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IN VITRO EFFECT OF *Purpureocillium lilacinum* (Thom) Luangsa-Ard *et al.* AND *Pochonia chlamydosporia* var. *catenulata* (Kamyschko ex Barron & Onions) Zare & Gams ON THE ROOT-KNOT NEMATODES [*Meloidogyne incognita* (Kofoid & White) Chitwood AND *Meloidogy nemayaguensis* Rammh & Hirschmann][*]

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Abstract

Purpureocillium lilacinum strain PL-11 and *Pochonia chlamydosporia* strain JL-1 fungal strains, are a biological alternative to reduce plant parasitic nematodes on the roots of plants. The objective of this research was to determine the most effective concentration of *P. lilacinum* strain PL-11 and *P. chlamydosporia* strain JL-1, for the management of root-knot nematode (*Meloidogyne* spp.). In the Plant Pathology laboratory, at University of Caldas, in a completely randomized design, 1 mL of *P. lilacinum* (1 x 10^9 spores / mL) were added to 32 bacteriological Petri dishes with agar; subsequently, 16 of them were inoculated with a 30 mL suspension containing 10 eggs of *Meloidogyne incognita* and *Meloidogyne mayaguensis* and, the other 16, were inoculated with a 30 mL suspension containing 10 juveniles (J2) of the two *Meloidogyne* species. Fungal infection of eggs and mortality of juveniles (J2) of the two species of *Meloidogyne* were evaluated at 24, 72, 120 and 168 h. The same procedure was performed with *P. chlamydosporia*, the combination *P. lilacinum* and *P. chlamydosporia*, *P. lilacinum* and *P. chlamydosporia* in combination with Carbofuran at concentrations between 1 x 10^3 and 1 x 10^9 spores / mL. The positive and negative controls were Carbofuran and water, respectively. Results demonstrated that mixing *P. lilacinum* and *P. chlamydosporia* (1 x 10^6 spores / L) in combination with Carbofuran, and the mixture *P. lilacinum* and *P. chlamydosporia* (1 x 10^8 spores / mL), caused the highest infections on eggs with 85% and 80%, respectively, and caused the highest mortality of juvenile (J2) of *M. incognita* and *M. mayaguensis* with 93% and 75%, respectively, compared to the water treated group, at 168 h.

Key words: biological control, infection, *Meloidogyne*, *Pochonia chlamydosporia*, *Purpureocillium lilacinum*.

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EFECTO *In vitro* DE *Purpureocillium lilacinum* (Thom) Luangsa-Ard *et al.* Y *Pochonia chlamydosporia* var. *catenulata* (Kamyschko ex Barron & Onions) Zare & Gams SOBRE EL NEMATODO DEL NUDO RADICAL [*Meloidogyne incognita* (Kofoid & White) CHITWOOD Y *Meloidogyne mayaguensis* Rammh & Hirschmann]

Resumen

Los hongos *Purpureocillium lilacinum*, cepa Pl-11 y *Pochonia chlamydosporia*, cepa Jl-1, son una alternativa biológica para reducir los nematodos fitoparásitos en las raíces de las plantas. El objetivo de esta investigación fue determinar la concentración más efectiva de *P. lilacinum*, cepa Pl-11 y *P. chlamydosporia*, cepa Jl-1, para el manejo del nematodo del nudo radical (*Meloidogyne* spp.). En el Laboratorio de Fitopatología de la Universidad de Caldas, en un diseño completamente aleatorio, a 32 cajas de Petri con agar bacteriológico se les adicionó 1 mL de *P. lilacinum* (1 x 10^9 esporas/mL); posteriormente, a 16 de ellas se les agregó 30 µL de suspensión con 10 huevos de *Meloidogyne incognita* y *Meloidogyne mayaguensis* y a las otras 16, se les agregó 30 µL de suspensión con 10 juveniles (J2) de las dos especies de *Meloidogyne*. La infección de los hongos sobre huevos y la mortalidad sobre juveniles (J2) de las dos especies de *Meloidogyne*, se evaluaron a las 24, 72, 120 y 168 h. El mismo procedimiento se realizó con *P. chlamydosporia*, la mezcla *P. lilacinum* y *P. chlamydosporia* y *P. lilacinum* o *P. chlamydosporia* en combinación con Carbofuran en concentraciones entre 1 x 10^3 y 1 x 10^9 esporas/mL. Los testigos fueron Carbofuran y agua. Se demostró que la mezcla de *P. lilacinum* y *P. chlamydosporia* (1 x 10^6 esporas/L) en combinación con Carbofuran, y la mezcla *P. lilacinum* y *P. chlamydosporia* (1 x 10^8 esporas/mL), causaron las mayores infecciones sobre los huevos con 85% y 80%, respectivamente, y produjeron las mayores mortalidades de juveniles (J2) de *M. incognita* y *M. mayaguensis* con 93% y 75%, respectivamente, en comparación con el testigo agua, a las 168 h.

Palabras clave: control biológico, infección, nematodos fitoparásitos.

INTRODUCTION

The root-knot nematode, *Meloidogyne* spp. Goeldi, causes great losses to the agricultural economy, due to its high adaptability, reproduction, survival and wide host range (AGRIOS, 2005; PERRY *et al.*, 2009). In Colombia, the losses caused by *Meloidogyne* spp. in the cultivation of guava (*Psidium guajava* L.) are above 60% (VILLOTA & VARÓN, 1997; BOLAÑOS *et al.*, 2007).

Guava is essential in the Colombian economy, as commercial activities generated with its cultivation support 9,000 families, representing $ 40,000 million pesos annually 15,000 planted hectares with a production of 145,000 tons yield (14.9 tons / ha) established in 22 states; of which Valle del Cauca, Meta, Caldas, Risaralda, Santander and Quindío, with 17,926, 12,988, 6,756, 6,577, 6,147 and 4,839 tons, respectively, stand out (FAO, 2008; DANE, 2011; TAFUR, 2012).

Generally, control of *Meloidogyne* spp. is performed through the use of fumigant nematicides (Metam sodium and Dazomet) or non-fumigants (Cadusafos and Carbofuran) (BARRES *et al.*, 2006; LIÑAN, 2009; ICA, 2014), perhaps by their effectiveness in reducing levels population and its availability on the market (ARAYA, 2003; PERRY *et al.*, 2009). However, the ability to detect concentrations of nematicides in the atmosphere has increased environmental monitoring and growing concerns about its use (GOWEN, 1997; CHITWOOD, 2003; WADA & TOYOTA, 2008).

