Investigation note

In vitro evaluation of the antifungal activity of *Heliopsis longipes* extract in strains of *Sclerotium cepivorum* and *Sclerotinia sclerotiorum*

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Abstract

White rot and soft rot caused by *Sclerotium cepivorum* and *Sclerotinia sclerotiorum* are the main cause of losses in garlic and lettuce crops, making their control difficult due to the production of sclerotia. An environmentally friendly alternative to disease control is the use of plant extracts; like *Heliopsis longipes*, which has the capacity to produce alcamidas like afinina, which are responsible for its effect as an insecticide and bactericide, it is worth mentioning that this compound is found in greater proportion in the roots of the plant. Therefore, the biological material was collected in Guanajuato in 2013, from which the fungus was isolated and in which the antifungal activity of the extract was evaluated and the viability of the sclerotia of *Sclerotium cepivorum* and *Sclerotinia sclerotiorum* was verified. through the technique of poisoned medium. Obtaining percentages of inhibition higher than 75% for the strains of *Sclerotium cepivorum*, while for *Sclerotinia sclerotiorum* less than 40%. In addition to showing statistically significant reduction in the production of sclerotia and only affect the vigor of growth in one of the strains evaluated. Therefore, the extract showed to be effective in the control of the parameters evaluated.

Keywords: *Heliopsis longipes*, *Sclerotium cepivorum*, *Sclerotinia sclerotiorum*, viability of sclerotia.

Reception date: May 2018
Acceptance date: June 2018

Introduction

Root diseases are the main causes of economic losses in onions and related crops, throughout the world. White rot caused by *Sclerotium cepivorum* Berk is one of the most important root diseases. The development of the disease occurs in different regions around the world where environmental conditions are favorable for the pathogen (Velázquez-Valle and Medina-Aguilar, 2004). In Mexico, this is the main cause of yield and quality losses in garlic Delgadillo *et al*. (2004), which has led to total losses in the Bajio (Pérez-Moreno *et al*., 2009), difficult to control due to sclerotia, reproductive structures of the fungus, which can remain viable for up to 20 years (Delgadillo *et al*., 2004).

Sclerotinia sclerotiorum (Lib.) Bary is the causal agent of white mold or soft rot, which has caused losses of up to 70% in the cultivation of lettuce. Symptoms in this are manifested in the final phase of the crop cycle, showing wilting of the outer leaves of the plant, with the presence of cottony white mycelial growth towards the basal or central part of the stem, from which compact bodies are formed called sclerotia (Arias *et al*., 2007).

A friendly alternative to the environment for disease control is the use of plant extracts González *et al*. (2011). Plant secondary metabolites or bioactive compounds with fungicidal properties are an option in the management of pathogens, since they act against a limited number of species, are biodegradable to non-toxic products, have a low impact on human health and can be incorporated into programs of integrated management of pests and diseases (Salgado-Galciglia *et al*., 2008).

Some genera of the *Heliantheae* tribe produce different types of alcamides, these compounds are hydrophobic amides or esters that contain unsaturated fatty acids as part of their chemical structure (García *et al*., 2004).

Heliopsis longipes is a member of the *Asteraceae* family, it presents different compounds such as afinin, belonging to the group of alkamides (Molina *et al.*, 1995; García *et al*., 2004). Affinin is the alkamide found in greater proportion in the roots of this plant and mainly responsible for the biological effects such as insecticidal and bactericidal activity; this compound has biocidal action on some Gram-positive and Gram-negative bacteria, as well as on some fungi of the Ascomicetes class (Molina *et al*., 1995; García *et al*., 2004; Montes-Belmont and Prado-Ligero, 2006; Salgado-Galciglia *et al*., 2008). The objective of this work was to evaluate the antifungal activity of the crude extract of *H. longipes* in strains of *S. cepivorum* and *S. sclerotiorum* and the viability of the sclerotia after the application of the extract.

Methodology

The research was carried out in the Department of Agricultural Parasitology of the Autonomous Agrarian University Antonio Narro in the Toxicology laboratory.

Sampling

The samples were collected during 2013 in Comonfort (PBGTO7) and Cortazar (PBGTO52 and PBGTO61) in the state of Guanajuato, Mexico, in garlic cultures, where plants were extracted that presented the following symptoms: initial yellowing in the basal leaves, wilting, necrosis of the

foliage and abundant presence of sclerotia in the bulbs of dead plants. The two strains of *S. sclerotiorum* (MBREP and MBLEC) isolated from cabbage and lettuce crops respectively; and the PBZAC strain of *S. cepivorum* from garlic cultures of the state of Zacatecas, Mexico, were provided by the Biologist Maria Mercedes Medina Aguilar.

