RESEARCH NOTE – NOTA INVESTIGATIVA

ISOLATES OF *POCHONIA CHLAMYDOSPORIA* VAR. *CHLAMYDOSPORIA* FROM MEXICO AS POTENTIAL BIOLOGICAL CONTROL AGENTS OF *NACOBBUS ABERRANS*

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ABSTRACT

Pérez-Rodríguez, I., A. Doroteo-Mendoza, F. Franco-Navarro, V. Santiago-Santiago and A. Montero-Pineda. 2007. Isolates of *Pochonia chlamydosporia* var. *chlamydosporia* from Mexico, as potential biological control agents of *Nacobbus aberrans*. Nematropica 37:127-134.

Pathogenicity of five Mexican isolates of *Pochonia chlamydosporia* var. *chlamydosporia* to eggs of *Nacobbus aberrans* was tested. A standardized method for mass production of the fungus was established and the potential of isolates as control agents of the nematode in glasshouse production was evaluated. Three isolates (MPc1-MPc3) parasitized 77.2%-89.0% of eggs using potato agar plates as fungal inoculum, and 72.0%-87.0% using colonized rice as inoculum. In glasshouse production, tomato plants inoculated with 15,000 chlamydospores/g of soil of isolate MPc3 showed less damage and fewer nematodes in roots compared to untreated soil. Chlamydospores and colony forming units (CFU)/g of substrate colonized by MPc3 were greater after 15 days of liquid and solid fermentation in rice. Biphasic fermentation in cracked maize for 21 days allowed chlamydospore yields and numbers of CFU/g of substrate that were greater than those of the fermentation in rice, even if the percent germination of chlamydospores was less than that in rice. Chlamydospore concentration decreased in both substrates by prolonging the fermentation period to 28 days. In Mexico, cracked maize is a cheaper substrate than rice and potentially as effective as rice for mass production of the fungus. Results confirm the potential of this fungus as a biological control agent of *N. aberrans*.

Key words: biological control, false root-knot nematode, growing substrates, Mexico, nematophagous fungi, plant-parasitic nematodes.

RESUMEN

Pérez-Rodríguez, I., A. Doroteo-Mendoza, F. Franco-Navarro, V. Santiago-Santiago y A. Montero-Pineda. 2007. Aislamientos de *Pochonia chlamydosporia* var. *chlamydosporia* de México como agentes potenciales de control biológico de *Nacobbus aberrans*. Nematropica 37:127-134.

Se probó la patogenicidad de cinco aislamientos mexicanos de *Pochonia chlamydosporia* var. *chlamydosporia* sobre huevos de *Nacobbus aberrans*; además, se estableció un método estandarizado para su producción masiva y se evaluó su potencial como agente de control biológico del nematodo en invernadero. Tres aislamientos (MPc1-MPc3) parasitaron 77.2%-89.0% de huevos al utilizar como fuente de inóculo placas de agar-papa, y 72.0%-87.0% al usar arroz colonizado por los hongos. En invernadero, las plantas de tomate inoculadas con 15,000 clamidosporas/g de suelo del aislamiento MPc3, presentaron menor daño y menos nematodos en raíces (Tukey, $P < 0.05$), comparado con las de suelo no tratado. Después de 15 días de fermentación líquida y sólida en arroz, las clamidosporas y unidades formadores de colonias (UFC)/g de sustrato colonizado por MPc3 fueron mayores. Con fermentación bifásica en maíz quebrado durante 21 días, la producción de clamidosporas y el número de

UFC/g de sustrato fueron mayores que con fermentación en arroz, aún cuando la germinación de clamidosporas fue menor que en arroz. La concentración de clamidosporas decreció en ambos sustratos cuando la fermentación se prolongó hasta 28 días. En México, el maíz quebrado es un sustrato más barato que el arroz y tan efectivo como éste para producir masivamente al hongo. Los resultados confirman el potencial del hongo como un agente de control biológico de *N. aberrans*.

Palabras clave: control biológico, hongos nematófagos, México, nematodo falso nodulador, nematodos parásitos de plantas, sustratos de crecimiento.

