

Trabajo Original

Ecotoxicología

Ecotoxicological evaluation of *Pochonia chlamydosporia* var. *catenulata* in terrestrial invertebrates.

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Abstract

The strain IMI SD-187 of *Pochonia chlamydosporia* var. *catenulata* is suggested a new nematocidal agent and its effects on terrestrial organisms are therefore being investigated. Effects of compatibility on several species of fungus that share the same habitat have previously been found, including *Pasteuria penetrans*, *Micorrizas*, *Rhizobium*, *Pochonia chlamydosporia* var *chlamydosporia*. The aim of this study was to examine a broad range of toxic effects and a wide concentration span to find the earliest and most relevant effects of this product on three invertebrates' species.

Key words: ecotoxicity, non-target terrestrial invertebrates, *Pochonia chlamydosporia* var. *catenulata*

Resumen

Evaluación ecotoxicológica de *Pochonia chlamydosporia* var. *catenulate* en invertebrados terrestres.

La cepa IMI SD-187 de *Pochonia chlamydosporia* var. *catenulata* se ha propuesto como un nuevo agente nematocida y por consiguiente sus efectos sobre los organismos terrestres deben ser estudiados. Las características de compatibilidad con varias especies de hongos que comparten su mismo hábitat han sido evaluadas previamente, incluyendo *Pasteuria penetrans*, *Micorrizas*, *Rhizobium* y *Pochonia chlamydosporia* var *chlamydosporia*. El objetivo de este estudio fue examinar un amplio rango de efectos tóxicos y de concentraciones para conocer los efectos más relevantes de este producto en tres especies de invertebrados.

Palabras claves: ecotoxicidad, invertebrados terrestres no-diana, *Pochonia chlamydosporia* var. *catenulata*

Introduction

The assessment of the effects and risks of chemicals for the terrestrial environment is a complex matter. Terrestrial systems are not associated with a single compartment, but with the interface between soil and the atmosphere. Although purely soil-dwelling organisms play a clear role, basic ecosystem functioning and biodiversity is associated with organisms, such as terrestrial plants, many invertebrates, and certain terrestrial vertebrates that are simultaneously or sequentially located in the soil or above-soil compartments. That is the reason why Directive 91/414/EC includes the need for specific assessments on certain terrestrial non-target groups, such as terrestrial vertebrates, bees, other non-target arthropods, earthworms or soil micro-organisms (Directive 91/414/EC).

Appropriate safety testing protocols need to be developed and tested to ensure the safety and efficacy of the agent prior to widespread use. Testing of direct toxicity and ecotoxicity to beneficial species are required by regulatory authorities around the world (Goettel et al., 2001). There are several major groups of beneficial production species, such as bees; pest control species, such as biological control agents and naturally occurring predators; and species involved in ecosystem processes such as earthworms.

Many previous workers have studied various aspects of the toxicity and ecotoxicity of this strain including vertebrates test with rats, rabbits and quail (García et al., 2004 and García et al., 2004a) and invertebrates ones using non-target beneficial plants (García et al., 2008). No toxic, irritants, pathogenic and infective effects were detected; it's suggest safety in the use of this fungus. However little is known about their effects on non-target invertebrates. Thus, as part of a test battery for ecotoxicity, it was considered appropriated to investigate a potential for toxicity in *Eisenia Andrei* Bouché, *Apis mellifera* Linneo and *Chrysopa exterior* Navas.

Materials and Methods

Test Material

In the studies, the fungus *Pochonia chlamydosporia* var. *catenulata*, IMI SD 187 was supplied by the Research and Development Unit of Biological Control for Agriculture, CENSA.

