min of dipping) but the nematodes recovered their mobility when transfered to fresh water if the period of dipping did not exced 48 h. All these results agree with the hypothesis of a neurotropic action upon the nervous receptors of the nematode. However, these are preliminary findings, and, the exact mechanism of a such neurotropic action is yet to be established. These results are very different from those generally obtained when studying insect mycotoxins which act by injection of the culture filtrate in the insect hemocoele (Roberts, 1966) or sometimes by ingestion in mixture with the insect food (Robert & Fargues, 1986) but never by epidermic way.

What is also interesting in these preliminary findings is the specificity of the toxic metabolite against a limited number of nematodes species. The reasons for such a specificity and the biochemical mechanisms of recognition involved on the nematode cuticle are also of interest. This specificity appears as an additional problem in the nematodical mycotoxins research. It is clear that it is absolutely necessary to test a lot of different nematodes species in order to reveal a possible toxic activity. In the future, if mycotoxins are to be used as nematicides, their specificity is a real advantage. It enables only the pest species to be killed, without destroying all the soil microfauna as current fumigants are doing.

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