As an alternative to chemical management of *Meloidogyne* spp., biocontrol agents such as *Pochonia chlamydosporia* var. *catenulata* (Kamyschko ex Barron & Onions) Zare & Gams, *Purpureocillium lilacinum* (Thom) Luangsa-Ard, Houbraken, Hywel-Jones & Samso and Gams, *Glomus clarum* Nicol. & Shenck, *Trichoderma harzianum* Rifai, *Cylindrocarpon destructans* (Zinsmeister) Scholten and *Athrobotrys oligospora*, are being used; though, *P. lilacinum* and *P. clamydosporia* var. *catenulata* are considered the most promising in the management of populations of *Meloidogyne* spp. (CARDONA & LEGUIZAMÓN, 1997; KERRY & JAFFEE, 1997; MONTES DE OCA *et al.*, 2005; PETEIRA *et al.*, 2005; PUERTAS *et al.*, 2006; SINGH *et al.*, 2013).

The fungus *P. lilacinum*, which belongs to the phylum Ascomycota, Sordariomycetes class, order Hypocreales and family Ophiocordycipitaceae (HIBBETT *et al.*, 2007; LUANGSA-ARD *et al.*, 2011), infects *Meloidogyne* spp. by contact, with conidia that attach and germinate on the cuticle and then penetrate the body of the nematode through appresoria; then takes its nutrients and reproduce massively invading the nematode's body until its death (MONZÓN *et al.*, 2009). In tomato (*Solanum lycopersicum* L.) *P. lilacinum* at a concentration of 2 x 10^6 spores / mL, infected 40% of females and 70% of eggs and juveniles of *Meloidogyne* spp. (KHAN & SAXENA, 1997, ESFAHANI & POUR, 2006). In American okra (*Abelmoschus esculentus*) *P. lilacinum* at a concentration of 2.3 x 10^8 spores / mL, reduced 78% of eggs and 81% of juveniles (J2) of *Meloidogyne* spp. (CRUZ, 2007). In limes (*Citrus aurantifolia* Christm. et Panz.) *P. lilacinum* at a concentration of 2 x 10^6 CFU / mL, mixed with *P. chlamydosporia* at a concentration of 2 x 10^6 CFU / mL, infected 49% of juveniles (J2) *M. javanica* and 54% of nematode eggs (RAO, 2005).

The fungus *P. chlamydosporia* var. *catenulata*, which belongs to the phylum Ascomycota, Sordariomycetes class, order Hypocreales and family Clavicipitaceae (HIBBETT *et al.*,

2007; ZARE *et al.*, 2001), acts by contact, infecting and parasitizing eggs of *Meloidogyne* spp., through appressoria developed from undifferentiated hyphae (MORGAN-JONES *et al.*, 1983; MONTES DE OCA *et al.* (2005) and PETEIRA *et al.* (2005) demonstrated that the fungus *P. chlamydosporia* var. *catenulata* in concentration of 5 x 10^6 spores / mL, is a potential agent for biological control of root knot nematodes in crops of beans [*Vigna unguiculata* (L.) Walp.] and infect 80% of the eggs of *Meloidogyne* spp. In tomato (*Solanum lycopersicum* L.) *P. chlamydosporia* in concentration of 5 x 10^6 spores / mL, reduces 72.83% of *M. javanica* eggs (DALLEMOLE-GIARETTA *et al.*, 2014). Also, *P. chlamydosporia* has great potential as a controller of *Meloidogyne* spp. by producing dictioclamidospores, which are resistant reproductive structures that allow it to survive, generate mycelium, colonize the rhizosphere and proliferate in the soil (GIRALDO & LEGUIZAMÓN, 1997; FLORES *et al.*, 2008; HERNÁNDEZ & DÍAZ, 2008).

The biocontrol effect of *P. lilacinum* and *P. chlamydosporia* var. *catenulata* on the root-knot nematode, without producing harmful effects to humans, animals and the ecosystem, has led to interest in developing commercial inputs based on these fungi (ATKINS *et al.*, 2003; GARCÍA *et al.*, 2004). Moreover, it has been found that the mycelial growth and the production of conidia of these fungi increase with increasing concentration (HERNÁNDEZ & DÍAZ, 2008; CABRERA *et al.*, 2011). Other research has reported that the effectiveness of *P. lilacinum* and *P. chlamydosporia* var. *catenulata* in handling *Meloidogyne* spp. varies depending on the strain, concentration of spores in the rhizosphere colonization and host specificity (STIRLING & WEST, 1991; KERRY & JAFFE, 1997; MORTON *et al.*, 2004; DALLEMOLE-GIARETTA *et al.*, 2014). Based on this information, the present research was conducted under conditions *in vitro*, in order to determine the most effective concentration of *P. lilacinum* strain PL-11 and *P. chlamydosporia* strain JL-1, alone or in combination, for use in future research in nurseries and established guava plantations in the management of root-knot nematode (*Meloidogyne* spp.).

MATERIALS AND METHODS

Location. The research was conducted in the laboratory of Plant Pathology Department of Agricultural Production, Faculty of Agricultural Sciences at the University of Caldas, Manizales, Caldas.

Preparation of inoculum of *Meloidogyne incognita* and *Meloidogyne mayaguensis*. Samples of guava tree roots of Palmira ICA-1 variety, at 5 years of age, with presence of root knot, were collected. Trees were located in the Taparcal farm, in the town of La Manuela, municipality of Palestina, department of Caldas. Root samples were taken to the laboratory for nematode extraction procedure based on the principle of nematode flotation on sucrose gradient described by JENKINS (1964) and MEREDITH (1973).

Initially, the roots were washed with tap water, allowed to dry at room temperature, 30 g of which were weighed on a balance Analytical Plus, Shimadzu® mark, and 1 cm rootstock were cut transversely with scissors. Subsequently, the pieces were placed into a glass vase of blender, Osterizer, model 565-15, with 500 mL of water, liquefied three times at high speed for 10 s. The liquefied solution was deposited on a 250 micron mesh sieve followed by a 106 micron mesh and finally a 25 micron mesh screen. The sample was washed with water to cause detachment of nematodes and the material left on the 25 microns mesh sieve was placed in centrifuge tubes of 50 mL capacity. Then, the tubes were centrifuged at 3,750 rpm for 5 minutes on a Labnet® centrifuge. Following centrifugation there was sedimentation of heavy particles at the bottom of the tube, and the supernatant was removed. Next, the tubes were filled again with a sucrose solution 50% and subjected again to centrifugation at 3,750 rpm for 5 min so that the nematodes remain floating in the sucrose solution by differential density and were separated from the heavier particles. Then, the supernatant was transferred to a 25 microns mesh sieve to wash sucrose with tap water at low pressure and prevent any negative effect on the nematodes.