Obtaining sclerotia

The 10 sclerotia were extracted directly from the garlic plants with the help of a stereoscopic microscope and dissection needles, then placed in petri dishes for disinfection.

Cultivation of the fungus

To obtain pure strains, the sclerotia were superficially disinfected with 3% sodium hypochlorite for three min, rinsed five times with sterile distilled water and planted in Petri dishes with solid medium of Papa Dextrose Agar (PDA) and incubated in darkness at 25 ±2 °C for three days.

Differentiation tests for *Sclerotium* spp.

For the identification of the species of *Sclerotium* isolates, the methodology used by Montes *et al*. (2003), in which mycelium discs of the different *Sclerotium* isolates were inoculated in five ripe tomato fruits (5 discs/fruit), with the purpose of differentiating the species that attack the onion *S. cepivorum* which affects only to the genus *Allium* and *S. rolfsii* that attacks a great variety of species.

Evaluation of the extract

To determine the minimum inhibitory concentration, five concentrations of 5 000, 10 000, 15 000, and 25 000 ppm were used, based on the concentration of affinin present in the extract (70 800 ppm), which was added to the potato dextrose agar medium (PDA) sterile before emptying in the plates, when it was at a temperature of 55 °C. From each of the isolates of *S. cepivorum* and *S. sclerotiorum*, discs of mycelium 1 cm in diameter were taken where the fungus was covering 80% of the surface of the disc, each of which were placed in the center of the boxes with poisoned medium. The boxes were incubated at 25 ±2 °C and the radial growth of the mycelium was measured every 24 h until the control covered the surface of the culture medium. With the percentages of inhibition, the average effective dose was determined by a PROBIT analysis with the SAS 9.1 program.

Number of sclerotia produced in the bioassay and growth vigor of the fungus

An average of the sclerotia produced in the petri dishes with the extract was obtained, counting the sclerotia that originated in each Petri dish after the comparison with the extract, in each of the four repetitions of each treatment.

The vigor of growth of the fungus was determined 14 days after sowing, a sample of 10 sclerotia was taken, using the scale proposed by the Horticultural Research International of Wellesbourne, in the United Kingdom (Ramírez *et al*., 2000) where: 0= sclerotia without growth; 1= sclerotia with first hyphae; 2= sclerotia with hyphae in 25% of the agar circle; 3= sclerotia with hyphae in 50%

of the agar circle; 4= sclerotia with hyphae in 100% of the agar circle; 5= formation of white sclerotia in the agar circle; 6= formation of brown sclerotia in the agar circle. The vigor of growth of the fungus was obtained with the average of the values in the four repetitions. The experiment was established under a completely randomized experimental design. An Anova and means comparison tests were performed.

Results

The *Sclerotium* isolates showed white mycelium, white sclerotia that turned from dark brown to black. The pathogenicity test to differentiate the *Sclerotium* species was (negative) in the tomato fruits, which indicates that the strains belong to the species *S. cepivorum* according to the methodology of Montes *et al*. (2003).

The results show a percentage of inhibition higher than 50% at the concentration of 15 000 ppm except for the strain from Zacatecas and the strains of *S. sclerotiorum*, being the strain PBGTO61 the one that showed the highest percentage of inhibition at the 25 000 ppm with a percentage of 77.01%. While strains of *S. sclerotiorum* (MBREP and MBLEC) showed the lowest percentages of inhibition of 39.58 and 25.09% in the concentration of 25 000 ppm, it should be mentioned that *S. cepivorum* strains showed a lower growth rate than strains of *S. sclerotiorum* (Table 1).

Table 1. Inhibition of mycelial growth by extract of *H. longipes*.

CONC. (PPM)	PBGTO7* (%)	PBGTO52* (%)	PBGTO61* (%)	PBZAC* (%)	MBREP** (%)	MBLEC** (%)
5 000	5.44	1.47	8.29	23.76	4.38	3.03
10 000	31.46	19.79	29.49	47.87	10.33	7.05
15 000	70.21	63.99	71.26	58.42	26.19	15.99
25 000	75.44	72.72	77.01	68.14	39.58	25.09

*= percentages of inhibition at 144 h; **= percentages of inhibition at 72 h.

Ramírez *et al*. (2008) evaluated the effect of the crude extract of *H. longipes* on the development of mycelium based on dry weight in the species of *S. cepivorum* and *S. rolfsii*, obtaining a decrease in dry weight of 90% and 80% respectively at concentrations of 25 µg mL^{-1}. It is worth mentioning that the highest inhibition percentage obtained in this study was 77.01% at 25 000 ppm of the PBGTO61 strain. Additionally, the mean lethal dose of affinin and the crude extract for *S. rolfsii* varied between 15 and 20 µg mL^{-1}, while for *S. cepivorum* it ranged between 5 and 10 µg mL^{-1}. In Table 2, it shows the average effective dose for each of the strains used in this study and their fiducial limits.

