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Antifungal effects of phenolic extract from industrial residues of *Aloe vera*

Beatriz Pintos¹, Luisa Martín-Calvarro¹, Dolores Piñón², Alberto Esteban¹, María L. Tello³, Elena Pérez-Urria¹ and Arancha Gómez-Garay¹

¹Universidad Complutense de Madrid, Facultad de Biología, Dept. Biología Vegetal I: Fisiología Vegetal. C/ José Antonio Novais 12, 28040 Madrid, Spain. ²Instituto Nacional de Investigaciones de la Caña de Azúcar. Dirección General de Investigación y Desarrollo. Ctra. del CAI Martínez Prieto, km 2. Boyeros, La Habana, Cuba. ³INIA, Ctra. de la Coruña km 7, 28040 Madrid, Spain.

Abstract

This research is concerned with the fungicidal properties of the phenolic extract from industrial residues of *Aloe vera* used for antifungal treatment of various plant pathogens (*Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Phaeoconiella chlamydospora*, *Phaeoacremonium aleophilum*, and *Sporisorium scitamineum*). Six phenolic compounds were identified in this extract from *A. vera* cortex: aloesin, α -barbaloin, chromone X, isoaloeresin D, β -barbaloin and aloeresin E. Phenolic extract was added to PDA medium at 20 concentrations from 0.32% to 10% and the growth of four different plant pathogenic fungi was tested. Fungal inhibition was calculated in order to evaluate the antifungal efficacy of phenolic extract against pathogens. Inhibition of *Sporisorium scitamineum* hyphal growth was observed after treatment with the phenolic extract at concentrations higher than 2.5% and a fungistatic effect with a 58.2% mycelia growth inhibition was detected at 3% extract concentration. Inhibition of *P. chlamydospora* and *P. aleophilum* hyphal growth was observed at concentrations higher than 4% and 3% respectively. A fungistatic effect with a 71.65% and a 19.87% mycelia growth inhibition was detected at 4.5% and 3.5% extract concentration respectively. About *F. oxysporum* f. sp. *radicis-lycopersici*, inhibition of hyphal growth was observed at concentrations higher than 2.5% and a fungistatic effect with a 32.07% mycelia growth inhibition was detected at 3% extract concentration. The results indicate that the tested extract possess antifungal activities against these pathogens at various concentration levels and could be used as a potential natural fungicide in order to control fungi pathogens providing a new use for the *A. vera* industrial residues.

Additional keywords: phytopathogenic fungi; phenolic compounds; fungicide.

Abbreviations used: FORL (*Fusarium oxysporum* f. sp. *radicis-lycopersici*); LOEC (lowest-observed-adverse-effect-concentration); MGI (mycelia growth inhibition); MIC (minimum inhibitory concentration); NOAEC (no-observed-adverse-effect concentration); PAL (*Phaeoacremonium aleophilum*); PCH (*Phaeoconiella chlamydospora*); PDA (potato dextrose agar); SS (*Sporisorium scitamineum*).

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Correspondence should be addressed to Beatriz Pintos: bpintos@ucm.es

Introduction

Direct crop losses in the field are caused by plant pathogens by reducing crop yield and quality. The control of plant diseases in traditional agricultural production systems is mainly based on the application of fungicides. However, fungicides application has led to problems and constraints in the control of these diseases by a loss in efficiency, due to an increased resistance to active ingredients, ecological damage

and a serious negative impact on the human health. An efficient alternative to inhibit the growth of several fungal pathogens and reach the control of crop diseases can be obtained by the use of bio-based products (Romanazzi *et al.*, 2012; De Corato *et al.*, 2016). Nevertheless, the commercial use of natural fungicides on the market is low, the 5th Annual Meeting of the Biological Control Industry reports a total of 55 biological fungicides registered in the US market and in the EU the registered biopesticides are much fewer:

21 fungicides to be used in Pome fruit, vines and tomato (Castillo *et al.*, 2012).

