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In vitro antifungal/fungistatic activity of manganese phosphite against soybean soil-borne pathogens

Actividad fungicida/fungistática *in vitro* del fosfito de manganeso contra hongos patógenos habitantes del suelo con soja

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Abstract. Soybean root and stem rots caused by soil-borne pathogens are diseases commonly found in soybean fields, and one of the most important causes of crop losses. In the present study, the mycelial sensitivity of *Fusarium virguliforme*, *F. tucumaniae*, *Sclerotinia sclerotiorum* and *Macrophomina phaseolina* was evaluated on potato dextrose agar media (25 mL) supplemented with different concentrations of manganese phosphite (MnPhi) diluted in water (0, 25, 37.5, 50, 100, 200, 300, 400, 500, 800 and 1000 µg/mL). Mycelial growth sensitivity was analyzed using logarithmic linear regression analysis. The MnPhi concentration needed to inhibit 50% of the mycelial growth (IC_{50}) ranged from 105 µg/mL (*Fusarium* spp.) to 409 µg/mL (*M. phaseolina*). Sclerotia were completely inhibited at 500 µg/mL. The results of our study represent the first report on the direct *in vitro* fungicidal/fungistatic action of MnPhi against fungi that are causal agents of soil-borne diseases.

Keywords: *Glycine max; Fusarium virguliforme; Fusarium tucumaniae; Macrophomina phaseolina; Sclerotinia sclerotiorum;* Inhibitory concentration.

Resumen. Las pudriciones de raíz y tallo (PRYT) en el cultivo de soja causadas por patógenos habitantes del suelo son enfermedades comúnmente encontradas en campos de soja, y son una de las causas más importantes de pérdidas económicas. La sensibilidad micelial de Fusarium virguliforme, *Fusarium tucumaniae*, *Sclerotinia sclerotiorum* y *Macrophomina phaseolina* fue evaluada en medio de cultivo agar papa glucosa (25 mL) suplementado con diferentes concentraciones (µg/mL) de fosfito de manganeso (PhiMn) diluido en agua (0; 25; 37,5; 50; 100; 200; 300; 400; 500; 800 y 1000). La sensibilidad del crecimiento micelial fue analizada usando análisis de regresión lineal logarítmico. Se calculó la concentración de PhiMn necesaria para inhibir el 50% del crecimiento micelial (CI_{50}). Los valores de CI_{50} fueron desde 105 µg/mL (*Fusarium* spp.) hasta 409 µg/mL (*M. phaseolina*). La formación de esclerocios fue completamente inhibida a 500 µg/mL. Los resultados del presente estudio representan el primer reporte de la acción fungicida/fungistática *in vitro* del Phi contra los hongos que causan las PRYT en el cultivo de soja.

Palabras clave: *Glycine max; Fusarium virguliforme; Fusarium tucumaniae; Macrophomina phaseolina; Sclerotinia sclerotiorum;* Concentración inhibitoria.

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INTRODUCTION

Soybean is the main crop in Argentina, with a planted area of 19.78 million hectares in the 2013/2014 growing season. Root and stem rots caused by soil-borne pathogens are diseases commonly found in soybean fields and one of the most important causes of crop losses (Gupta et al., 2012; Leandro et al., 2012; Walters et al., 2013). In addition to killing plants, root and stem rots can slow down or stop plant growth, causing premature defoliation and decreasing the number and weight of seeds. *Rhizoctonia solani* Kühn, *Fusarium tucumaniae* Aoki, O´Donnell, Homma & Lattanzi, *F. virguliforme* O'Donnell & T. Aoki, *Macrophomina phaseolina* (Tassi) Goidanish, *Pythium* spp., *Phytophthora sojae* (Kaufman & Gerdman) and *Sclerotinia sclerotiorum* (Lib.) De Bary, are among the most important soil-borne pathogens causing root and stem rots, and yield-reducing diseases (Hartman et al., 1999; Scandiani et al., 2010; Ploper et al., 2011; Carmona et al., 2015; Grijalba & Gally, 2015). All these soybean soil-borne pathogens attack many species of cultivated plants, and develop different survival structures such as chlamydospores, microsclerotia, sclerotia and oospores. This is why the common management strategies, such as genetic resistance, seed treatment with fungicides and crop rotation, generally fail to provide adequate control of soil-borne diseases (Dorrance et al., 2009; Gupta et al., 2012; Leandro et al., 2012).

Phosphites (PO_3^{-3}; Phi) are phloem-mobile oxyanions of phosphoric acid (H_3PO_3) able to elicit systemic acquired resistance in some host species such as apple, potato, and *Arabidopsis* (Percival et al., 2009; Eshraghi et al., 2011 Machinandiarena et al., 2012), and to exhibit direct toxicity against certain pathogens such as *Venturia inaequalis*, *Phytophthora cinnamomi* and *Phytophthora infestans* (Smillie et al., 1989; Guest & Grant, 1991; Wilkinson et al., 2001; Thao & Yamakawa, 2009). Thus, Phi could be an alternative to manage soybean diseases caused by soil-borne pathogens.