Test species

- Earthworm, *Eisenia andrei* Bouché (Oligochaeta: Lumbricidae); adults (older than two month old with clitellum), weight of 300 to 600 mg, without deformations in their body and without manifesting physiologic and behaviour dysfunctions. They were purchased from the System of standardized cultivation of the Research group of Terrestrial Organisms from the Department of Investigations of the National Center of Toxicology (CENATOX).
- Honey bee: *Apis mellifera* Linneo (Hymenoptera: Apidae); young from 1 to 7 days old, were purchased from an in situ colony of the UBPC in San José de las Lajas, Agriculture Ministry. They were feeded with free access to a solution at 50% of sugar in steril destiled water.
- Insect: predaceous neuropteran, larvae of *Chrysopa exterior* Navas (Neuroptera: Chrysopidae), from the insects lab in the Institute of Vegetable Sanity. They were feeded with free access to eggs from lepidopterans *Corcyra cephalonica* Station.

A negative control nondosed, a vehicle and an infectivity control group treated with the final product inactivated (PAI) were used in all studies.

Tests were performed on the laboratory level and they were based on standard methodologies as described in international OECD (1996) and EPA (1996) guidelines.

Study designs

Earthworm, acute toxicity test. Filter paper test, OECD 207, 1984.

48 adult earthworm were distributed in four groups: control, placebo (Tween 80 al 0,1%), treated with 10^3 clamidospores/ml (PA_{LD}: clamidospores powder/ low dose) and

treated with con 10^7 clamidospores/ml (PA). Ten replicates, each consisting of one worm per vial, for each treatment were used. The test temperature was $20 \pm 2^\circ\text{C}$. Test was done in the dark and for a period of 96 hours with mortality and any behavioural or pathological symptoms assessments.

Earthworm, Artificial soil test, OECD 207, 1984.

In the artificial soil test, ten worms were kept in 750g weight of the soil and three replicates for each treatment were kept under the test conditions for 14 (acute) and 30 days (sub-chronic). Evaluated groups: control, treated with 1g of inactivated clamidospores powder (PAI), other group with $2,2 \times 10^8$ clamidospores/kg of sustrato (1g of PA, clamidospores powder), another with 9×10^5 clamidospores/kg of substrate (0,04g of PF_{LD} , colonized substrate / low dose) and with $2,2 \times 10^7$ clamidospores/kg of sustrato (1g of PF, colonized substrate). It was used static médium without renovation. Mortality and any behavioural or pathological symptoms were assessed. For the infectivity analysis, another replica was included by group, where 1g of substrate and 1g of worms at 7, 14, 21 and 30 days under study, were put in semiselective medium (Kerry et al., 1993) on petri dish and incubated at 25°C for 10 days. Samples were examined under the light microscope (Zeiss-Axiolab, Magnification 40x) to detect the presence of the fungus.

Honey bee, acute test, OPPTS 885.4380 EPA, 1996.

75 bees per group, distributed in three replicates of 25 were used. They were treated with powder of clamidospores applied in 0.05% tween 80 to the pronotum of individual bees with previous anaesthesia for freezing (Hajek and Goettel, 2000). Groups of treatment: control, vehicle (Tween 80 to 0,05%), inactivated powder of clamidospores (PAI), 5×10^3 clamidospores / bee (PA_{LD}) and 5×10^4 clamidospores / bee (PA). The test was done in the dark with $25\text{-}35^\circ\text{C}$ of temperature and 50-80% of a relative humidity. At 4, 24, 48, 72 hours and 7 days mortality and any behavioural or pathological symptoms were assessed. The infectivity analysis was done like in the earthworm study.

Predaceous insect, acute toxicity for direct and residual exposure, OPPTS 885.4340, EPA 1996.

65 larvae were kept during 7 days in vials in an individual way and distributed 10 in each experimental group: control, vehicle (Tween 80 to 0,05%), inactivated powder of clamidospores (PAI), 10^3 clamidospores/ml (powder of clamidospores, low dose PA_{LD}), 10^5 clamidospores/ml (medium dose, PA_{MD}) and 10^7 clamidospores/ml (PA). The treatment was done for direct exposure in filter paper and for residual exposure, microinmersion method described for Dennehy et al (1993), was used.