The biological compounds most useful in fungi control have different effects on the development of mycelia growth and the effect on sporulation rate and inhibition of germination ranging from a fungistatic effect to complete inhibition. Plant extracts applied as a crude state or as a fraction inhibit partially or totally fungal growth, affecting the development of colonies in laboratory tests when applied at low concentrations of bioactive compounds (Mahlo *et al.*, 2010; Castillo *et al.*, 2012; Cerqueira *et al.*, 2016). Among plant metabolites, plant phenolics are therefore active in biological systems and probably the capacity or biological value explains its abundance in plant tissues (Meckes *et al.*, 2004). Synthesis of aromatic substances is a major defense mechanism of plants. Biochemicals and their oxidation products are implicated in disease resistance (Sukand & Kulkarni, 2006; Patzke & Schieber, 2018). Phenolic compounds and related oxidative enzymes are considered as one of the most important biochemical parameters for disease resistance (Pradeep & Jambhale, 2002; Osorio *et al.*, 2010; Patzke *et al.*, 2017).

In natural plant defense, accumulation of phenolic compounds at the infection site has been correlated with the restriction of pathogen development, since such compounds are toxic to pathogens (Benhamou *et al.*, 2000). This mechanism can be exploited in the fight against plant pathogens by the use of exogenous phenolics. The amphipathicity of these compounds can explain their interactions with the membrane and thus the antimicrobial activity (Veldhuizen *et al.*, 2006). Phenolic extracts show, among other properties, antifungal activity by reducing or totally inhibiting fungal growth in a dose-response manner.

One of the plant species whose use has spread over in recent years for various purposes is the aloe. *Aloe vera* L. (also referred by Miller as *Aloe barbadensis*) has a wide spectrum healing qualities, which vary in terms of its components: enzymes, monosaccharides, polysaccharides, anthraquinones, amino acids, minerals and vitamins (Capasso *et al.*, 1998; López *et al.*, 2013). Although the main composition is water and aloe represents 99-99.5% of the plant, the remainder, between 0.5% and 1%, is a high concentration of sugars (up to 60% of dry matter), vitamins, proteins and amino acids, organic acids, minerals and phenolic compounds. Among these components, there are more than 150 that theoretically could have potential activity, of which 75 are biologically active compounds. Many *A. vera* active ingredients have been identified (Jasso de Rodríguez *et al.*, 2005; Debnath *et al.*, 2018).

Diverse extractives (including phenolics) of *A. vera* fresh leaves show antifungal activity against

different plant pathogenic fungi (Jasso de Rodríguez *et al.*, 2005; Subramanian *et al.*, 2006; Nebedum *et al.*, 2009; Nidiry *et al.*, 2011; Zapata *et al.*, 2013; Flores-López *et al.*, 2016). However, these works were based on the application of *A. vera* gel, pulp and liquid fraction from leaves. In the present study, we have reported the efficacies of the phenolic extract obtained from leave residues resulted from the *A. vera* extractive industry.

In this work four plant pathogenic fungi were selected because these produce severe losses in crops. Sugarcane smut, caused by the fungus *Sporisorium scitamineum*, is considered the most severe disease of sugarcane in the world. Disease affects germination, tiller counts, smut incidence, plant girth, millable stalks per hectare and tons cane per hectare.

Fusarium crown and root rot is also a soil-borne disease, with the potential to limit productivity in glasshouse and field tomato crops. The causal agent is *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Fungicides are of little use on most *Fusarium* diseases (Sierotzki & Ulrich, 2003).

Phaeoconiella chlamydospora and *Phaeoacremonium aleophilum* are both fungi frequently associated with esca and grapevine decline. Esca is a destructive disease that affects grapevines worldwide.

Processing of *A. vera* gel extraction leads to the formation of several by-products and residues that can have a nowadays not exploited economical potential. The aim of this study was the evaluation of the *in vitro* antifungal activity of phenolic extract from *A. vera* leaf residues against four different plant pathogenic fungi: *Sporisorium scitamineum*, *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *Phaeoconiella chlamydospora* and *Phaeoacremonium aleophilum*.