Subsequently, 20 mL of water with nematodes were collected in a petri dish, which is then mounted on a stereoscope Leica® brand increased 30 X to remove eggs and juveniles (J2) of *Meloidogyne* spp. using a micropipette brand Biohit® 100 µL. Immediately after, the quantification of eggs and juveniles (J2) was made *Meloidogyne* spp. in 100 g of roots, using a box counting 36 cells divided into (6 x 6) each of 1 cm^2. Eggs and juveniles (J2) of *Meloidogyne* spp. were disinfected separately a solution of sodium hypochlorite 0.5% for 3 min, and then washed with sterile distilled water (ADE).

Additionally, *Meloidogyne incognita* and *Meloidogyne mayaguensis* species were identified through the morphological characterization of perineal patterns of adult females and morpho-metrics tests of juveniles (J2), following taxonomic keys of TAYLOR & SASSER (1983), EISENBACK (1985), JEPSON (1987) and PERRY *et al*. (2009).

Biological and chemical actives used for control of *M. incognita* and *M. mayaguensis*. For the experiment, two biological products, formulated by Laverlam International Corp., Butte, Montana, USA, were used. The first was *Purpureocillium lilacinum*, strain PL-11 formulated as a wettable powder (WP) at a concentration of 4 x 10^9 spores / g, (trade name Biostat®), and the second was *Pochonia chlamydosporia* var. *catenulata*, strain JL-1, (provided by National Coffee Research Center - Cenicafé, strain Cenicafé Jl-1) formulated as wettable powder at a concentration 1.58 x 10^8 spores / g and 2 x 10^6 chlamydospores / g. The chemical active Carbofuran was used at concentration of 330 g / L (trade name Furadan® 3 SC). This input comes as a concentrated solution and toxicological category I.

Preparation of biological and chemical concentrations actives. The fungi were diluted in water to a solution containing 1 x 10^9 spores / mL determined by the following procedure: 1 g of *P. lilacinum* or *P. chlamydosporia* was weighed on a balance Analytical Plus brand Shimadzu® then deposited into a beaker containing 1 L of tap water and homogenized with a magnetic stirrer Arec® brand. Then the count of spores / mL was carried out through a hemocytometer Boeco® mark, following the procedure described by CASTAÑO-ZAPATA (1998). After performing various spore counts, the concentration of 1 x 10^9 spores / mL of *P. lilacinum* was achieved with 2.5 g / L of water and the concentration of 1 x 10^9 spores / mL of *P. chlamydosporia* was obtained with 2 g / L of water.

From the concentration of 1 x 10^9 spores / mL *P. lilacinum* or *P. chlamydosporia*, sequential concentrations were obtained up to 1 x 10^3 spores / mL, by applying the formula C1 = V2.V1/C2 suggested by CASTAÑO-ZAPATA (1998), where V1 = initial volume of the suspension; C1 = initial concentration of spores / mL; V2 = final volume of the suspension; C2 = final concentration of spores / mL.

The concentrations 125, 250 and 500 ppm of Carbofuran were achieved by adding 378.78, 757.56 and 1,137 µL, respectively, using a micropipette brand Biohit® 1000 µL into a liter of water of the chemical product Furadan®, to be used as the standard chemical positive control.

Preparation of culture medium. Bacteriological agar Oxoid® Brand No.1 1.5%, was used as culture medium. Prepared by weighing 7.5 g added to 1 L of distilled water in a beaker. The mixture was homogenized on a magnetic stirrer Arec® mark and then placed on a hot plate until boiling. Subsequently, the mouth of the beaker was covered with aluminum foil and autoclaved 60 120°C for 15 min at 18 psi. When the medium reached a temperature of 60°C, 5 mL of 25% lactic acid was added, under laminar flow hood, in order to inhibit the growth of any bacteria. Then, the mixture was homogenized using a sterile glass stirrer. Then, 6 mL of the prepared medium was poured into each Petri dish of 60 x 15 mm and then allowed to stand for 3 h until the medium became slurry.

Application of treatments. To thirty-two Petri dishes containing the semi bacteriological agar medium, 1 mL of *P. lilacinum* solution containing 1 x 10^9 spores / mL were added. Then, to 16 of these Petri dishes, 30 µL of a suspension containing 10 eggs of *M. incognita* and *M. mayaguensis* were added using a micropipette Biohit® capacity of 100 µL; and to the other 16 Petri dishes a 30 µL suspension containing 10 juveniles (J2) of *M. incognita* and *M. mayaguensis* were added. Subsequently, fungal infection on eggs and mortality (infection) of juveniles (J2) of *Meloidogyne* species was evaluated at 24, 72, 120 and 168 h. A water treated, negative control, group of 32 Petri dishes (16 boxes with eggs and 16 boxes with juveniles) was also tested.

The above procedure was performed with the other treatments: *P. lilacinum* (1 x 10^8 to 1 x 10^6 spores / mL), *P. chlamydosporia* (1 x 10^9 to 1 x 10^6 spores / mL), the mixture of *P. lilacinum* and *P. chlamydosporia* (1 x 10^5 to 1 x 10^8 spores / mL), *P. lilacinum* (1 x 10^7 to 1 x 10^4spores / mL) combined with Carbofuran (125 ppm), *P. chlamydosporia* (1 x 10^7 to 1 x 10^4 spores / mL) combined with Carbofuran (125 ppm), *P. lilacinum* and *P. chlamydosporia* (1 x 10^6 to 1 x 10^3 spores / mL) in combination with Carbofuran (125 ppm), and Carbofuran (125, 250 and 500 ppm). Regardless, for eggs and juveniles (J2) of *M. incognita* and *M. mayaguensis* 448 experimental units, determined by 28 treatments corresponding to concentrations of actives, four replicates and four exposure times were evaluated.

Statistical analysis. The experimental design used was completely random. The data obtained were subjected to normality test of Kolmogorov-Smirnoff, complying with the hypothesis of normality (p-value > 0.01), an analysis of variance was performed, and a comparison Tukey test at a 5% level of probability with the Statistical Analysis System (SAS, 2009).

Variables evaluated. Immediately after completion of exposure time of eggs and juveniles (J2) of *M. incognita* and *M. mayaguensis* to a given treatment, two drops of Lactophenol blue were added to each Petri dish; contents being deposited on a slide of 2.54 x 7.62 cm, using a micropipette Biohit ® brand of 100 µL, in order to evaluate the following variables:

Infection (%): defined as the invasion and multiplication of the fungus on eggs of *M. incognita* and *M. mayaguensis* and expressed in percentage as the number of infection per 100 eggs on the initial population. The corrected infection (I), was calculated using the following formula Schneider-Orelli (COSTA *et al.*, 1974): I (%) = [(Infection in treatment (%) - Infection control (%) / (100 - Infection control)] x 100, where, Infection treatment = (Number of eggs infected x 100) / Initial population.

