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SCREENING OF SOME INDIGENOUS PLANTS FOR IDENTIFYING THE INHIBITORY EFFECT AGAINST PHYTOPHTHORA INFESTANS

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ABSTRACT. The present study aims to complete the first step towards finding a viable solution for controlling the oomycete *Phytophthora infestans*, the phytopathogenic agent responsible for the emergence of potato and tomato late blight. The authors have proposed a screening of some indigenous plants, both from wild and cultivated flora in order to highlight the antifungal effect of the hydroalcoholic extracts, obtained from these plants, by percolation. The experiment was conducted in vitro and involved the evaluation of antifungal effect of ivy (*Hedera helix*), cocklebur (*Xanthium strumarium*), wormwood (*Artemisia spp*), sage (*Salvia officinalis*) and rosemary (*Rosmarinus officinalis*). To identify the antifungal capacity were used the well diffusion method, and the radial growth method. The best results were obtained with rosemary, wormwood and cocklebur.

Keywords: oomycete, *Phytophthora infestans*, vegetal extracts, antifungal activity

INTRODUCTION

Phytophthtora infestans is one of the most important phytopathogen which exists worldwide. The oomycete *P. infestans* is considered to originate from Mexico, along with other species belonging to the genus, such as *P. mirabilis, P. ipomoea* and possibly *P. phaseoli* (Grünwald, 2005). Currently, it is not known the whole mechanism of action of *P. infestans* (Hadwiger et al., 2006). Although it has been shown that the virulence of the oomycete is given to a certain extent by the presence of effector molecules encoded by avirulence genes which allow for the rapid infection and colonization of host tissues. In addition, there are a number of protease inhibitors which prevent the immune response of the plant, so that the time between infection and sporulation of the oomycete does not take longer than 3 days (Mizubuti et al., 2007).

The oomycete was responsible for the Great Famine in Ireland in the mid nineteenth century (Donnelly, 2005), which drew the attention of the scientific world, and though there have been efforts to control it, there are only synthetic products which provided reliable results. (Hadwiger et al., 2006). The question of the remanence of the harmful chemicals in soil, water, plants and thus the human and animal body could not be solved. Currently, are investigated some environmentally friendly methods to combat the disease, both by agricultural practices and by applying preemergent and emergent bioproducts (Lourenco Jr et al., 2006; Haverkort et al., 2008; Ghorbani et al., 2005). The use of bioproducts obtained from fresh or dry pvegetal material is the subject of extensive research worldwide. Studying the native plants in order to identify the active compounds responsible for inhibition of this phytopathogenic agent was an important step in identifying a mechanism of action against P. infestans (Blaeser et al., 1998; Zaller, 2006). Therefore, were highlighted a number of biocompounds as isoflavones, alkaloids, terpenes, tannins, etc., as potential antifungal substances.

One of the easiest methods of extracting the compounds of interest from plants is percolation, a method which involves the extraction of active principles from the vegetal material at room temperature, using countercurrent solvent (Handa et al., 2008). In this study, were used 5 indigenous plants, four from wild flora and one from cultivated flora. These five plants were studied for their antifungal activity and are as follows: wormwood, cocklebur, rosemary, sage and ivy.

MATERIALS AND METHODS

The vegetal material

The vegetal material was purchased from S.C. Plafar S.R.L., a local supplier of medicinal plants.

Preparation of the vegetal extracts

The plant material has been processed in order to obtain the plant extracts, by carrying out the percolation process. The solvent used was 50o ethanol and the ratio of plant material - solvent was 1:10. The solvent action on the plants was for 24 hours, the percolation rate being of one drop every 5 seconds. After percolation, each extract was filtered with Buchner funnel, under vacuum, using Whatman 1 filter paper. The plant extracts obtained were stored at 4oC.

Test microorganism

The strain of P. infestans belongs to the collection of the microbiology laboratory of NIRDBS.

Antifungal screening of the vegetal extracts

The antifungal screening of the vegetal extracts selected against *P. infestans* was performed in two stages.

The first stage consisted of using the agar well diffusion method (Bobbarala et al., 2009). This method has been customized for optimal performance in this

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experiment due to the presence of *P. infestans*. The experiment began by cultivating the oomycete a period of seven days on a specific medium (pea - agar). Several 8 mm discs were cut from the periphery of a *P. infestans* mycelium of seven days old innocullum cultivated on specific medium (pea-agar). These were placed on potato dextrose agar (PDA) nutrient medium, on the center of the Petri dishes (70 mm diameter). In the PDA medium were cut wells at a distance of about 1.5 from the plate center and 2 cm from one to another and from the edge. After five days of subculturing the oomycete, the wells were filled with the hydroalcoholic extracts from the native plants. The amount of extract per well was 70 µl. The extracts were used as such, and as dilution of 1:2 and 1:3 v/v with sterile distilled water. The experiment had three replications and the contol used was 50o ethanol and sterile distilled water. Duration of the experiment was 11 days after filling the wells with the hydroalcoholic extracts and 15 days after the oomycete subculturing. The plates were incubated at 20° C throughout the experiment.

