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Original article

Effect of systemic fungicide on the presymbiotic growth of *Rhizophagus irregularis* (INCAM 11), *in vitro*

Efecto de un fungicida sistémico en el crecimiento presimbiótico de *Rhizophagus irregularis* var. INCAM 11, *in vitro*

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ABSTRACT: Fungicides are widely used in current culture systems to control or eliminate fungal plant pathogens. However, these chemicals can affect indigenous soil microorganisms, including those that promote plant growth, such as arbuscular mycorrhizal fungi (AMF). Considering the above exposed, the present study set out to determine the effect of different concentrations of the systemic fungicide Previcur energy 84 sl on the presymbiotic stage of *Rhizophagus irregularis* (INCAM 11), under *in vitro* culture conditions. Modified Strullu and Romand culture medium (SRM) was employed to evaluate percentage of germination, growth of germinating tube, as well as its percentages of increase and decrease, when studying four concentrations of the Previcur Fungicide (0,1; 1; 10 and 100 mg L^{-1}). At 10 mg $^{-1}$ a stimulating effect on germination and on the growth of the germ tube of the fungus. The present work constitutes the first evidence on the effect of a systemic fungicide in the presimbiotic stage of mycorrhizal fungus on in *vitro* conditions reported in Cuba.

Key words: mycorrhizas, germination of spores, culture medium, pesticides.

RESUMEN: Los fungicidas se utilizan ampliamente en los sistemas de cultivo actuales para controlar o eliminar los fitopatógenos fúngicos. Sin embargo, estos productos químicos pueden afectar a los microorganismos autóctonos del suelo, incluidos los que promueven el crecimiento vegetal, como los hongos micorrízicos arbusculares (HMA). Teniendo en cuenta lo anterior, el presente estudio se propuso determinar el efecto de diferentes concentraciones del fungicida sistémico Previcur energy 84 sl en el estadio presimbiótico de *Rhizophagus irregularis* (INCAM 11), en condiciones de cultivo *in vitro*. Para ello, se evaluó en el medio de cultivo SRM (Strullu y Romand Modificado), el porcentaje de germinación, el crecimiento del tubo germinativo, así como sus porcentajes de incremento y decremento, al estudiar cuatro concentraciones del fungicida Previcur (0,1; 1; 10 y 100 mg L^{-1}). Se observó que, a la concentración de 10 mg L^{-1} este fungicida tiene un efecto estimulador en la germinación y en el crecimiento del tubo germinativo del hongo. El presente trabajo constituye la primera evidencia en Cuba sobre el efecto de un fungicida sistémico en el estadío presimbiótico de un hongo micorrízico arbuscular, *in vitro*.

Palabras clave: germinación de esporas, medio de cultivo, micorrizas y pesticidas.

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INTRODUCTION

Arbuscular mycorrhizal fungi (*Phyllum Glomeromycota*) are an integral part of numerous ecosystems and are considered particularly advantageous because they are associated in symbiosis with most of the vascular plants studied (1). These fungi are obligate biotrophs, so they cannot complete their life cycle in the absence of a host plant (2); hence, the differentiation of a functional mycorrhiza is a complex process that demands the participation of both organisms (3).

On the other hand, in modern agriculture, crop protection against the attack of pathogenic organisms is mainly carried out with agrochemicals, which can be applied directly at certain stages of the crop, through seed treatment or applied to the soil. These include fungicides, which are widely used to control or eliminate fungal plant pathogens. However, these products also affect other fungi indigenous to the soil, including those that promote plant growth, such as AMF (4). The intensive use of chemicals, especially fungicides and fertilizers, is one of the main causes of the reduction of diversity in soils, including AMF (5,6). However, recent studies report that the application of fungicides can also have a positive effect on mycorrhizal establishment; this depends on their mode of action and the AMF species involved.