In the studies the variables statistically analyzed were the mortality and the toxicity of the controls with regard to the treaties, for that was carried out an analysis of multiple comparisons of proportions with a level of significance of 95%.

Results and Discussions

In the study of worms in filter paper, 96 hours after application, only a worm of the treated group with powder of clamidospores died, for 10% of mortality. Related with the physiologic and behaviour alterations; in the first hours there were not apparent damages. At 96 hours, constrictions appeared along the body in 50% of the worms of this same group and in one of the placebo group that represents 10% of alterations. They appear as a defence mechanism in front of adverse situations (Hernández et al., 1997). In spite of the alterations, all the worms stayed alive during the assays and it is in fact the mortality the final point of the study.

In the acute study in artificial substrate didn't happen deaths and a worm was only observed broken into fragments in the treated group with the low dose of the final product (3,3% of alterations). In the sub-chronic there were 6,6% of deaths and 3,3% of alterations (broken into fragments worms) from the day 14 of the study in the groups of the biggest dose of powder of clamidospores and of the colonized substrate (Table 1).

In the evaluation of the infectivity it was demonstrated that the presence of the fungus in the substrate was in correspondance with the quantities applied initially. The [RETEL](#)

worms were capable of translocate the clamidospores without the occurrence of mortality neither physiologic alterations related with the ingestion of the fungus during the 30 days of the study. This result is related with the capacity that they have of ingest enormous quantities of soil and thus must have a high exposure to the soil pollutants (Srivastava et al., 2005).

It is considered that didn't occur replication, because the recovery either of worms or substrates was in the same order of UFC of the fungus in all groups (Table 2). Then is demonstrated that the strain IMI SD 187 was not toxic for *E. andrei* in these conditions, with a CL_{50} above 10^8 clamidospores/ kg substrate, that is 100 times bigger than the dose of application in the field. Similar results were obtained when exposing *Lumbricus terrestris* L. to conidios of *M. anisopliae* (Hozzank et al., 2002).

Since the fungus has a high persistence in the soil (Hidalgo et al., 2000); high concentrations in the worms could appear, according to the results obtained in this work without any acute and sub-chronic toxic effect.

The susceptibility of the bee's *A. mellifera* to the strain IMI SD 187 was evaluated in the traditional topical bioassay. The mortality of the treated groups was inside the range of validity of the study, which should not exceed 20% in the control and neither there were differences with regard to him (Table 3). The longevity of the workers is 4-5 weeks, while in those that stay in boxes it happens great mortality in only seven or 10 days, as it indicates 20% of the mortality in the controls non treaties (Vandenberg, 1990).

The bees didn't present toxic signs neither alteration in the behaviour related with the application of the fungus, in this species of high sensibility in ecotoxicologics studies. In the infectivity study, the fungus of the body of the bees was not isolated; what indicates that this host is not suitable for the colonization.

The results indicate that the DL_{50} in *A. mellifera* of this bionematicide is above 10^5 clamidospores/ bee. Reinecke et al. (1990) obtained similar results when evaluating conidios of *M. anisopliae* and not find adverse effects neither mortality associated to the microorganism.

The mortality in the study in *C. exterior*, although in low percents, appeared indistinctly in all experimental groups (Table 4). It was without a direct relationship with the doses neither the variants of the product evaluated with regard to the control, what indicates the absence of toxicity in these predators with a CL_{50} above 10^7 clamidospores/ml.

In the infectivity analysis the presence of the fungus was demonstrated in *C. exterior*. This confirms the absence of toxicity since the necessary condition to achieve a harmful effect on non-target organisms is in fact, the combination of toxic and infective effects and in this case the predators were able to survive in presence of high concentrations of the fungus. Then any toxic effect should not be expected during the application in the form recommended in the field. Related with this, several experiments demonstrate that some insects can be refractory to the infection (Goettel and Jaronski, 1997).