Material and methods

Plant material and extraction of phenols

Phenolic compounds were extracted from the dried residue of aloe cortex as follows: a mass of 150 g dried powder was placed in an Erlenmeyer flask with 750 mL of distilled water. The flask was covered with aluminum foil to avoid light exposure. This mixture was refluxed at 60 °C for 12 h. The pH of the sample was adjusted to 3.0 by adding glacial acetic acid for the stabilization of the phenolic compounds. After this process, the sample was filtered using Whatman filter paper no. 41 and centrifuged at 3500 rpm for 15 min (Osorio *et al.*, 2010). The supernatant (phenolic extract) was used for further experiments.

HPLC analysis of phenolic compounds

HPLC analysis was carried out in a Beckman system (San Ramon, CA, USA) comprising a Programmable Solvent Module 126 pump, Scanning Detector Module 167 and manual injector. The data were processed with the GOLD system. Reverse-phase HPLC (RP-HPLC) of phenolic compounds was carried out following Esteban-Carrasco *et al.* (2001): the system operated at 45 °C on a Beckman ultrasphere 5 µm ODS column (25 cm × 4.6 mm I.D.) using a flow rate of 1 mL/min. Solvent A was 2.5% acetic acid in water and solvent B was acetonitrile. The elution conditions applied were a linear gradient from 12 to 26% in 30 min, from 26 to 70% in 15 min, and finally, washing and re-conditioning of the column. Monitoring was set at 290 nm for identification. In order to quantify phenolics compounds, peak heights were correlated with the concentration according to the calibration curve of aloin (β-barbaloin), which was purchased from Sigma (Saint Louis, MO, USA).

Test fungi

Sporisorium scitamineum (SS), *Phaeoconiella chlamydospora* (PCH), *Phaeoacremonium aleophilum* (PAL) and *Fusarium oxysporum radialis-lycopersici* (FORL) used were grown on potato dextrose agar (PDA, Merck Germany) medium. The stock cultures plates (50 mm ø) were incubated at optimum temperature (40 ± 2 °C for SS; 22 ± 2 °C for PCH and PAL and 25 ± 2 °C for FORL) for 4 weeks to allow mycelium growth into the medium.

In vitro antifungal assay

The antifungal properties of phenolic extract were evaluated for assessing in contact phase effects towards mycelia growth. *In vitro* assay was performed in PDA media treated with different concentrations of phenolic extract. PDA medium was autoclaved at 121°C for 15 min and cooled to 40 ± 2 °C. The control treatment contained only PDA culture medium. Different concentrations of phenolic extract (from 0.32 to 10%) were prepared by mixing in the flasks with warm sterile molten medium to obtain final concentrations. The PDA agar with phenolic extract was poured into sterile 50 mm Petri plates (6 mL/plate). Mycelial plugs (5 mm ø) obtained from the actively growing margin of four weeks-old cultures of each species were obtained and placed aseptically at the centre of each treatment Petri plate and cultivated in the dark at optimum temperature. In order to reach the minimum inhibitory concentration (MIC) each phenolic extract concentration was tested

in triplicate in each assay and the assays were repeated to confirm results.

The antifungal activity of the phenolic extract was tested using radial growth technique. The mean radial mycelia growth of each fungus was determined by measuring the diameter of the colony in two directions. The growth was compared to the control plate 7 days after inoculation in SS and FORL and 14 days after inoculation in PCH and PAL. For each concentration, three replicate plates were used.

Data analysis

All experiments were performed with three replications of each phenolic extract concentration. The mean growth values were obtained and then the radial inhibition was calculated. The percentage of mycelia growth inhibition (MGI) in relation to the control treatment was calculated by using the formula $MGI (\%) = ((R-r)/R) \times 100$, where R is the radial growth of fungal mycelia on the control plate and r is the radial growth of fungal mycelia on the treated plate. Data were analyzed using a one-way analysis of variance at the significance level of $p < 0.05$ and the Duncan's test. The Dunnett's test was used to calculate the minimum difference between the control and the treatment means detected as being statistically significant. The no-observed-adverse-effect concentration (NOAEC), lowest-observed-adverse-effect-concentration (LOEC) and MIC for mycelial growth of the four plant pathogens were calculated. Significant difference was considered as that with a p value < 0.05 in all statistical analyses. All the statistical analyses were implemented using the statistical package Statistica v. 9.