Mortality (%): defined as the rate of deaths in juveniles (J2) of *M. incognita* and *M. mayaguensis*, during the time of exposure to a particular treatment, and expressed in percentage as the number of J2 dead for each 100 individuals of the initial population. The corrected mortality (M), was calculated by the formula of Schneider-Orelli (COSTA *et al.*, 1974) described below: M (%) = [Treatment Mortality (%) - Control Mortality (%) / 100 - Mortality in the control)] x 100; wherein Treatment Mortality = (Number of dead J2 x 100) / Initial population.

Lethal Concentration ninety (LC_{90}) in spores / mL: defined as the concentration of a biological, physical or chemical agent which killed 90% of the organisms in a population (REPETTO, 1997). This variable was determined by relating the mortality data of juveniles (J2) of *M. incognita* and *M. mayaguensis* and their respective

concentrations, through linear regressions in Microsoft Excel 2010 program. The criterion for a dead juvenile stage (J2) of *Meloidogyne* spp. was immobility, when stimulated with a bristle, after been placed in Petri dishes containing sterile distilled water for 24 h (PINKERTON & KITNER, 2006).

RESULTS AND DISCUSSION

Effect of *P. lilacinum* and *P. chlamydosporia* on eggs and juveniles (J2) of *M. incognita* and *M. mayaguensis*. Variance analysis for the variables infection of eggs and mortality of juvenile stages (J2) of *M. incognita* and *M. mayaguensis*, showed highly significant statistical differences between treatments at 168 h after application. The high coefficients of determination R2 = 0.93 and 0.95, respectively, as the low coefficients of variation CV = 11.78% and 12.75%, respectively, demonstrated the reliability of the results obtained (Table 1).

Table 1. Analysis of variance for infection of eggs (%) and mortality (%) of juveniles (J2) of *M. incognita* and *M. mayaguensis*, at 168 h after the application of treatments.

Source of Variation	Degrees of Freedom (D.F.)	Evaluated variables	
		Infection of Eggs (%)	Mortality of juvenile stages (J2) of *M. incognita* and *M. mayaguensis* (%)
Treatment	27	60946,428**	46471,428**
Error	84	2650,000	3400,000
Corrected Total	111	63596,428	49871,428
R^2		0,958	0,931
C. V. (5%)		11,780	12,457

** Denotes highly significant differences.

By performing a Tukey's test ($p < 0.05$) on the variables infection of eggs and mortality of juveniles (J2) of *M. incognita* and *M. mayaguensis*, it was determined that the mix *P. lilacinum* and *P. chlamydosporia* (1×10^6 spores / L) in combination with Carbofuran (125 ppm) was the best treatment, as it caused the highest infection of eggs with 85%

and increased mortality of J2 of both species with 93% compared to the water treated group where no mortality occurred (Figures 1 and 2). The result of the mortality of juveniles (J2) of both species of *Meloidogyne*, obtained with such combination, had no statistical difference (p = 0.05) with that obtained with the Carbofuran at the highest concentration (500 ppm), but with the other treatments (Figures 1 and 2). The result of infection on eggs of *M. incognita* and *M. mayaguensis* obtained in this experiment with the *P. lilacinum* and *P. chlamydosporia* (1 x 10^6 spores / L) mixture in combination with Carbofuran (125 ppm) coincides with that reported by DHAWAN & SINGH (2009) who found that the mixture *P. lilacinum*, *P. chlamydosporia* and cake neem (*Azadirachta indica* A. Juss) in combination with Carbofuran in okra (*Abelmoschus esculentus* L.) infested with *M. incognita* caused 90% infection of eggs.

After the above treatment, the mixture of *P. lilacinum* and *P. chlamydosporia* (1 x 10^8 spores / mL) was the best treatment, being significantly different from other treatments (p = 0.05) since 80% infection was obtained on eggs and 75% mortality on J2 of *M. incognita* and *M. mayaguensis* in comparison to the water treated control (Figures 1 and 2). The result of infection of these fungi on the eggs of *Meloidogyne* species was greater than that reported by RAO (2005), who found that *M. javanica* eggs extracted from roots of limes (*Citrus aurantifolia* Christm. et Panz.), and exposed to *P. lilacinum* and *P. chlamydosporia* (2 x 10^6 CFU / mL) for 96 hours, showed an infection of 54% compared to that obtained in water treated groups. The results of infection of eggs and mortality of J2 of *M. incognita* and *M. mayaguensis* obtained in this study confirm what was stated by CANNAYANE & RAJENDRAN (2001), that the combination of *P. lilacinum* with other biocontrol agents such as *P. chlamydosporia*, a greater potential for the biological management of *Meloidogyne* spp.

Comparing the fungal infection caused by the fungi individually, it was found that *P. chlamydosporia* (1 x 10^9 spores / mL) was significantly greater (p = 0.05), as it caused a 68% infection of eggs on *M. incognita* and *M. mayaguensis* while *P. lilacinum* infected 63% at same spore concentration (Figures 1 and 2). The result of infection *P. chlamydosporia* on both eggs of *Meloidogyne* species obtained in this study was similar to that reported by ATKINS *et al.* (2003), who proved that the same fungus at the concentration of 1 x 10^6 spores / mL, under semi-controlled conditions, reached 68% infection on *M. incognita* eggs, after six months of application in a succession of crops of tomato (*Solanum lycopersicum* L.) and lettuce (*Lactuca sativa* L.).

However, in this study with *P. chlamydosporia* (1 x 10^9 spores / mL) 65% mortality of juveniles (J2) of *M. incognita* and *M. mayaguensis* was obtained, with no statistical difference with that obtained with *P. lilacinum* (1 x 10^9 spores / mL) (Figures 1 and 2). This result was similar to that obtained by VERGARA *et al.* (2012) with *P. chlamydosporia* (1.03 x 10^8 spores / mL) in conditions *in vitro* achieving 66% mortality of juveniles (J2) of *R. similis* after 120 h.

In this study, it was also found that infection of *P. chlamydosporia* (1 x 10^9 spores / mL) on eggs of *M. incognita* and *M. mayaguensis* had no statistical difference (p = 0.05) with that obtained with *P. lilacinum* (1 x 10^9 spores / mL) and with treatments where *P. chlamydosporia* and *P. lilacinum* (1 x 10^4 or 1 x 10^5 spores / mL) were mixed in combination with Carbofuran (125 ppm). The mortality of J2 of the two species of *Meloidogyne* with *P. chlamydosporia* (1 x 10^9 spores / mL) showed no statistical difference (p = 0.05) with those achieved with treatments where *P. lilacinum* (1 x 10^6 or 1 x 10^7 spores / mL) were combined with Carbofuran (125 ppm) (Figures 1 and 2).