The fungus, *Pochonia chlamydosporia*, is a facultative parasite present in suppressive soils and recognized as a promising biological control agent for cyst (Kerry *et al.*, 1984) and root-knot nematodes (de Leij *et al.*, 1992, 1993; Atkins *et al.*, 2003a, b; Kerry and Hidalgo-Diaz, 2004). The fungus is found in soil, colonizes the rhizosphere of some plants, and proliferates in the egg masses formed by root-knot nematodes on the roots of their host plants (Hirsch *et al.*, 2001). The fungus may be applied to soil as a chlamydospore suspension to reduce root-knot nematode populations and their damage to susceptible plants (Bourne *et al.*, 1996).

Integrated and sustainable strategies such as the application of the fungus with poor hosts for the nematode to reduce infestations in soil before the next fully susceptible crop is grown, have been developed to maximize the potential of *P. chlamydosporia* as biological control agent in different agricultural systems (Hidalgo *et al.*, 2000; Kerry and Bourne, 1996; Kerry and Hidalgo, 2004). This strategy has been very useful for the management of root-knot nematodes, especially in organic crop production systems (Atkins *et al.*, 2003b; Kerry and Hidalgo, 2004).

Different isolates of the fungus have been found and isolated from suppressive soils around the world and have shown differences in their control efficacy (Bourne *et al.*, 1994; de Leij and Kerry, 1991; de Leij *et al.*, 1993). In Mexico, some native isolates of *P. chlamydosporia* var. *chlamydosporia* from three states (Mexico State, Tlaxcala and Puebla), have been found and tested for the management of nematode pests in vegetables (Flores, 2003). One of the most important of these nematodes in Mexican agriculture is the false root-knot nematode *Nacobbus aberrans* (Thorne, 1935) Thorne & Allen 1944, which causes severe damage in crops such as tomato (*Lycopersicon esculentum*), chili (*Capsicum annuum*) and bean (*Phaseolus vulgaris*) (Manzanilla-López *et al.*, 2002). Chemical control is effective, but costly, and causes environmental and human health concerns. As a consequence, non-chemical control approaches have become more attractive to Mexican growers in production systems where the false root-knot nematode is present. The need for alternative strategies to chemical management has prompted a study to determine i) the *in vitro* parasitic ability of five Mexican isolates of *P. chlamydosporia* var. *chlamydosporia* against *N. aberrans* eggs; ii) their biological efficacy against this nematode on tomato cv. Rio Grande in glasshouse conditions; and iii) the suitability of new and more affordable substrates under the conditions in Mexico for the growth of this beneficial fungus. The results of this work are reported in this note.

Organisms and Growth Conditions

The nematophagous fungi used in this study included five isolates of *P. chlamydosporia* var. *chlamydosporia* (MPc1-MPc5) collected from soils naturally infested with

N. aberrans (Flores, 2003), and stored after freeze drying in the nematophagous fungi collection at the Colegio de Postgraduados. The isolates were cultured on potato-agar (PA) at 27°C in the dark.

Eggs Parasitism

Two experiments were established to test the parasitic ability of the five fungal isolates cultured on two different substrates (colonized rice and potato-agar (PA) plates) to parasitize the eggs of two populations of *N. aberrans* (Tecamachalco, Puebla and Montecillo, Mexico State). In the first experiment, colonized rice was used to produce the fungal inoculum using standard techniques (Hidalgo *et al.*, 2000, modified by Pérez, 2004). A suspension of 5×10^3 chlamydospores/ml for each isolate was prepared through the dilution of colonized rice in water-agar (WA) 0.05% to be applied onto WA 1.5% plates + antibiotics. In the second experiment, approximately 5 ml of WA 0.05% were added to PA plates where the fungus was grown for 21 days, and the colony surface scraped using a glass rod. From each plate, aliquots of 0.2 ml of the fungal suspension were taken and poured onto WA 1.5% plates + antibiotics. *Nacobbus aberrans* eggs were collected from egg masses extracted from tomato cv. Rio Grande roots infected by *N. aberrans* and maintained in a glasshouse at 35°C. Eggs were placed in WA 0.05% to obtain a concentrated suspension. Two groups of Petri dishes with the five fungal isolates from colonized rice and PA were incubated for 48 hours at 27°C and then 500 nematode eggs were added to each of the five isolates. Eggs were exposed to the fungus in the dishes for four days at 27°C and then examined under a compound microscope. Fungal parasitism was evaluated by examining 100 eggs selected randomly in the Petri dishes to estimate the percent of parasitism. Each experiment was randomly arranged and consisted of ten treatments replicated five times (5 isolates × 2 nematode populations) and two controls without fungi, one for each population of the nematode. All data were analyzed statistically and means were compared using Tukey's test ($P < 0.05$). Statistical procedures were performed using Statistical Analysis System (SAS) software.