The second stage was performed by using the radial growth method (Banso et. al., 1999) for testing the antifungal effect of the selected plant extracts. This method consisted in the introduction and mixing into the culture medium (pea - agar) of the plant extracts. For each tested plant were used 20 µl extract / 1 ml medium. The plates were left to solidify under the laminar flow and then a 8 mm disc of seven days old culture of *P. infestans* was inoculated in the center of each Petri plate. As controls were used both 50° ethyl alcohol and sterile distilled water. The experiment was performed in 3 repetitions at an incubation temperature of 20°C, for a period of 10 days. The second phase of the experiment lasted less because of the specific cultivation medium for the *P. Infestans* isolate, the pea – agar.

The Petri dishes were monitored every 24 hours, aiming to observe both the presence of the halo (antifungal activity of the extract) and the stage of development of the mycelium (fungistatic effect of the extract). The experiment was cansidered completed when the developing stage of the oomycete used as control reached the Petri plate edges. In measuring the mycelium was taken into account the size of the initial inoculum disc.

RESULTS AND DICUSSIONS

In the first stage of the experiment, the oomycete development was inhibited completely under the action of extracts used as such and the previously developed mycelium did not retain any viability (Fig. 1). When observing the plant extracts diluted at the ratios 1:2 and 1:3 v/v with sterile distilled water, the oomycete developed considerably less by comparing to the control, but the total inhibition has not occurred. In Table 1 is shown the *P. infestans* susceptibility to the action of the hydroalcoholic plant extracts. This is determined by identifying the presence or absence of contact inhibition halo of mycelium of the oomycete with the vegetal extracts spread into the nutritive media around the wells (0-5 mm = +, 5 to 10 mm = + +, ≥ 10 mm = + + + (Table 1)).

The measurements were performed from the 5th day because of the oomycete capacity to develop under the circumstances. Following the recorded data, it was observed that the extracts diluted in the ratios 1:2 or 1:3 their had an inhibitory effect significantly decreased. All the 5 extracts tested had inhibitory effect on the fifth day of development at 1:2 ratio, but from the seventh day the oomycete susceptibility to the action of plant extracts decreased. In this report, the plants showing the strongest effect are wormwood, cocklebur and rosemary. The 1:3 ratio extracts have little effect compared to the ratio 1:2, as from the seventh day only the wormwood and rosemary had inhibitory effect, while the cocklebur no longer has inhibitory activity.

Table 1. Inhibitory activity of the vegetal extracts against *P. Infestans* tested by well diffusion method

Period of observation		5th day		7th day		9th		11th day	
Dilution		1:2	1:3	1:2	1:3	1:2	1:3	1:2	1:3
Vegetal extracts	Wormwood	+++	+	+	+	+	-	-	-
	Cocklebur	++	+	+	-	+	-	-	-
	Rosemary	++	+	+	+	+	-	-	-
	Sage	++	+	+	-	-	-	-	-
	Ivy	++	+	+	-	-	-	-	-
Control	50° ethanol	-	-	-	-	-	-	-	-
	S.D.W.	-	-	-	-	-	-	-	-

Furthermore, it was identified a slower development of the mycelium in the presence of the vegetal extracts diffused in the medium. The susceptibility was determined by measuring the micelyum size compared with the control (Table 2). To determine the fungistatic effect, as a result of processing the data, significant differences were observed in the activity of plant extracts diluted 1:2 and 1:3 for the first 5 days of the experiment. Subsequently, plant extracts diluted by 1:2 with S.D.W. slowed the development of the oomycete up to 6 mm more than in the case of the plant extracts diluted by 1:3. Plant extracts showed fungistatic effect measured in the range of 7-10 mm compared to the control.

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Table 2. Determination of the fungistatic effect of the hydroalcoholic vegetal extracts against *P. infestans*

Period of observation		1st day		3rd day		5th day		7th day		9th day		11th day	
Dillution		1:2	1:3	1:2	1:3	1:2	1:3	1:2	1:3	1:2	1:3	1:2	1:3
The size of the micellyum on the interaction with plant extracts (mm)	Sample 1	14	14	30	31	34	35	47	50	55	56	64	70
	Sample 2	14	14	29	30	34	35	47	50	55	56	64	70
	Sample 3	14	14	28	30	33	34	46	49	55	55	64	70
	Control 1	14		35		40		54		65		82	
	Control 2	14		35		40		54		64		82	
	Control 3	14		35		39		54		64		82	