The objective of the present study was to evaluate the *in vitro* mycelial sensitivity of root and stem rot pathogens in soybean to manganese phosphite (MnPhi), at different concentrations.

MATERIALS AND METHODS

The mycelial sensitivity of *F. virguliforme* (11-390-5), *F. tucumaniae* (11-389-3 and 248-11), *S. sclerotiorum* (LARP 56) [all from the Culture Collection of Centro de Referencia de Micología (CEREMIC), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Santa Fe, Argentina] and *M. phaseolina* (INTA Estación Experimental Agropecuaria Salta Culture Collection) were tested in 9-cm plastic Petri dishes. These dishes contained 25 mL of potato dextrose agar media (PDA, Merck) supplemented with different concentrations of MnPhi (ULTRA PLUS Mn -SPRAYTEC Fertilizantes Ltda. Brazil), 40% w/w P_2O_5 (520 mg P_2O_5/mL) diluted in water (0, 25, 37.5, 50, 100, 200, 300, 400, 500, 800 and 1000 µg/mL). Manganese phosphite was filtered through 0.22 µm MILLEX®GP filter units (Millipore) and then added to the autoclaved medium. Mycelial discs of each pathogen isolate, 6 mm in diameter, taken from 7-day-old PDA colonies, were placed in the center of each Petri dish supplemented with the different MnPhi concentrations. The plates were sealed with PVC plastic film and incubated in a growth chamber at 22 ± 2 ˚C, 12 h photoperiod, provided by three fluorescent lamps (Osram daylight 40 W), positioned 50 cm above the plates.

Colony diameters were measured with a precision ruler in two perpendicular directions, when the mycelial growth in the control (0 MnPhi µg/mL) reached the dish edge three days after the inoculation with *S. sclerotiorum*, and six days after the inoculation with *M. phaseolina*. For slow-growing pathogens, such as *Fusarium* species, the diameter of the fungal colony was measured 26 days post-inoculation, even though the mycelium did not reach the dish edge.

The influence of pH on growth inhibition due to MnPhi addition was evaluated for each treatment. The pH of the PDA either supplemented or not with each concentration of MnPhi was measured with a pH meter before the addition of mycelial discs of each pathogen isolate. An additional control treatment was carried out by seeding mycelial discs of *all pathogens* in PDA acidified up to pH 3 with tartaric acid 10% v/v.

A completely randomized experimental design was used for the *in vitro* tests with three repetitions within each assay. Experimental units were represented by a Petri dish. The assays were repeated three times. The mycelial growth sensitivity bioassays were analyzed using logarithmic linear regression analysis. The MnPhi concentrations needed to inhibit 50% of the mycelial growth (IC_{50}) were calculated from the generated equations. Data from the pH tests were analyzed using ANOVA, and differences between means were tested using Fisher's LSD test. Statistical analyses were carried out using INFOSTAT software (professional version 1.1).

RESULTS

The results from the *in vitro* bioassay showed that while the concentration of MnPhi increased, the mycelial growth of all pathogens decreased (Fig. 1). The coefficients of determination for the IC_{50} equations ranged from 0.79 to 0.98 (Table 1). The highest IC_{50} was determined for *M. phaseolina* followed by *S. sclerotiorum*. The *Fusarium* spp. isolates were the most sensitive to MnPhi, with an IC_{50} ranging from 105 to 114 µg/mL (Fig. 1).

In the Petri dish, formation of sclerotia of *S. sclerotiorum* was completely inhibited over 500 µg/mL and mycelial growth was null at 1000 µg/mL (Fig. 1). A similar result was