In both experiments, differences (Tukey $\alpha < 0.01$) where observed in the parasitic ability of the isolates tested, independent of the inoculum source used. In the first experiment (colonized rice), increased parasitism of nematode eggs from the Montecillo and Tecamachalco populations was induced by the isolates MPc1-MPc3, and to a lesser extent by MPc5 and MPc4 (Table 1). Similar results were obtained in the second experiment using the isolates grown in PA plates (Table 1).

Glasshouse Experiment

A soil naturally infested with *N. aberrans* was collected from a tomato field in Tecamachalco and stored in a greenhouse at 30°C. The inoculum source for each isolate was colonized rice (Hidalgo *et al.*, 2000, modified by Pérez, 2004), from which viability and quality (concentration of chlamydospores/g of substrate, germination of chlamydospores and CFU/g of substrate) were assessed. The five isolates were applied to the nematode-infested soil in two concentrations, one medium (7,500 chlamydospores/g of soil) and one high (15,000 chlamydospores/g of soil), and using vermicompost (15 ton/ha) as a carrier, which was incorporated by mixing it into the soil. Inoculated soil was then poured in 1.6 kg pots and planted with two plants per pot of nematode-free tomato

Table 1. Parasitism of different isolates of *Pochonia chlamydosporia* var. *chlamydosporia* on eggs of two populations of *Nacobbus aberrans* using two inoculum sources (Experiment 1 = colonized rice. Experiment 2 = potato agar plates).

	Experiment 1		Experiment 2	
Isolate	Montecillo ($\alpha < 0.01$; $R^2 = 0.99$)	Tecamachalco ($\alpha < 0.01$; $R^2 = 0.99$)	Montecillo ($\alpha < 0.01$; $R^2 = 0.99$)	Tecamachalco ($\alpha < 0.01$; $R^2 = 0.98$)
Control (no fungus)	0.0 e[y]	0.0 d	0.0 e	0.0 d
MPc1	87.0 a	81.2 a	86.0 a	78.2 ab
MPc2	79.0 b	74.0 a	77.2 b	79.4 ab
MPc3	77.0 b	79.4 a	89.0 a	83.4 a
MPc4	34.8 d	10.2 c	70.2 c	61.6 c
MPc5	52.0 c	51.0 b	44.4 d	71.4 b
MSD[z]	7.1	8.5	6.8	9.2

[y]Values are means of five replicates. Numbers with similar letters are not different significantly (Tukey $\alpha < 0.01$).
[z]Minimum significant difference.

seedlings var. Rio Grande obtained from clean tomato cultures. There were ten treatments (5 isolates × 2 rates) and one untreated control replicated four times. Fungi were applied twice, the first time at planting and the second one 30 days after planting. At 50 days after planting, plant growth parameters (leaf dry weight and fresh weight of roots) were recorded. Nematode root galls, egg masses, vermiform and sedentary nematode stages/g of roots were counted. Fungal parasitism was assessed and expressed as percent of infected egg masses and colony forming units (CFU)/g of soil and roots. Data were analyzed statistically as mentioned above.

There were significant differences between the treatments for most of the variables tested. The gall index in plants treated with isolate MPc3 at the high application rate was the lowest in comparison with the remaining treatments ($P < 0.05$), whereas the leaf dry weight was approximately 80.6% higher than that observed in control plants ($P < 0.05$) (Table 2). The number of vermiform stages, mature females and eggs masses/g of roots was lower ($P < 0.05$) in plants treated with the isolate MPc3 at a rate of 15,000 chlamydospores/g of soil. This isolate was one of the two isolates re-isolated from soil and from roots (Table 2).

Substrates for Mass Production of the Fungus

Rice is the most common substrate used for the growth of *P. chlamydosporia* in a combination of liquid and solid fermentation phases (Lfr+Sfr) (Hidalgo *et al.*, 2000; Pérez, 2004). Two variants of this methodology were tested and compared to Lfr+Sfr. These variants included liquid fermentation with rice and solid fermentation with cracked maize (Lfr+Sfcm), and both fermentations with cracked maize (Lfcm+Sfcm). Glass flasks were used to produce fungal inoculum in broth of each substrate (liquid fermentation) during three days at 27°C and 130 rpm; solid fermentation was achieved using autoclaved grains of each substrate, which were contained in plastic bags (200 g of substrate/plastic bag) and inoculated with 25 ml of

Table 2. Effect of the application of different isolates of *Pochonia chlamydopsoria* var. *chlamydosporia*—two rates—on tomato plants grown in naturally infested soil by *Nacobbus aberrans* (Tecamachalco population).