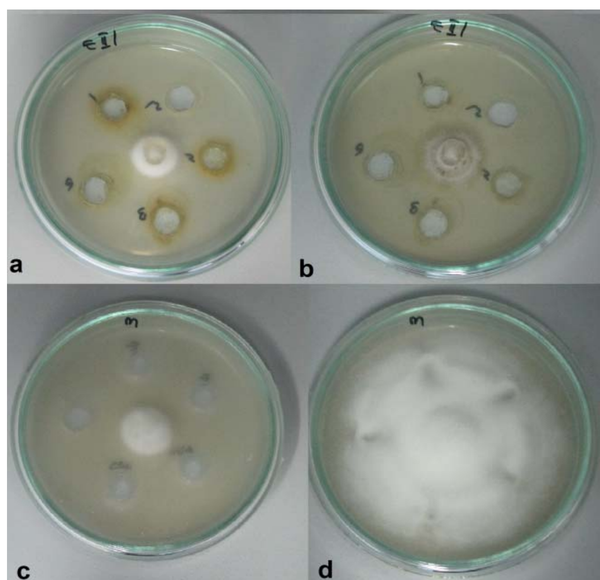

Figure 1. Representation of the antifungal effect of plant extracts tested by well diffusion method (a: vegetal extracts on day 1, b: vegetal extracts on day 11; c: control on day 1, d: control on day 11);

The second stage of the experiment, carried out by radial growth method, showed a fungistatic effect against *P. infestans* of the plant extracts obtained from rosemary, wormwood and cocklebur (Fig. 2). The plant extracts that showed the highest capacity of inhibition is rosemary, being observed a mycelium diameter of 49 mm, on the tenth day of the experiment. This was followed by wormwood and cocklebur, with a diameter of 55 mm of the mycelium. In the case of the inocullum samples tested with extract of rosemary it was registered a daily increase of the mycelium between 1mm (between first and second day) and 9mm (between the seventh and eighth day). The cocklebur and the wormwood had a similar effect seen in the daily development of mycelium in the range of 1-9 mm between the first and second day, respectively between the sixth and eighth day. A reduced inhibitory effect had the sage extract with a mycelium final size of 68 mm. The growth rate of the mycelium was between 1mm/day between the first and second day and 11 mm / day between the sixth and the seventh day. Ivy recorded a growth rate of at least 1 mm mycelium / day (between the first and second day) and a maximum of 9 mm / day (between the sixth and seventh day), with a final size of the mycelium in the tenth day of 66 mm.

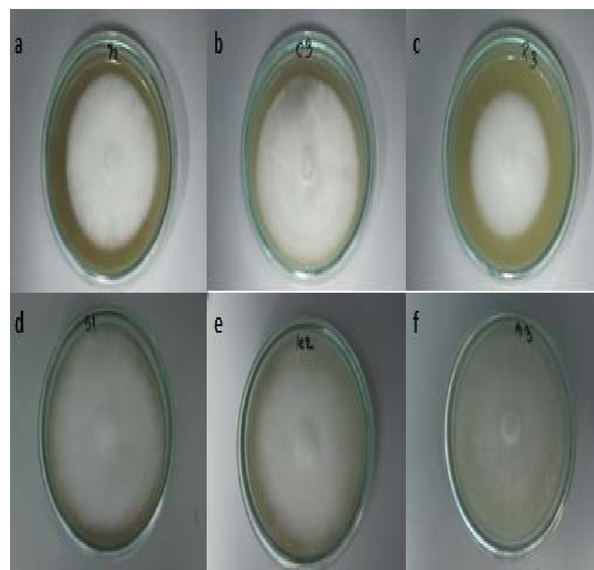

Figure2. Representation of the antifungal effect of the vegetal extracts in the 10th day of testing (a: wormwood extract; b: cocklebur extract; c: rosemary extract; d: sage extract; e: ivy extract; f: control)

CONCLUSIONS

In vitro experiments carried out showed an antifungal potential of all the five plants used adepending upon the concentration and the quantity of the extract used, and also on the species of the plant used for obtaining the hydroalcoholic extracts.

The well diffusion method showed antifungal and fungistatic effect of hydroalcoholic extracts obtained with ethanol 50o from wormwood, rosemary, sage, cocklebur and ivy. These extracts have an inhibitory effect on *P. infestans* when used as such. If the plant extracts are diluted in sterile distilled water at a ratio of 1:2 or 1:3, the extracts lose their inhibitory capacity of about 65 % and 70 %.

The radial growth method in which were used 20 µl plant extract in 1 ml medium showed fungistatic effect of three extracts, namely rosemary, wormwood and cocklebur. Thus it was demonstrated that at low concentration only this three plants retain their inhibitory effect over *P. infestans*.

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