Considering the scarce knowledge on the effect of fungicides on AMF under *in vitro* conditions and that the use of these fungi as biofertilizers is becoming more and more frequent in different agricultural production systems, the present research has the objective of evaluating the effect of different concentrations (0.1, 1, 10 and 100 mg L^{-1}) of the systemic fungicide Previcur energy 84 sl on the pre-symbiotic stage of *Rhizophagus irregularis* (INCAM 11), under *in vitro* conditions.

MATERIALS AND METHODS

The research was conducted between 2017 and 2018, at the National Institute of Agricultural Sciences (INCA), belonging to the Ministry of Higher Education and located in the municipality San José de las Lajas, Mayabeque province.

Biological material

The inoculum used was the AMF *Rhizophagus irregularis* (Blaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler (INCAM 11), from the INCA stock. The inoculum had an average concentration of 25 spores g^{-1} of fresh soil.

SRM (Strullu and Romand Modified) culture medium (7). The medium was composed of (g L^{-1}): macroelements-$MgSO_4.7H_2O$-73.9, KNO_3-7.6, KCl-6.5, KH_2PO_4-0.41; Ca $(NO^3).2.4H_2O$-35.9; NaFeEDTA-0.16; microelements-$MnSO_4.4H_2O$-1.225, $CuSO_4.5H_2O$-1.1, $ZnSO_4.7H_2O$-0. 14, H_3BO_3-0.925, $Na_2MoO_4.2H_2O$-0.12, (NH_4) $6Mo_7O_2 4.4H_2O$-1.7; Vitamins (g L^{-1})-Calcium pantothenate-0.09, Biotin-0.0001, Nicotinic acid-0.1, Pyridoxine-0.09, Thiamin-0.1 and Cyanocobalamin-0.04; Sucrose-10. The pH was adjusted to 7.5; before adding 4 g L^{-1} of Gellam Gum. The medium was sterilized at 121 ºC for 15 min.

Effect of different concentrations of the systemic fungicide Previcur energy 84sl on the pre-symbiotic stage of *Rhizophagus irregularis* (INCAM 11), under *in vitro* conditions

In order to evaluate the effect of a systemic fungicide on the *in vitro* presymbiotic growth of INCAM 11, this experiment was carried out using the commercial fungicide Previcur energy 84 sl, whose active component is: propyl 3-(dimethylamino) propylcarbamate 53 %, ethylhydrogen phosphonate 31 % and inert ingredients 16 % (8).

Spores grouped in clusters were used as propagules. These were extracted from the soil, using the technique of wet sieving and decantation and subsequent extraction through centrifugation of a sucrose+Tween 80 solution at 2000 rpm for five minutes, according to Gerdemann and Nicolson (9); modified by Herrera et al. (10). Subsequently, they were disinfected using the methodology of some authors (11), modified by others (12).

From the extracted propagules, spores grouped in clusters were selected, placed on a membrane (0.44 µm pore diameter) and washed three times with sterile distilled water. Subsequently, Chloramine T 2 % (m/v) and two drops of Tween 20 were added for 10 minutes. Next, the propagules were washed three times with sterile distilled water and treated with an antibiotic solution (streptomycin sulfate (0.02 %) (m/v) and gentamicin sulfate (0.01 %) (m/v), for 10 minutes, previously sterilized with the aid of a millipore filter (type HA, 4.0 cm diameter and 0.22 µm pore size). Finally, the membrane with the propagules was transferred to the same antibiotic solution, previously filtered in a sterile Petri dish (90 mm diameter) for 24 hours (12).

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After this time, five propagules per 90 mm diameter Petri dish containing SRM medium (Strullu and Romand Modified) were inoculated (7). The pH of the culture medium was adjusted to 7.5 (13). After sterilizing the culture medium at 121 °C for 15 min, the commercial systemic fungicide Previcur energy 84 sl was added at four concentrations (0.1; 1; 1; 10 and 100 mg L^{-1}). Ten plates

were used for each of the fungicide concentrations. A completely randomized design was used for the experiment.