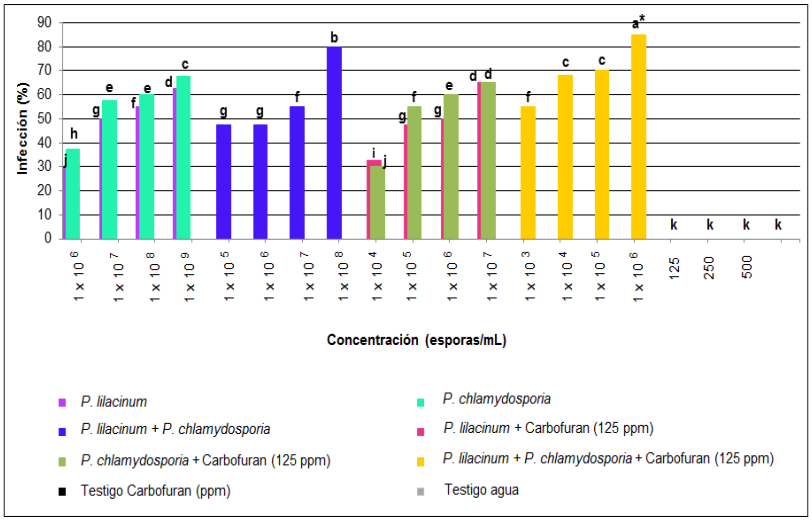
After the above treatments, median comparisons with a Tukey's test (p = 0.05), showed that *P. lilacinum* alone (1 x 10^9 spores / mL) caused 63% infection of eggs and 65% mortality of J2 of *M. incognita* and *M. mayaguensis*, at 168 h of exposure, showing above 30% in both variables compared with *P. lilacinum* alone and *P. chlamydosporia* alone, at the lowest concentration (1 x 10^6 spores / mL) (Figures 1 and 2). The result of infection of eggs on the two species of *Meloidogyne* obtained in this study was similar to that reported by AYATOLLAHY & FATEMY (2010), who indicate that *P. lilacinum* (isolate 8.1) at a concentration of 2.3 x 10^8 spores / mL, caused a 60% infection of *Heterodera schachtii* eggs at 48 h after application. Furthermore, the result of mortality of J2 of *M. incognita* and *M. mayaguensis*, seen with *P. lilacinum* (1 x 10^9 spores / mL), was similar to that reported by VERGARA *et al.* (2012), who obtained 62% mortality of J2 of *R. similis* with *P. lilacinum* (1.03 x 10^8 spores / mL) after 120 h of being applied in conditions *in vitro*.

In this research, 63% of infection caused by *P. lilacinum* (1 x 10^9 spores / mL) on eggs of *M. incognita* and *M. mayaguensis* had no statistical difference with that achieved by *P. lilacinum* or *P. chlamydosporia* (10^7 spores / mL) in combination with Carbofuran at low concentration (125 ppm). Similarly, 65% mortality of J2 of the two species of *Meloidogyne* achieved with *P. lilacinum* (1 x 10^9 spores / mL) was not statistically different from that obtained with *P. chlamydosporia* (1 x 10^9 spores / mL) and *P. lilacinum* (1 x 10^6 or 1 x 10^7 spores / mL) in combination with Carbofuran (125 ppm) (Figures 1 and 2).

Furthermore, the positive control Carbofuran at its maximum concentration (500 ppm) resulted in a 100% mortality of J2 of *M. incognita* and *M. mayaguensis* 168 h after application (Figures 1 and 2). However, Carbofuran had no effect on the eggs of both species of *Meloidogyne* in three concentrations (125, 250 and 500 ppm) and presented no statistical differences from the water treated control (Figures 1 and 2). A similar result was reported by VERGARA *et al.* (2012), with Carbofuran (330 g / L) in eggs of *R. similis*, were no effect of the chemical substance was found after 120 h of exposure. This result is due to the protective function of the egg cover, such as the vitellin, chitin and especially an internal glycolipid layer, which provides resistance to chemicals and prevents the entry of foreign substances (STIRLING & WEST, 1991; PERRY *et al.*, 2009).

VERGARA *et al*. (2012) mention that the application of Carbofuran is not efficient to handle eggs of *R. similis*; contrary to what happens with the fungi *P. lilacinum* and *P. chlamydosporia*, which have high potential for biocontrol. However, the results of this study showed that Carbofuran (125 ppm) applied in combination with *P. lilacinum* and *P. chlamydosporia* (1 x 10^6 spores / mL) caused eggs of *M. incognita* and *M. mayaguensis* to be infected exceeding 5% of that produced when the two fungi were applied as a mixture, in concentration of 1 x 10^8 spores / mL (Figures 1 and 2). This happened due to the compatibility of the two fungi with the chemical input, which causes a synergistic effect, which allows for greater control of *Meloidogyne* spp. (MAHENDRA *et al*., 2009).

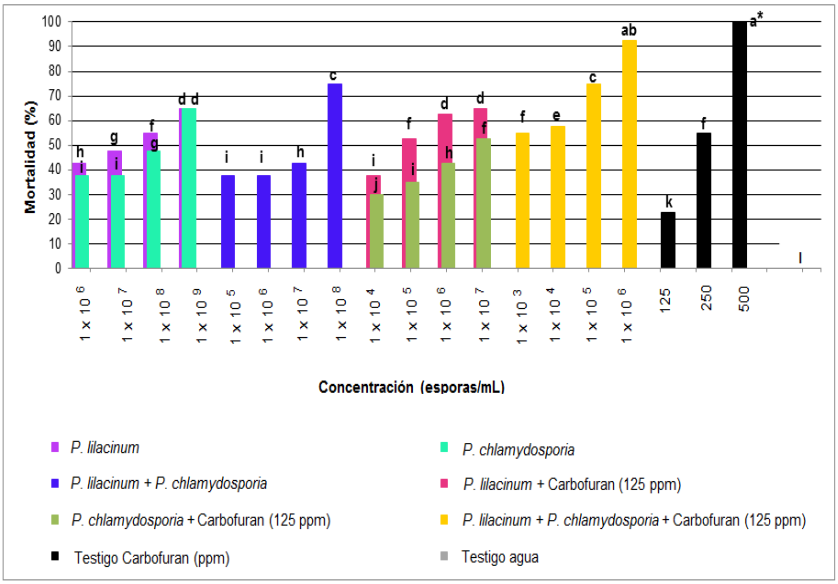
The results obtained in this investigation, allowed us to prove that the infection of the fungus on eggs and mortality of J2 of *M. incognita* and *M. mayaguensis* were higher when the concentration (spores / mL) of *P. lilacinum* and *P. chlamydosporia* also increased (Figures 1 and 2). In consequence, it was found that when fungi were applied (individually, in mixture or in combination with Carbofuran) with a greater concentration, the values of infection of eggs of the two *Meloidogyne* species are higher, which was equal or above 63%. Also the highest mortality values of both J2 of the *Meloidogyne* species, were obtained which were higher than 65%. However, when the same fungi, were applied in lower concentrations smaller infection values occurred, which were at or below 55%; similarly, mortality values of J2 were recorded at or below 58% (Figures 1 and 2).