Results and discussion

In this work, the phenolic extract obtained from industrial residues of *A. vera* was tested against important plant pathogens. Six phenolic compounds were identified in this extract (Fig. 1, Table 1): aloesin, α-barbaloin, chromone X, isoaloeresin D, β-barbaloin and aloeresin E. These compounds have also been identified in previous studies in methanolic extracts from *A. vera* cortex (Esteban-Carrasco *et al.*, 2001). Anthraquinones are the phenolic compounds present in our extract, the most prominent being chromone X (0.119 mM) and aloesin (0.108 mM). The aloin/barbaloin (10-β-D-glucopyranosyl-1, 8-dihydroxy-3-hydroxymethyl-9) is considered to be the most important phytoconstituents found in aloe species (Tyler, 1994; Dubey, 2015). It is one of the main biologically active constituents of aloe which is found in nature as a mixture of two diastereoisomers, aloin A (10R) and aloin

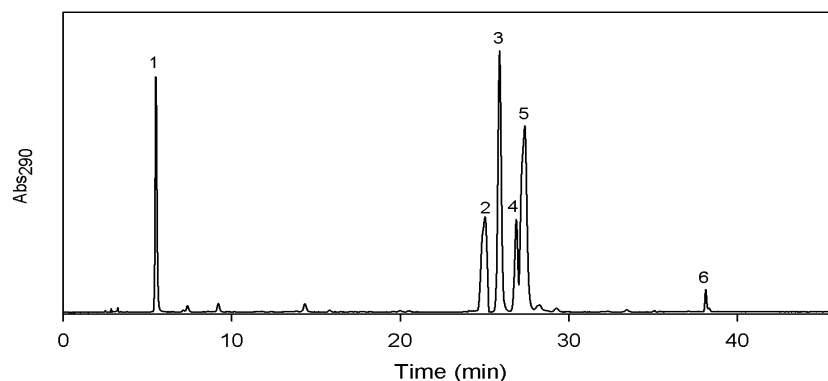


Figure 1. Profile of RP-HPLC phenolics compounds from the extract of the industrial residues of *A. vera*. 1, Aloesin; 2, α -Barbaloin; 3, Cromone X; 4, Isoaloeresin D; 5, β -Barbaloin and 6, Aloeresin E.

B (10S). Aloin is generally contained in the exudate seeping out from freshly cut leaves, whilst very low amounts of aloin exist in Aloe gel obtained from the internal mass of aloe leaf (Fanali *et al.*, 2010). Aloin is a good antifungal agent; previous reports demonstrate such effect (Zapata *et al.*, 2013; Dubey, 2015).

Four chromones have been identified in the extract: Aloesin (Haynes *et al.*, 1970), isoaloeresin D and aloeresin E (Saccu *et al.*, 2001), and another chromone X (Esteban-Carrasco *et al.*, 2001). Chromones have shown previously antifungal activity with approximately 50% inhibition for *FORL* growth (Prakash *et al.*, 2008).

Results of biological activity of this extract against four fungal strains showed a strong fungicide effect on the growth of *SS*, *PCH*, *PAL* and *FORL*. For all four phytopathogenic fungi a total inhibitory concentration was reached, while lower concentrations showed a fungistatic activity of the extract. Fungicide activity was considered when no fungal growth was observed in the plates and fungistatic activity was considered when fungal growth was delayed.