The percentage germination of the spores of strain INCAM 11 was determined. The dynamics of germ tube growth was determined from the moment at which growth was observed, with origin in the sporophore or in the sustentation hyphae. Measurements were taken from the base of the new hypha to its apex, every seven days for one month and a micrometer coupled to a stereomicroscope (NOVEL N-800M, Nanjing Jiangnan Novel Optics Co., Ltd; China, 40X) was used. The percentage increase in the variable germ tube length was calculated with respect to the control not treated with the fungicide.

Statistical Analysis

Once normality was verified, the confidence interval of the means was calculated at 95 % probability, taking into account the number of repetitions and the reproducibility of the data. SPSS version 19 (14) was used for statistical processing of the data.

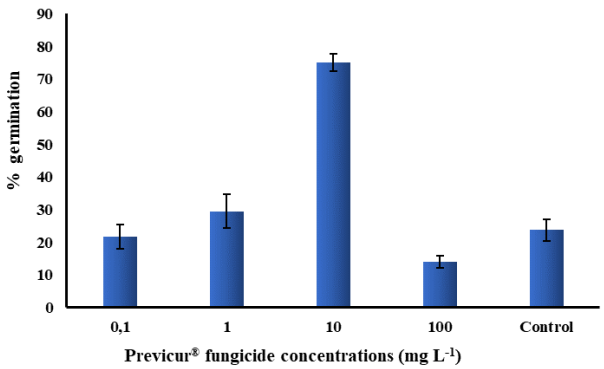
RESULTS AND DISCUSSION

In this experiment, four concentrations of the systemic fungicide Previcur were studied on the *in vitro* growth of *R. irregularis* in SRM medium at pH 7.5. This pH value was chosen taking into account that 7.5 was found to be the pH at which the presymbiotic stage of this strain developed best (Figure 1).

When analyzing the behavior of this variable, it can be seen that the highest germination percentage values were reached when applying the 10 mg L^{-1} concentration of the fungicide, which was significantly different from the rest of the treatments. On the other hand, when analyzing the effect of the 0.1 mg L^{-1} and 1 mg L^{-1} concentrations of the systemic fungicide on the germination of INCAM 11, no statistical differences were found between these and the control, but with the 100 mg L^{-1} concentration, which presented the lowest germination percentage values (Figure 1).

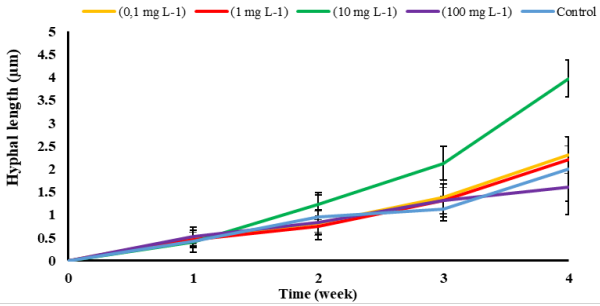
Figure 2 shows that there was an increase in the length of the germinative tube over time, appreciable in all the treatments studied. During the first three weeks of the experiment, no significant differences were observed among the treatments; however, in the fourth week the values of the variable began to disperse, with the length of the germinative tubes of spores treated with the 10 mg L^{-1} concentration of the Previcur fungicide being greater.

Figure 3 shows that in the concentration of 10 mg L^{-1} the percentage increase of this variable, with respect to the untreated control, was 50 %. In the case of the 0.1 and 1 mg L^{-1} concentrations, they only reached a 10 % increase, with no significant differences between them, except for the 100 mg L^{-1} concentration, which decreased by 10 % with respect to the control.