* Values in the same column followed by the same letter are statistically equal, according to the Tukey test at 5% probability.

Figure 1. Infection (%) of eggs by *P. lilacinum* and *P. chlamydosporia* on *Meloidogyne incognita* and *Meloidogyne mayaguensis*, 168 h after application.

Similar to what happened with the effect of greater concentration of *P. lilacinum* and *P. chlamydosporia* on the variables infection of eggs and mortality of juveniles (J2) of *Meloidogyne* spp., it was also determined that these variables were directly related to the time of exposure to a particular treatment. Consequently, the infection of eggs and mortality of J2 of the *Meloidogyne* species caused by the fungal isolates was low after 24 hours (≤ 10%) but at 168 h it reached a range between 30 and 93% (Figures 1 and 2). This behavior was also reported by VERGARA *et al.* (2012), who found that in laboratory conditions *P. lilacinum* (1.03 x 10^8 spores / mL) caused an infection of11% on eggs of *R. similis* after 12 h of application, whereas after 120 h, this increased to 79%.



* Values in the same column followed by the same letter are statistically equal, according to the Tukey's test at 5% probability.

Figure 2. Mortality (%) of *P. lilacinum* and *P. chlamydosporia* on juveniles (J2) of *Meloidogyne* spp. 168 h after application.

Infection with *P. lilacinum* and *P. chlamydosporia* on the egg vitellin layer and the outer layer of the cuticle of juveniles (J2) of *M. incognita* and *M. mayaguensis* was observed under a microscope with a 10X objective at 24, 72, 120 and 168 h after exposure (Figures 3 and 4).

At 24 h, both the vitelline layer of eggs and the cuticle of juveniles (J2) of *M. incognita* and *M. mayaguensis*, were surrounded by conidia of fungi. Subsequently, after 72 h of exposure, the same vitellin layers expressed fungal hyphae. After 120 h, the layers

were partially covered by masses of hyphae and at 168 h these hyphae covered the whole eggs and juveniles (J2) of the species listed (Figures 3 and 4). Furthermore, at 168 h, a rupture of the egg cover, formed by the outer vitelline layer, median chitinase layer and the glycolipid inner layer, important for the development of the embryo, generally occurred (PERRY *et al*., 2009).

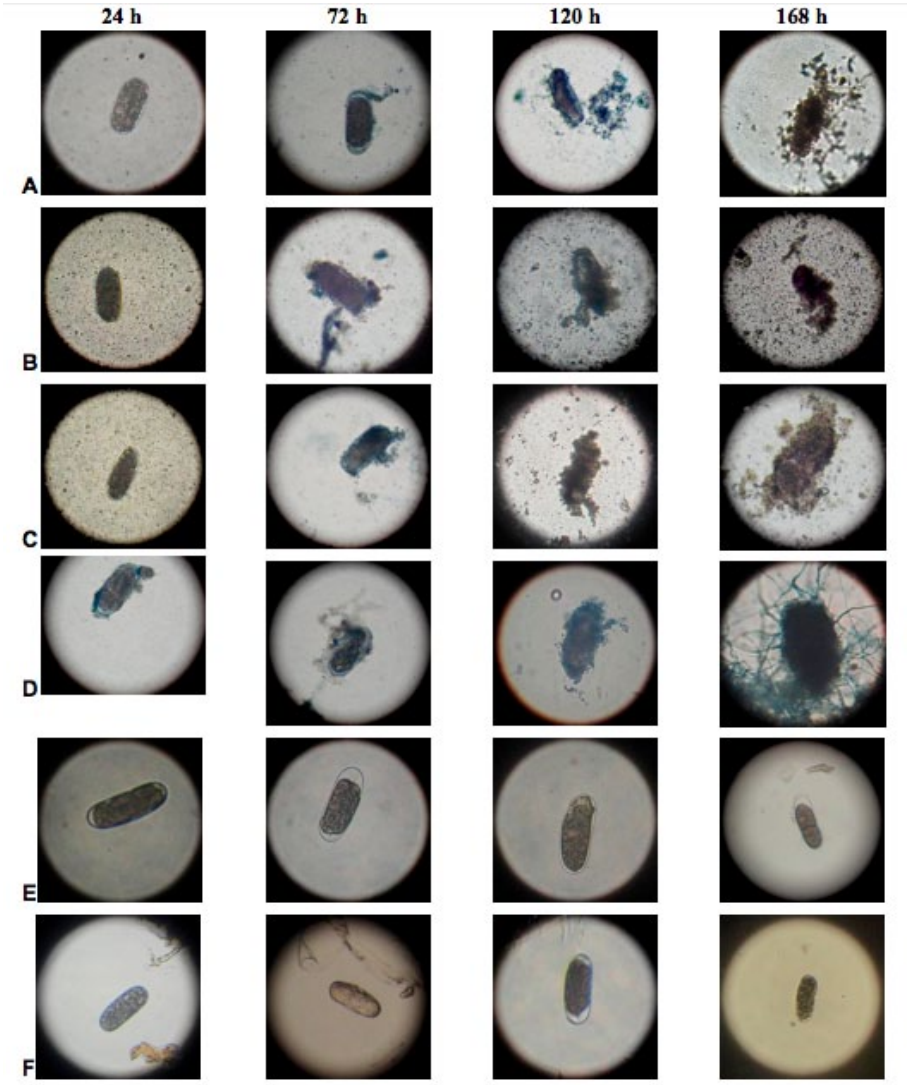


Figure 3. Eggs of *Meloidogyne incognita* and *Meloidogyne mayaguensis* infected with *Purpureocillium lilacinum* (1 x 10^9 spores / mL) (A), *Pochonia chamydosporia* var. *canetulata* (1 x 10^9 spores / mL) (B), *Purpureocillium lilacinum* and *Pochonia chamydosporia* var. *canetulata* (1 x 10^8 spores / mL) (C), *Purpureocillium lilacinum* and *Pochonia chamydosporia* var. *canetulata* (1 x 10^6 spores / mL) with Carbofuran (125 ppm) (D), Carbofuran, positive control (500 ppm) (E) and water, negative control (F), at 24, 72, 120 and 168 h of exposure. Magnification 10X.