The contact phase effects of different concentrations of phenol extract on the mycelia growth of *SS*, *PCH*, *PAL* and *FORL* strains are shown in Table 2. The antifungal effects of the extract were diverse for the four tested fungi. These results showed that

phenolic extract of *A. vera* industrial residue was highly inhibitory to mycelia growth of *SS* among the pathogens tested. Inhibition of *SS* hyphal growth was observed after treatment with the phenolic extract at concentrations higher than 2.5% (Table 3). For *SS* a fungistatic effect with a 58.2% MGI was detected at 3% extract concentration (LOEC, Table 3) while complete inhibition (100%) was observed at 3.5% extract concentration (MIC, Table 3 and Fig. 2A). The sensitivity or resistance of sugarcane to smut can be related to changes in sugarcane leaf levels of phenolic compounds. Thus an increase in both caffeic and syringic acids was detected in the resistant cultivar Mayarí 55-14 when it was inoculated with *SS* (Santiago *et al.*, 2010). Thus, *SS* is susceptible to phenolic compounds, but not only to sugarcane ones because present results show the efficiency of *A. vera* phenolic compounds against *SS* too.

On the esca and grapevine decline pathogens *PCH* and *PAL* different results were obtained. An effective concentration range was obtained narrower in *PCH* (between 4 and 5%), while in *PAL* it ranged from 3% to 9% (Table 3). Inhibition of *PCH* and *PAL* hyphal growth was observed following treatment with the phenolic extract at concentrations higher than 4% and 3% respectively (Table 2). For *PCH* a fungistatic effect with

Table 1. Identification of phenolic compounds from the extract of the industrial residues of *Aloe vera* by HPLC. The retention times (R_t) and peak wavelength of each compound are shown. The concentration was calculated from the aloine (β -barbaloin) calibration curve.

Peak	R_t (min)	Compound	Area (%)	Concentration (mM)	Peak wavelength (nm)
1	4.81	Aloesin	27.01	0.108	212-216, 244-248, 252-254, 296-297
2	23.64	α -Barbaloin	10.75	0.041	226, 270, 298, 360
3	24.48	Cromone X	29.95	0.119	205-207, 210-217, 225-228, 242-244, 252, 283-300
4	25.40	Isoaloeresin D	10.14	0.038	213, 228, 242, 252, 300
5	25.99	β -Barbaloin	20.43	0.080	226, 270, 298, 360
6	37.83	Aloeresin E	1.71	0.006	205, 217, 223, 244, 252, 281

Table 2. Antifungal activity of phenolic extract from *Aloe vera* industrial residues against growth of four plant pathogenic fungi: *Fusarium oxysporum f. sp. radicle-lycopersici* (*FORL*), *Phaeoconiella chlamydospora* (*PCH*), *Phaeoacremonium aleophilum* (*PAL*), and *Sporisorium scitamineum* (*SS*); (n=3).

Concentration (%)	Mycelial growth inhibition (%)			
	<i>FORL</i>	<i>PCH</i>	<i>PAL</i>	<i>SS</i>
0	0 a	0 a	0 a	0 a
0.315	2.06±3.14 a	2.78±1.60 a	3.61±5.47 a	11.89±4.24 a
0.625	4.58±5.58 a	10.53±5.78 a	2.84±3.01 a	12.11±4.95 a
1.25	1.33±2.04 a	11.46±6.10 a	3.61±0.48 a	23.49±3.05 a
2.5	6.02±1.21 a	10.84±5.33 a	8.62±3.43 ab	45.42±3.78 ab
3	32.07±2.86 b	11.8±1.15 a	9.61±0.49 abc	58.20±2.77 b
3.5	22.09±2.56 b	19.2±2.93 a	19.87±4.10 d	100
4	34.01±0.74 bc	22.62±1.36 a	16.27±0.10 bcd	100
4.5	45.44±2.31 c	71.65±4.31 b	17.64±0.12 bcd	100
5	51.01±8.83 c	100	19.88±0.08 cd	100
5.5	100	100	21.81±6.96 d	100
6	100	100	21.71±1.67 d	100
6.5	100	100	19.03±0.05 bcd	100
7	100	100	25.37±6.30 d	100
7.5	100	100	39.32±7.08 e	100
8	100	100	56.99±3.96 f	100
8.5	100	100	78.34±10.24 g	100
9	100	100	100	100
9.5	100	100	100	100
10	100	100	100	100

a 71.65% MGI was detected at 4.5% extract concentration (LOEC, Table 3), while complete inhibition (100%) was observed at 5% extract concentration (MIC, Table 3 and Fig. 2B). For *PAL* a fungistatic effect with a 19.87% MGI was detected at 3.5% extract concentration (LOEC, Table 3) while complete inhibition (100%) was observed at 9% extract concentration (MIC, Table 3 and Fig. 2C). It has been described an increase in phenolic compounds in esca-diseased plants (Amalfitano *et al.*, 2000; Agrelli *et al.*, 2009; Lima *et al.*, 2010). Nevertheless, there are *Vitis vinifera* susceptible varieties to esca disease, which are able to activate phenolic production as a defense