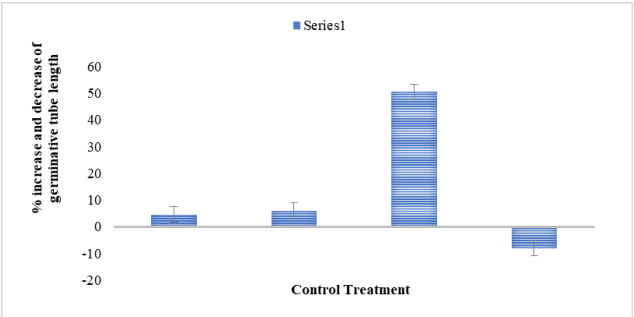
Bars represent the means of 10 plates containing 5 propagules each

Figure 1. Effect of applying different concentrations of the fungicide Previcur to the SRM culture medium on the germination of spores of *Rhizophagus irregularis* species (INCAM 11) for 28 days



Bars represent the confidence intervals of the mean of treatments for p≤ 0.05 (n=10)

Figure 2. Dynamics of germ tube growth of propagules of *Rhizophagus irregularis* species (INCAM 11) inoculated on SRM medium for 4 weeks, with different concentrations of the fungicide Previcur (0.1; 1; 10; 100 mg L^{-1})



Bars represent the confidence intervals of the mean of treatments for p ≤ 0.05 (n=10)

Figure 3. Percentage increase and decrease in germ tube length of *Rhizophagus irregularis* (INCAM 11) propagules inoculated on SRM medium with different concentrations of the fungicide Previcur (0.1; 1; 10; 100 mg L^{-1}), with respect to the control, calculated at the end of the experiment

It is evident that the application of Previcur fungicide to the SRM culture medium significantly stimulates the growth of germinating hyphae of *R. irregularis.*

These *in vitro* results are related to previous research (15), where the stimulatory effect of some systemic fungicides on the germination percentage, germ tube growth and root colonization of *Glomus intraradices* was reported at concentrations between 0.1 and 10 mg L^{-1}. Other studies have also shown that systemic fungicides can stimulate or inhibit mycorrhizal functioning, depending on the active ingredient concentration used (16).

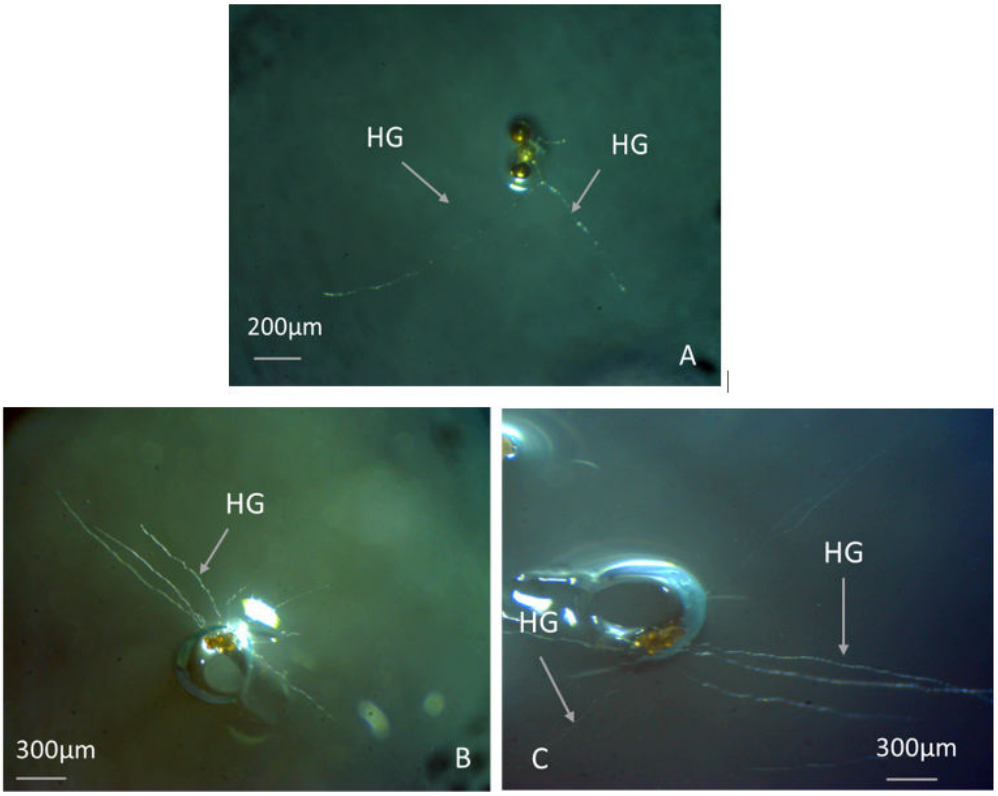
Interestingly, germination and hyphal growth of *Rhizophagus irregularis* spores were observed at all concentrations studied, indicating that there was no inhibitory effect on these variables, even when their values were lower than the concentration of 100 mg L^{-1}. This could be explained on the basis that the toxicity of Previcur, when applied, even at higher concentrations (100 mg L^{-1}), does not prevent the reactions that are triggered for mycelial development in the culture medium used.