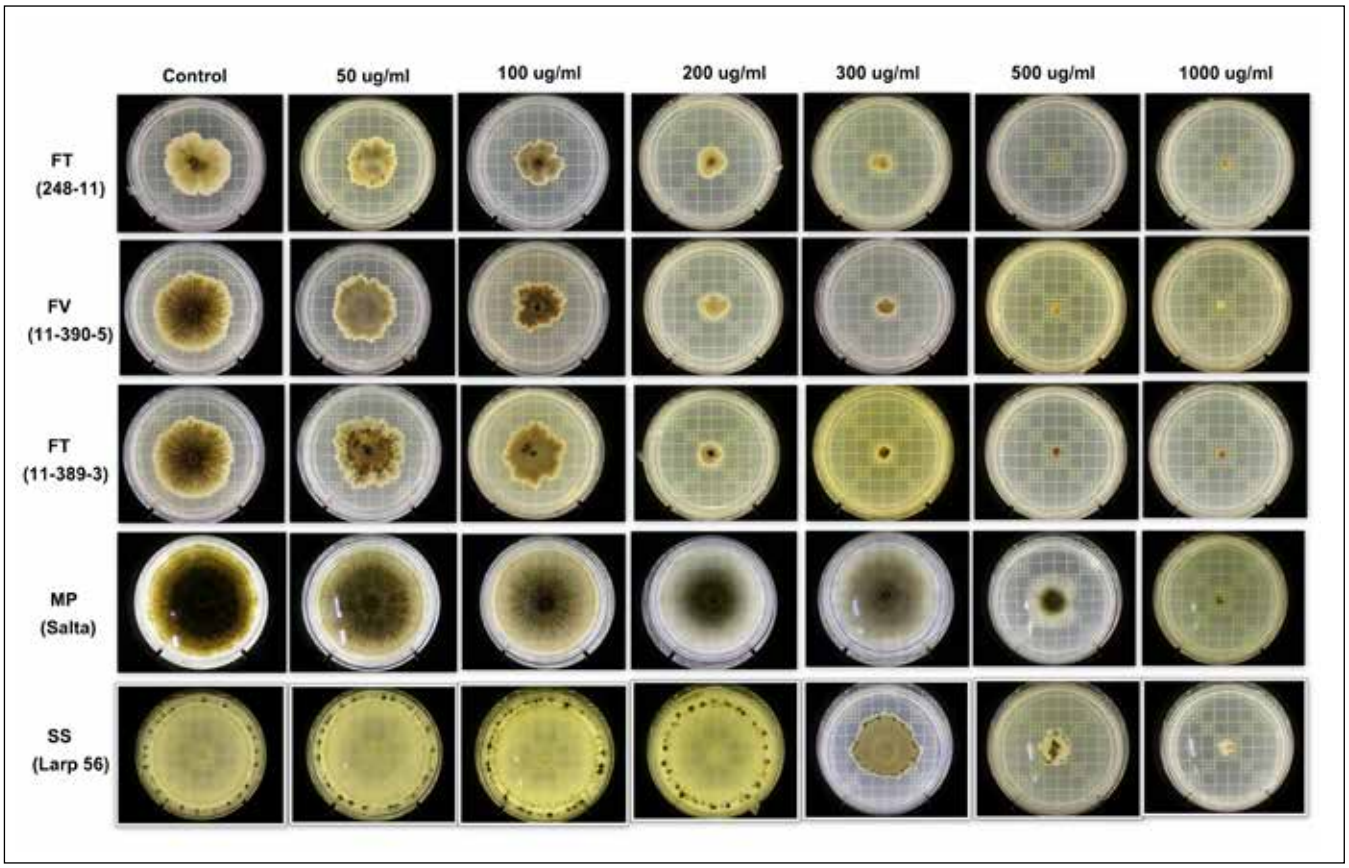

Fig. 1. Effect of manganese phosphite (MnPhi) on *in vitro* mycelial growth of *Sclerotinia sclerotiorum* (LARP 56), *Macrophomina phaseolina* (Salta province), *Fusarium virguliforme* (11-390-5), *Fusarium tucumaniae* (CCC 248-11) and *Fusarium tucumaniae* (11-389-3) using different concentrations.

Fig. 1. Efecto *in vitro* del fosfito de manganeso (PhiMn) sobre el crecimiento micelial de *Sclerotinia sclerotiorum* (LARP 56), *Macrophomina phaseolina* (Provincia de Salta), *Fusarium virguliforme* (11-390-5), *Fusarium tucumaniae* (CCC 248-11) y *Fusarium tucumaniae* (11-389-3), usando diferentes concentraciones.

Table 1. Regression equations, coefficients of determination (R^2) and 50% inhibitory concentrations of mycelium growth (IC_{50}) of *Macrophomina phaseolina*, *Sclerotinia sclerotiorum*, *Fusarium virguliforme* and *Fusarium tucumaniae*.

Tabla 1. Ecuaciones de regresión, coeficientes de determinación (R^2) y concentraciones inhibitoria del 50% del crecimiento micelial (CI_{50}) de *Macrophomina phaseolina*, *Sclerotinia sclerotiorum*, *Fusarium virguliforme* y *Fusarium tucumaniae*.