Table 3. The no-observed-adverse-effect concentration (NOAEC, %), lowest-observed-adverse-effect-concentration (LOEC, %) and minimum-inhibitory-concentration (MIC, %) for mycelial growth of four plant pathogens: *Fusarium oxysporum f. sp. radicle-lycopersici* (*FORL*), *Phaeoconiella chlamydospora* (*PCH*), *Phaeoacremonium aleophilum* (*PAL*), and *Sporisorium scitamineum* (*SS*) exposed to phenolic extract of *Aloe vera* industrial residues.

Test species	NOAEC ^a	LOEC ^a	MIC
<i>FORL</i>	<2.5	3	5.5
<i>PCH</i>	<4	4.5	5
<i>PAL</i>	<3	3.5	9
<i>SS</i>	<2.5	3	3.5

^a Dunnett's procedure with $p < 0.05$.

response, but this may be not sufficient to successfully fight the disease (Lima *et al.*, 2010). In this way, the phenolic extract studied in this work offers a new possibility in order to act against these both pathogens, *PCH* and *PAL*.

About the other studied pathogen, *FORL*, a fungistatic effect with a 32.07% MGI was detected at 3% extract concentration (LOEC, Table 3) while complete inhibition (100%) was observed at 5.5% extract concentration (MIC, Table 3 and Fig. 2D). Total phenol accumulations in the host tomato plant induced by the plant pathogen or by applying chemical inducers as salicylic acid and biocontrol agents as *Trichoderma harzianum* play an important role in resistance and defense against *F. oxysporum* (Ojha & Chatterjee, 2012). The current study indicates that applying phenolic extract from *A. vera* industrial residue can be a novel strategy in plant disease management.

The explanation of the antifungal activity of this extract can be the capacity of phenols to form complexes with polysaccharides and proteins of external layers of the fungal cells destabilizing the functions of cell walls and membranes, causing the death of the microorganism (Aguilar *et al.*, 2007). The hydrophilic part of the molecule interacts with the polar part of the membrane, while the hydrophobic benzene ring and the aliphatic side chains are buried in the hydrophobic inner part of the membrane (Viuda-Martos *et al.*, 2008). Furthermore, the hydroxyl group in the formation of hydrogen bonds