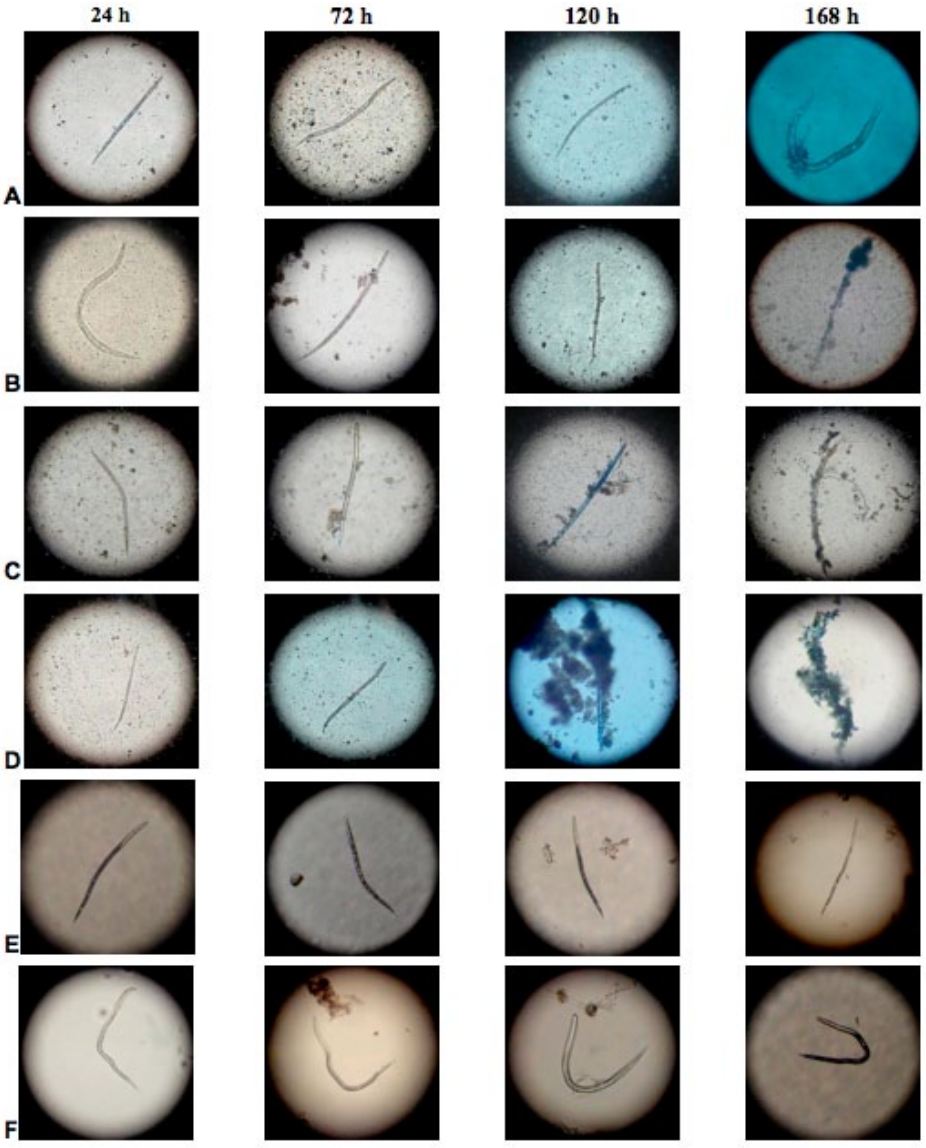


Figure 4. Juveniles (J2) of *Meloidogyne incognita* and *Meloidogyne mayaguensis* dead by *Purpureocillium lilacinum* (1 x 10^9 spores / mL) (A), *Pochonia chamydosporia* var. *canetulata* (1 x 10*9* spores / mL) (B), *Purpureocillium lilacinum* and *Pochonia chamydosporia* var. *canetulata* (1 x 10^8 spores / mL) (C), *Purpureocillium lilacinum* and *Pochonia chamydosporia* var. *canetulata* (1 x 10^6 spores / mL) with Carbofuran (125 ppm) (D), Carbofuran, positive control (500 ppm) (E) and water, negative control (F), at 24, 72, 120 and 168 h of exposure. Magnification 10X.

The results obtained in this research demonstrated that under *in vitro* conditions, the highest values of infection on mortality of eggs and juveniles (J2) of *M. incognita* and *M. mayaguensis*, which were between 63 and 93%, respectively, were achieved with the treatments listed below in descending order:

- Mixing *P. lilacinum* and *P. chlamydosporia* (1 x 10^6 spores / mL) in combination with Carbofuran at lower concentration (125 ppm) resulted in 85% egg infection and 93% mortality of juveniles (J2) of *M. incognita* and *M. mayaguensis*.
- Mixing *P. lilacinum* and *P. chlamydosporia* (1 x 10^8 spores / mL) resulted in 80% egg infection and 75% mortality of juveniles (J2) of the two *Meloidogyne* species.
- *P. chlamydosporia* (1 x 10^9 spores / mL) resulted in 68% infection of eggs and 65% mortality of juveniles (J2) of both species of *Meloidogyne*.
- *P. lilacinum* (1 x 10^9 spores / mL) resulted in 63% infection of eggs and 65% mortality of juveniles (J2) of *M. incognita* and *M. mayaguensis*.

Lethal concentration ninety (LC_{90}). The lethal concentrations (LC_{90}), caused by *P. lilacinum* and *P. chlamydosporia*, alone, in mixture or in combination with Carbofuran, in the mortality of juveniles (J2) of *M. incognita* and *M. mayaguensis* were calculated by relating the values mortality of juveniles (J2) of *Meloidogyne* species, obtained in *in vitro* conditions at 168 h, in each of the concentrations described above. These values were adjusted to linear equations of the form Y = a + BX, where "Y" corresponded to the mortality of J2 (%); "X" to the concentration of each treatment; and "a" and "b", the coefficients calculated in the linear regression analysis (Figure 5).

The high determination coefficients R2 = 0.90 to 0.96, obtained demonstrated the probabilistic reliability thereof; for this reason, we proceeded to clear the LC_{90} for each treatment. The concentrations of fungi alone, in mixture or in combination with the low concentration of Carbofuran (125 ppm), which caused 90% (LC_{90}) of mortality of juveniles (J2) of *M. incognita* and *M. mayaguensis* is presented below (Table 2).

Table 2. Lethal concentration ninety (LC_{90}) for *P. lilacinum*, *P. chlamydosporia* and Carbofuran for juvenile stages (J2) of *M. incognita* and *M. mayaguensis*.