Pathogens	Linear regression equations	n	R^2	P values	IC_{50} (µg/mL)
Macrophomina phaseolina (Salta province)	y = -70.20 Ln(x) + 459.22	12	0.80	0.0001	409
Sclerotinia sclerotiorum (LARP 56)	y = -42.08 Ln(x) + 280.47	18	0.79	<0.0001	326
Fusarium virguliforme (11-390-5)	y = -19.89 Ln(x) + 122.19	15	0.98	<0.0001	114
Fusarium tucumaniae (248-11)	y = -15.26 Ln(x) + 95.51	15	0.96	<0.0001	111
Fusarium tucumaniae (11-389-3)	y = -21.57 Ln(x) + 130.58	15	0.94	<0.0001	105

observed for *M. phaseolina*, since a concentration of 800 µg/mL completely inhibited the mycelial growth and a concentration of 500 µg/mL inhibited microsclerotia formation (Fig. 1). For the three isolates of *Fusarium* spp., the mycelial growth was very slow, and even in the control it failed to cover the entire plate at 26 days post-seeding (Fig. 1). Manganese phosphite concentrations of 500 µg/mL or above completely inhibited the mycelial growth of the three isolates of *Fusarium* spp. (Fig. 1).

The pH ranged from 5.5 (0 µg/mL MnPhi) to 3 (1000 µg/mL MnPhi). The mycelia of *M. phaseolina* and *S. sclerotiorum* growing on media acidified with tartaric acid showed no dif-

ferences (P<0.0001) with the control. By contrast, *Fusarium* spp. were partially affected (up to 30%) (P<0.0001) by acidification, showing total inhibition of mycelial growth at pH 4.

All pathogens were completely inhibited by MnPhi (Fig. 1). The inhibitory effect of MnPhi was more evident on *Fusarium* spp., with the lowest IC_{50} (105-114 µg/mL) compared to the other pathogens (326-409 µg/mL) (Fig. 1, Table 1).

DISCUSSION

The results obtained are consistent with those reported by Lobato et al. (2010) and Araújo et al. (2010) in relation to *in vitro* inhibition of true fungi such as *Fusarium solani*, *Rhizoctonia solani*, *Streptomyces scabies* and *Colletotrichum gloeosporioides* by Phi. Lobato et al. (2010) found higher IC_{50} for *F. solani*, being 1280 µg/mL for CaPhi, >3560 µg/mL for KPhi and 680 µg/mL for CuPhi. In the case of oomycetes such as *P. infestans* and *P. plurivora*, IC_{50} were lower. Dalio et al. (2014) found an IC_{50} of 34 µg/mL for mycelial growth inhibition and of 2.9 µg/mL for zoospore inhibition of *P. plurivora* by Phi.

The media supplemented with 25 and 1000 µg/mL of MnPhi had a pH of 5 and 3, respectively. At pH values between 3 and 5, *M. phaseolina* and *S. sclerotiorum* were not significantly inhibited (Csöndes et al., 2011). Therefore, the inhibition of mycelial growth observed in the present study is mainly attributed to the effect of MnPhi and not to the acidification of the medium. In contrast, the effect of acidification by MnPhi could partially contribute to the mycelial growth inhibition of *Fusarium* species causing sudden death syndrome (Sanogo & Yang, 2001). According to Lobato et al. (2010), the activity of Phi cannot be attributed to a single reason, and probably would result from a combination of several factors such as the concentration of phosphite anion, nature of cation, acidification of the medium and type of pathogen. In this way and according to Lobato et al. (2010), when acidification is not important, the concentration of phosphite anion plays the main role in inhibiting the growth of pathogens. Most fungi tolerate a wide pH range, but the optimum range of growth is between 5.0 and 6.5. Thus, microorganisms that are in media at pHs above or below the optimum range will have impaired development (Araújo et al., 2010). However, the type of pathogen or fungal species would also be an important influential factor. In this regard, Lobato et al. (2010) determined that the pH could partially contribute to mycelial growth inhibition in other fungal species (up to 30%), including those of the *Fusarium solani* species complex.

There is still no conclusive explanation regarding the biochemical mode of action of Phi against pathogens (Dalio et al., 2014). It might be possible that *in vivo* interactions of Phi act in a direct way (Smilie et al., 1989), reducing fungal growth or generating hyphal disruption (King et al., 2010). We found no studies on the effects of Phi on the formation of sclerotia produced by pathogens.

The results of the present study represent the first report of direct *in vitro* fungicidal/fungistatic action of Phi against soybean fungi, which are causal agents of soil-borne diseases. Further *in vivo* research is needed to assess the impact of Phi applied on the soil or plant to analyze whether the control achieved in this work *in vitro* is also attainable in the plant. In this regard, Simonetti et al. (2015) tested MnPhi alone or in combination with PGPR bacteria to control *M. phaseolina* in soybean under greenhouse conditions; it was the first work to report effective control of *M. phaseolina* (up to 37%) using MnPhi as seed treatment.

In the same way, some preliminary results related to the effect of MnPhi seed treatment on field development of soybean sudden death were reported (Carmona et al., 2013).

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