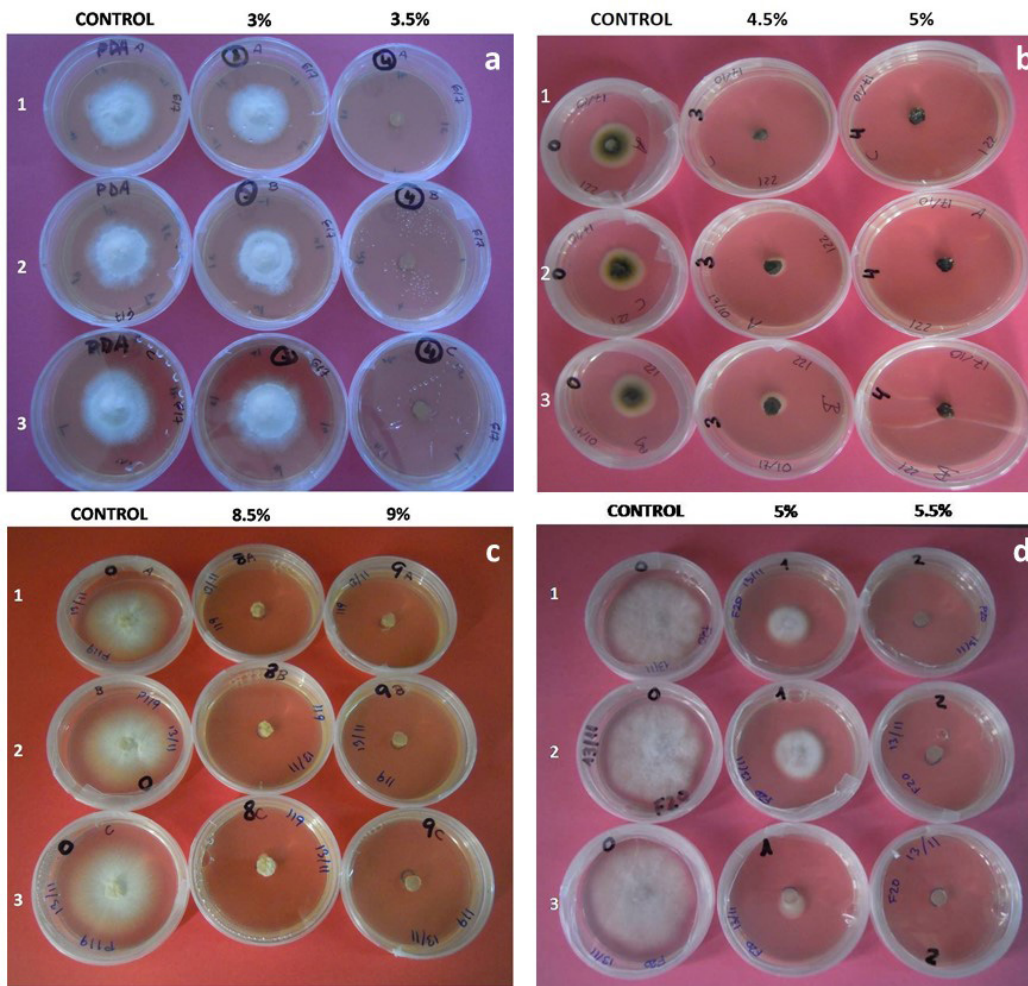


Figure 2. Effect of phenol extract on radial hyphal growth on PDA medium in three replicates against four fungal strains: a) *Sporisorium scitamineum*, 7 days after inoculation; b) *Phaeoconiella chlamydospora*, 14 days after inoculation; c) *Phaeoacremonium aleophilum*, 14 days after inoculation; and d) *Fusarium oxysporum* f. sp. *radicis-lycopersici*, 7 days after inoculation.

and the acidity of these phenolics compounds may have other possible explanations (Cristani *et al.*, 2007). Thus, a factor to be considered is the slightly acidic nature of phenols and the possible relevance of pH effects in regulating the growth of these fungi. Growth may be profoundly affected by a number of physical factors like temperature, pH, light, aeration, pressure, etc. Most fungi generally prefer slightly acidic conditions for their growth (Maharshi & Taker, 2012). To study the possible pH impact over the fungi growth produced by the addition of the phenolic extract to PDA media, the pH of the media at the MICs were determined. PDA media were elaborated at the same MIC determined pH by adding HCl (1N) and the fungi were grown on this medium. Although a slight reduction was detected in the rate of growth (data not shown), in any case there was total inhibition thereof. We have not found any data on the effect of pH over the *in vitro* mycelia growth of *SS*. The growth of *PCH* and *PAL* varies at pH between 4.1

and 8.0 but in any case it results totally inhibited (Valtaud *et al.*, 2009) and it is reported that acidic pH favors growth of all *Fusarium* sp. although the fungus could grow and sporulate under a wide range of pH from 4.0 to 8.0 (Agarwal & Sarbhoj, 1978; Gupta *et al.*, 2010).

In summary, this study has clearly showed potential use of this extract on diseases suppression *in vitro* providing a new use for the *A. vera* industrial residues. The inclusion of such natural products as this phenolic extract in crop protection strategies, as alternative to synthetic fungicides, will help to maintain the balance of agroecosystems and the safety of the harvested products.

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