Treatments	LC_{90} (esporas / mL)
P. lilacinum	3,163 x 10^{12} (Figure 5A)
P. chlamydosporia	1,682 x 10^{13} (Figure 5B)
Combination of *P. lilacinum* and *P. chlamydosporia*	1,313 x 10^{10} (Figure 5C)
*P. lilacinum*in combination with Carbofuran (125 ppm)	2,249 x 10^9 (Figure 5D)
P. chlamydosporia in combination with Carbofuran (125 ppm)	1,468 x 10^{12} (Figure 5E)
Both *P. lilacinum* and *P. chlamydosporia* in combination with Carbofuran (125 ppm)	1,093 x 10^6 (Figure 5F)
Carbofuran	443,570* (Figure 5G)

* Concentration of Carbofuran = parts per million (ppm).

It was found that the equations obtained showed a positive correlation between the concentration and the percent mortality of juveniles (J2) of *M. incognita* and *M. mayaguensis*, and defined the lines that have a positive slope and are of increasing order, confirming that with increasing concentration of spores and ppm of Carbofuran, also increases mortality (%) of J2 nematodes.

The results showed that an increase of 1 x 10^1 spores / mL in *P. lilacinum* concentration causes an increase of at least 7.50% mortality in juveniles (J2) of *Meloidogyne* species; *P. chlamydosporia* an increase of 7.75%; *P. lilacinum*in mix with *P. chlamydosporia* 12.04%; *P. chlamydosporia* combined with 125 ppm Carbofuran 7.50%; *P. lilacinum* combined with 125 ppm of Carbofuran 9.25%; *P. chlamydosporia* combined with 125 ppm of Carbofuran 7.50%; and *P. chlamydosporia* mixed with *P. lilacinum* and combined with 125 ppm of Carbofuran 13%. In addition, an increase of 100 ppm Carbofuran, produces a 20% increase in mortality of J2 nematodes.

CONCLUSIONS

• In *In vitro* conditions, the fungi *P. lilacinum* alone and *P. chlamydosporia* alone at a concentration of 1 x 10^9 spores / mL, as did the mixture of these fungi in the concentration 1 x 10^8 spores / mL, infected between 63 and 80% of eggs and juveniles (J2) of *M. incognita* and *M. mayaguensis*, at 168 h of exposure.

• The mixture of *P. lilacinum* and *P. chlamydosporia* in concentration of 1 x 10^6 spores / mL in combination with Carbofuran at a concentration of 125 ppm, caused 93% mortality of juveniles (J2) of *M. incognita* and *M. mayaguensis* under *in vitro* conditions at 168 h exposure.

• The evaluation of the *Purpureocillium lilacinum* strain PL-11 and *Pochonia chlamydosporia* var. *catenulata* strain JL-1, in *in vitro* conditions, confirmed their high potential in the control of *M. incognita* and *M. mayaguensis*, for promoting their use in conditions of nursery and field within an integrated management program for *Meloidogyne* spp. as an environmentally safe, affordable, accessible and user-friendly alternative.

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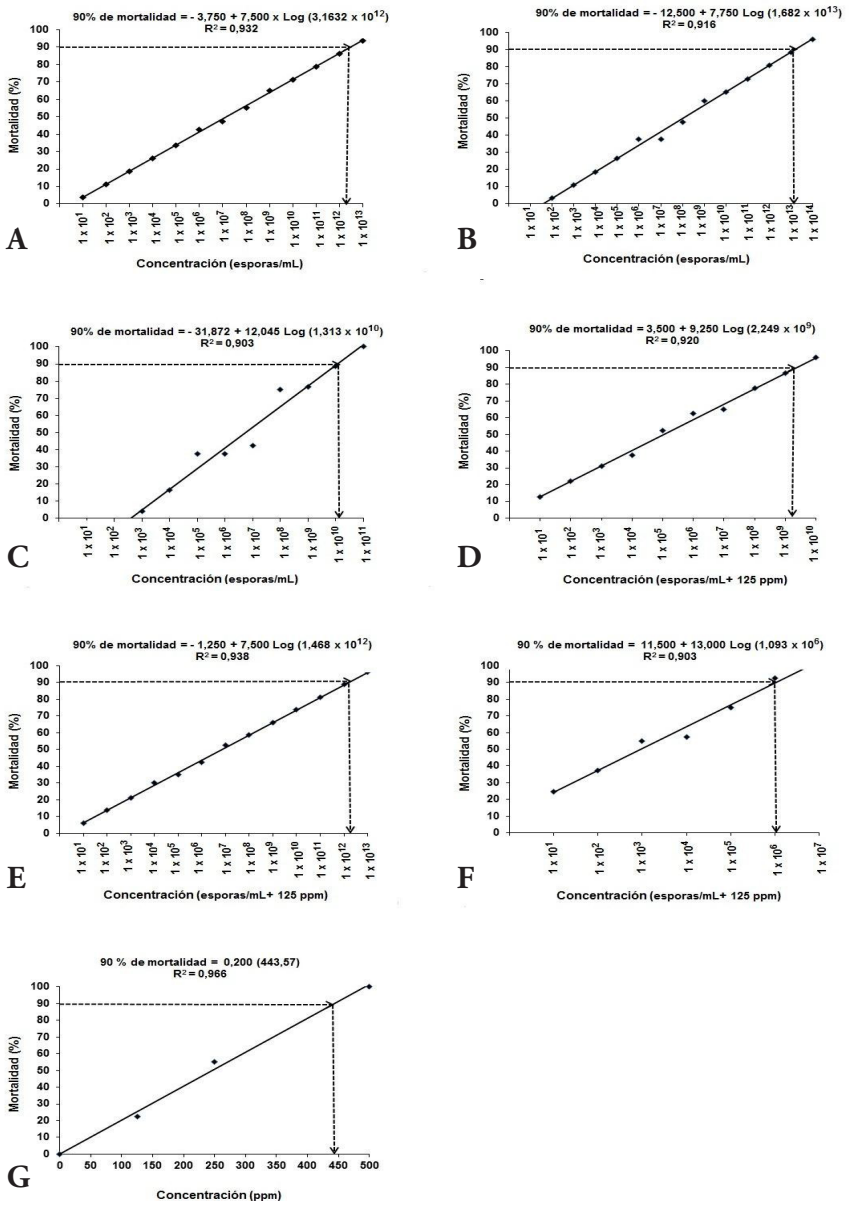

Figure 5. Equations showing the concentration that kills 90% of juveniles (J2) of *M. incognita* and *M. mayaguensis* and corresponding treatment. Treatments: **A**. *P. lilacinum*. **B**. *P. chlamydosporia*. **C**. *P. lilacinum* + *P. chlamydosporia*. **D**. *P. lilacinum* + 125 ppm Carbofuran. **E**. *P. chlamydosporia* + 125 ppm Carbofuran. **F**. *Plilacinum* + *P. chlamydosporia* + 125 ppm Carbofuran. **G**. Carbofuran.

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