Likewise, Ramírez *et al*. (2008) evaluated the ergosterol content in the isolates when exposed to affinin and the crude extract, with reductions in ergosterol in both species and in the two presentations (raw root extract and purified affinin), stating that the inhibition of ergosterol is not specific so it is parallel with the dry weight of the mycelium, which could be taken as an index of growth. While Pérez-Moreno *et al*. (2000) states that the use of chemical products such as

tebucanzole for the control of *S. rolfsii* isolates which were collected in 2003, have not developed the resistance to the mode of action of this type of products; however, it may be possible that the use of this product is affecting the effect of other compounds such as afinin.

Table 2. Effective mean dose (ED_{50}) on mycelial growth of *S. cepivorum* and *S. sclerotoirum*.

CEPA	ED_{50}	Fiducial limits 95 (%)	Fiducial limits 95 (%)
PBGTO7	13 259	2 788	12 7976
PBGTO52	6 533	5 709	8 053
PBGTO61	12 951	3 326	75 446
PBZAC	10 891	5 873	16 293
MBREP	32 326	25 625	48 020
MBLEC	57 900	37 555	154 043

In Table 3, it can be observed that in the six strains there is a tendency to decrease the production of sclerotia as the concentrations of the extract increase in the culture medium, showing significant difference in all strains in the production of sclerotia, PBZAC being the only strain to show no difference in the production of sclerotia.

Table 3. Sclerotium production.

CONC. PPM	Number of sclerotia					
	PBGTO7	PBGTO52	PBGTO61	PBZAC	MBREP	MBLEC
Test	2445.5 a	2352 a	2331.5 a	2318.5 a	33.25 a	38 a
5 000	2187.3 a	1862.3 ab	1923.8 ab	2011.5 a	39.5 a	31 ab
10 000	1013.8 b	1481.8 ab	1848 ab	1527.5 a	29.5 ab	22.5 ab
15 000	1352.8 b	727.5 b	1790.5 ab	1457 a	27.25 ab	19.75 b
25 000	1204.3 b	694 b	1408.3 b	1385.5 a	18 b	17.75 b

Values with the same letter in the same column are statistically equal (LDS, $p > 0.01$).

These results coincide with those reported by Ochoa *et al.* (2012a) in the antifungal evaluation of four plant extracts (tabaquillo, cinnamon, pirul and chirimoya) in the control of three species of *Fusarium*, of which the extracts of pirul and tabaquillo showed an increase in the production of conidia. Like Montes and Prado (2006) who report the increase in the production of sclerotia of *S. cepivorum* when exposed to four extracts (parsley, alfalfa, black pepper and marjoram) of the 15 evaluated extracts; showing increases of 296.41%, 192.67%, 140.6% and 117.35%.

The vigor of *in vitro* growth of the sclerotia showed no significant difference between the treatments in the strains PBGTO7, PBGTO52, PBGTO61, PBZAC and MBREP (Table 4). While in the MBLEC strain, it was reduced to 4.75 at the 25 000 ppm concentration; it should be mentioned that the ED_{50} calculated for this strain is higher (57 900 ppm).

Table 4. Vigor of growth of sclerotia *in vitro*.

CONC. PPM	Strains					
	PBGTO7*	PBGTO52*	PBGTO61*	PBZAC*	MBREP*	MBLEC*
Test	5.5 a	4.5 a	4.5 a	5.5 a	6 a	6 a
5 000	5.25 a	4.5 a	4.5 a	5.25 a	6 a	5 ab
10 000	5.5 a	4.25 a	4.25 a	5.25 a	5.5 a	6 a
15 000	5 a	4.25 a	4 a	5.25 a	5.5 a	5.25 ab
25 000	5 a	4 a	4 a	5 a	5 a	4.75 b

Values with the same letter in the same column are statistically equal (LDS, $p > 0.05$).

Pérez-Moreno *et al*. (2009) evaluated the ability of five fungicides to inhibit growth, sclerotia production, viability and growth vigor, with Tebuconazole and TCMTB, which showed the lowest growth vigor (without growth), while Procimidone, Thiabendazole and Iprodione showed a growth vigor of 4.87, 5.58 and 5.73 respectively. It should be mentioned that in the strain PBGTO52, PBGTO61 and MBLEC showed a vigor of growth lower than those reported by Pérez-Moreno *et al*. (2009) for Procimidone, Thiabendazole and Iprodione products.

Conclusions

The extract of *H. longipes* showed an effect on mycelial growth, reduced the number of sclerotia and the vigor of growth was only affected in one of the strains used in this study.

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