Conclusion

The absence of toxic effects and deaths in *Eisenia andrei* B. indicate that the bionematicide constituted by the strain IMI SD 187 is innocuous for these terrestrial organisms and was considered non toxic for *Apis mellifera* L. and for larvae of *Chrysopa exterior* N.

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Table 1. Toxic effects and mortality in *E. andrei* treated with *P. chlamydosporia* at the end of each study.

Alterations (A) and mortality (M) in <i>E. andrei</i> ^a .						
Groups	Filter paper		Acute		Sub-chronic	
	M	A	M	A	M	A
Control	0	0	0	0	0	0
Vehicle	0	1/10 (10)	-	-	-	-
PAI	-	-	0	0	-	-
PA _{LD}	0	0	-	-	-	-
PA	1/10 (10)	5/10 (50)*	0	0	0	1/30 (3,3)
PF _{LD}	-	-	0	1/30 (3,3)	-	-
PF	-	-	0	0	2/30 (6,6)	1/30 (3,3)

*Statistical differences with regard to the control ($p < 0,05$)

(-) groups not included in the studies

^a Value as Frequency of Deaths or Alterations (%)

PAI: inactivated clamidospores powder

PA_{LD}: powder of clamidospores, low dose

PA: clamidospores powder

PF_{LD}: colonized substrate, low dose

PF: colonized substrate

Table 2. Presence of the strain IMI SD 187 in *E. andrei* and in substrate.

Groups	Values of UFC of the fungus / g			
	Acute (14 days)		Sub-chronic (30 days)	
	Worms	Substrate	Worms	Substrate
Control	0	0	0	0
PAI	0	0	0	0
PA	$2,4 \times 10^4$	6×10^5	$1,92 \times 10^4$	$5,25 \times 10^5$
PF _{LD}	$2,5 \times 10^2$	$1,7 \times 10^3$	1×10^2	$4,45 \times 10^3$
PF	$3,1 \times 10^3$	$1,4 \times 10^4$	$2,7 \times 10^3$	$5,7 \times 10^4$

PAI: inactivated clamidospores powder

PA: clamidospores powder

PF_{LD}: colonized substrate, low dose

PF: colonized substrate

Table 3. Mortality in *A. mellifera*.

Groups	Mortality in <i>A. mellifera</i>			
	72 h		96 h	
	Dead/Total	%	Dead/Total	%
Control	7/75	9,3	11/75	14,6
Vehicle	8/75	10,6	10/75	13,3
PAI	11/75	14,6	12/75	16
PA _{LD}	13/75	17,3	13/75	17,3
PA	11/75	14,6	12/75	16

*Statistical differences with regard to the control ($p < 0,05$)

PAI: inactivated clamidospores powder

PA_{LD}: powder of clamidospores, low dose

PA: clamidospores powder

Table 4. Mortality in *Chrysopa exterior*.

Mortality in <i>C. exterior</i>^a				
Groups	Direct exposure		Residual exposure	
	72 h	7 days	72 h	7 days
Control	1/10 (10)	1/10 (10)	0	2/10 (20)
Vehicle	2/10 (20)	2/10 (20)	0	2/10 (20)
PAI	1/10 (10)	1/10 (10)	1/10 (10)	3/10 (30)
PA _{LD}	0	1/10 (10)	0	2/10 (20)
PA _{MD}	1/10 (10)	1/10 (10)	2/10 (20)	3/10 (30)
PA	2/10 (20)	3/10 (30)	1/10 (10)	3/10 (30)

* Statistical differences with regard to the control ($p < 0,05$)

^a Value as Frequency of Deaths or Alterations (%)

PAI: inactivated clamidospores powder

PA_{LD}: powder of clamidospores, low dose

PA_{MD}: powder of clamidospores, medium dose

PA: clamidospores powder

Recibido: 28/03/08

Aceptado: 08/04/08