Isolate	Rate (chl/g soil)	GI[q]	LDW[r] (g)	FWR[s] (g)	JUV[t] (per g roots)	MF[u] (per g roots)	EM[v] (per g roots)	CFUR[w] (per g roots)	CFUS[x] (per g soil)
No fungus		5.9 a[y]	0.9 b	8.8 b	78 a	29 ab	21 b	0 b	0 b
MPc1	7500	4.3 bc	1.8 b	15.3 ab	86 a	25 ab	16 c	0 b	0 b
MPc1	15000	5.6 a	2.2 ab	19.4 a	31 ab	23 b	20 b	0 b	0 b
MPc2	7500	4.8 b	2.4 ab	13.2 ab	74 a	27 ab	16 c	0 b	0 b
MPc2	15000	5.6 a	1.8 b	16.5 ab	73 a	29 ab	16 c	633 a	250 a
MPc3	7500	4.3 bc	2.2 ab	17.0 ab	39 ab	24 b	26 a	0 b	0 b
MPc3	15000	3.3 c	4.7 a	14.9 ab	3 b	5 c	1 d	667 a	100 a
MPc4	7500	4.5 b	2.1 ab	10.1 ab	68 a	26 ab	22 b	0 b	0 b
MPc4	15000	5.5 a	1.7 b	18.6 a	63 a	25 ab	21 b	0 b	0 b
MPc5	7500	5.6 a	1.8 b	16.2 ab	61 a	24 b	21 b	0 b	0 b
MPc5	15000	5.6 a	2.1 ab	19.3 a	45 a	33 a	21 b	0 b	0 b
MSD[z]		0.6	2.8	9.7	41	8	3	213	45

[q]Gall index.
[r]Leaf dry weight.
[s]Fresh root weight.
[t]Third and fourth stages juveniles.
[u]Mature females.
[v]Eggs masses.
[w]Colony forming units on roots.
[x]Colony forming units in soil.
[y]Values are means of four replicates. Numbers with similar letters are not different significantly (Tukey $\alpha < 0.05$).
[z]Minimum significant difference.

fungal inoculum obtained in liquid fermentation (Pérez, 2004). The most promising *P. chlamydosporia* isolate from the previous experiments was used in this test (MPc3). There were three treatments (Lfr+Sfr, Lfr+Sfcm and Lfcm+Sfcm) replicated five times (five plastic bags with 200 g of substrate per treatment). At 15, 21 and 28 days after inoculation, number of chlamydospores/g of substrate, CFU/g of substrate, and chlamydospore germination (%) were evaluated. Data were analyzed statistically as mentioned above.

Number of chlamydospores, CFU/g of substrate and percent of chlamydospore germination observed at different fermentation periods in liquid and solid rice and maize and/or their combination are shown in Table 3. The results of this experiment indicated that the growth of this fungal isolate expressed as concentration of chlamydospores/g of substrate was greater after a short period (15 days) of liquid and solid fermentation in rice. However, the biphasic fermentation in cracked maize for a period of 21 days allowed excellent chlamydospore yield that was greater than that of the fermentation in rice, even though the percent germination of the chlamydospores was less than that

Table 3. Product quality variables obtained by a biphasic method for mass production of *Pochonia chlamydosporia* var. *chlamydosporia*, using different growth substrates at different days after inoculation.

	Chlamydospores/g of substrate (10^6) ($\alpha < 0.01$)			Germination (%) ($\alpha < 0.01$)			CFU[u]/g of substrate (10^7) ($\alpha < 0.01$)		
	Days after inoculation			Days after inoculation			Days after inoculation		
Treatment	15 $R^2 = 0.95$	21 $R^2 = 0.45$	28 $R^2 = 0.57$	15 $R^2 = 0.51$	21 $R^2 = 0.42$	28 $R^2 = 0.80$	15 $R^2 = 0.93$	21 $R^2 = 0.94$	28 $R^2 = 0.98$
Lfr-Sfr[v]	7.7 a[w]	9.5 b	5.3 b	66.2 b	75.0 ab	68.4 c	1.5 a	1.8 b	2.6 c
Lfr-Sfcm[x]	5.3 b	10.3 ab	8.1 a	74.8 a	80.0 a	77.0 b	5.7 b	7.3 a	5.2 b
Lfcm-fcm[y]	2.1 c	13.1 a	5.2 b	68.6 ab	73.6 b	82.8 a	7.0 c	8.4 a	7.6 a
MSD[z]	1.0	3.2	2.2	6.8	6.1	5.6	1.2	1.4	5.2 10^6