Although *in vitro* research in this regard is scarce, reports have been found indicating that some systemic fungicides such as Metalaxyl can cause an increase in germination and mycelial length of AMF spores *in vitro* (17). This information coincides with what was found in this study, in which Previcur, with the same mode of action as Metalaxyl, stimulated germ tube growth of INCAM strain 11 at the 10 mg L^{-1} concentration (Figure 4).

In the literature consulted, some systemic fungicides have also been reported to have different effects, depending on the AMF species used. For example, the fungicide Fenhexamide, presents a fungistatic effect at concentrations higher than 20 mg L^{-1}, when applied to the culture medium by reducing the germination of *Rhizophagus irregularis* spores and affecting the growth, architecture and physiology of the extraradical mycelium (18,19). However, the same fungicide at a concentration similar to that of the present study (10 mg L^{-1}) does not affect germination, nor the anastomosis formation of *Funneliformis mosseae* hyphae in SRM culture medium, *in vitro* (20).

According to *in vitro* studies (20,21), some systemic fungicides, when used in SRM culture medium and in the presence of *Rhizophagus irregularis* and *Funneliformis mosseae* strain, tested even at higher concentrations than in this study (200 mg L^{-1}), showed a positive effect on AMF. Although there is very little literature available on the direct effects of fungicides on AMF under these experimental conditions of monoxenic culture, hence the novelty and importance of this research, it can be appreciated, according to these results, which the effect of some systemic fungicides depends fundamentally on the concentration of the fungicide and the species of AMF under study.

Similar results to those of this work were found by other researchers (22), who observed, under in vitro conditions,



A: without fungicide and B: with fungicide. C: INCAM 11 spores treated with Previcur at equal concentration, week 4. Photos taken under a stereomicroscope (NOVEL N-800M, Nanjing Jiangnan Novel Optics Co., Ltd.; China, 40X)

Figure 4. Germination hyphae (GH) of INCAM 11 spores treated or not with Previcur at a concentration of 10 mg L^{-1}

that the fungicide Myclobutanil in a concentration range of 0.2 mg L^{-1} to 20 mg L^{-1}, did not affect the biomass, nor the viability of *R. irregularis* hyphae. The results of this experiment are supported by data obtained in the field, in which AMF colonization was reduced only when Myclobutanil produced changes in host plant physiology.

On the other hand, in the absence of a host plant, chemicals containing Chlorotoluron appear to have no effect on AMF spore germination (4), whereas those containing Bifenox and Mecoprop, loxynil, and Clopyralid inhibit spore germination at low concentrations, but stimulate germination at concentrations considered high. These results correspond with the effect of Previcur on INCAM 11 strain stimulating its germination and germ tube growth at a concentration of 10 mg L^{-1}.

The results of this research show how Previcur fungicide has a stimulatory effect on INCAM 11 strain at a concentration of 10 mg L^{-1}. It should be noted that in the SRM culture medium, only the chemical was used in the culture medium and the AMF species *Rhizophagus irregularis* was inoculated, showing a direct effect of this fungicide on AMF.

CONCLUSIONS

The 10 mg L^{-1} concentration of the systemic fungicide Previcur energy 84 sl had a positive effect, stimulating germination and germ tube growth of *Rhizophagus irregularis* under *in vitro* culture conditions.

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