[u]Colony forming units.
[v]Liquid fermentation with rice+solid fermentation with rice.
[w]Values are means of five replicates. Numbers with similar letters are not different significantly (Tukey $\alpha < 0.01$).
[x]Liquid fermentation with rice+solid fermentation with cracked maize.
[y]Liquid fermentation with cracked maize+solid fermentation with cracked maize.
[z]Minimum significant difference.

in rice. Chlamydospore concentration decreased in all treatments by prolonging the fermentation period to 28 days.

In the first of the tests in this study, and independently of the inoculum source of the fungus used, isolates MPc1-3 were most virulent against both populations of the false root-knot nematode. The parasitism rate of these isolates was similar to, or slightly greater than, that observed in a study carried out by Hidalgo *et al.* (2000) with isolates of *P. chlamydosporia* var. *catenulata* on *Meloidogyne incognita* eggs.

Despite the three isolates having caused a high percentage of parasitism of nematode eggs *in vitro*, only MPc3 isolate showed good results when it was applied into naturally infested soil in glasshouse conditions. This isolate at a high concentration, applied with vermicompost as a source of organic matter, was associated with lower gall index, greater leaf dry weight, and fewer eggs masses, juveniles and mature females/g of roots, compared to the control plants. This MPc3 isolate was the only one which could be re-isolated from egg masses, soil and roots at the end of the experiment, whereas the other isolates tested, with the exception of MPc2 re-isolated only from soil and roots, did not persist. That only one isolate of *P. chlamydosporia* was effective against *N. aberrans* eggs can be explained by the wide host preference of the isolates of this fungus (de Leij and Kerry, 1991; Hidalgo *et al.*, 2000). Although isolate MPc3 was effective in glasshouse conditions, more extensive testing is necessary to determine the effectiveness of the isolate in a range of conditions before its development as a potential biological control agent of the false root-knot nematode.

P. chlamydosporia is able to become established more easily in soil from chlamydospores than from an external food source, which may support the growth of other soil microorganisms successfully competing with the fungus, limit-

ing its proliferation in soil (de Leij and Kerry, 1991; Kerry *et al.*, 1984). In fact, chlamydospores have been used as the preferred propagule for application to soil because they do not require an additional energy source to establish the fungus in the rhizosphere of plants. Different yields of chlamydospores have been obtained from solid media to obtain sufficient inoculum for treatments in the field because, in liquid fermentation systems, chlamydospore production is poor. Although of the biphasic fermentation process devised for the production of *P. chlamydosporia* using cracked rice reduces the production costs (Hidalgo *et al.*, 2000; Kerry and Hidalgo, 2004), this method is still expensive in Mexico. The results of our laboratory study indicate that a good alternative to improve the growth of *P. chlamydosporia* is the use of cracked maize, which is 2.5 times cheaper in Mexico than rice, the conventional medium used to mass produce *P. chlamydosporia*. In this study, when the biphasic method with cracked maize (broth and grain) was used, most of the chlamydospores were produced at 21 days after inoculation, which is similar to the inoculum period estimated by others (Hidalgo *et al.*, 2000; Pérez, 2004). In all treatments, there was a reduction in the number of chlamydospores 28 days after inoculation, despite an increase in chlamydospore germination and the number of CFU. These findings indicate that prolonged incubation under the conditions tested in this study does not result in greater chlamydospores production.

These results are the first in an extensive study of the parasitic relationship (eggs parasitism and biological efficiency) between *P. chlamydosporia* var. *chlamydosporia* and *N. aberrans*, which was initiated by Flores (2003), and confirm the potential importance of this fungus as a control agent of the false root-knot nematode. Results from *in vitro* and glasshouse experiments are indicative and not conclusive, so it is necessary as to improve and standardize large scale production of the fungus to corroborate parasitic potential of the best Mexican isolate by field trials.

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