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CRUDE EXTRACTS OF *Drimys winteri* BARK TO INHIBIT GROWTH OF *Gaeumannomyces graminis* VAR. *tritici*

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ABSTRACT

The aim of this study was to assess the effect of *Drimys winteri* J.R. Forst. & G. Forst. bark and its extracts, obtained sequentially with n-hexane, acetone, and methanol, against *Gaeumannomyces graminis* var. *tritici* (Ggt). Ground bark of *D. winteri* was mixed with potato dextrose agar growth media at concentrations of 250, 500, 1000, 2000, and 4000 mg L^{-1} in Petri plates. Each extract was mixed at concentrations of 100, 200, 400, and 800 mg L^{-1}. Petri plates were inoculated in the center with a 5-mm mycelium disk and were incubated at 24 ± 1 ºC. Daily measurements of mycelium radial growth were taken to determine growth rate and growth inhibition (%). A ground bark concentration of 978 mg L^{-1} was needed to inhibit Ggt growth by 50%, while extracts obtained with n-hexane and acetone only required 198 and 234 mg L^{-1}, respectively. The methanol extract only inhibited Ggt growth by 33% when tested at the highest concentration. In conclusion, ground bark and crude extracts, obtained sequentially with n-hexane, acetone, and methanol, from *D. winteri* bark inhibit Ggt growth when applied *in vitro*. The n-hexane extract showed the highest inhibitory growth activity.

Key words: Wheat take-all, soil borne, plant extracts, Ggt.

INTRODUCTION

Take-all disease caused by the ascomycete fungus *Gaeumannomycete graminis* (Sacc.) von Arx & Olivier var. *tritici* Walker (Ggt) (Wiese, 1987) is among the most serious fungal diseases affecting wheat (*Triticum aestivum* L.) worldwide. This disease is particularly severe in Chilean Andisols with the highest organic matter contents (Campillo *et al*., 2001). This fungus presents a challenging phytopathological problem since it is rarely identified early and is evident only in advanced stages of cultivation (Madariaga and McMahon, 1981). General plant development is affected, and its production is reduced by the depletion of all yield components (Hornby, 1998). Yield losses can be as high as 50% (Wiese, 1987; Agrios, 2004).

Plants affected by this disease exhibit chlorosis, stunted growth, early maturity, formation of whitened spikes, and sterile or hollow kernels (Latorre, 2004). This phytopathogen, which has enzymes that degrade cell walls, destroys much of the plant's root system and basal stem (Dori *et al*., 1995; Agrios, 2004). Partially or totally damaged stems cannot conduct water and nutrients needed for normal plant development and growth (Apablaza, 1999). Affected plants have few roots, which are short, black-brown in color, and dry; dark Ggt hyphae growths are evident on the root tissue (Apablaza, 1999). This fungus can spread from diseased to healthy plants through root contact, the residue in the soil after harvest, soil lodged in agricultural equipment, and in soil water runoff after rain (Cook, 2003; Latorre, 2004). Additionally, ascospores of this pathogen can be dispersed by wind and rain (Latorre, 2004).

Seed disinfection and soil fumigation with diverse antifungal chemicals are usually recommended to control this disease. However, these methods are not satisfactory given their high cost and marginal effectiveness (Hornby, 1998; Apablaza, 1999; Cook, 2003). Various biological agents have been examined to control Ggt (Wong *et al*., 1996; Kim *et al*., 1997; Cook *et al*., 2002), though, none have been determined to be appropriate for a variety of soil conditions and production systems (Capper and Higgins, 1993; Cook, 2003).

Crop rotation with non-susceptible species appears to be the only effective control method to prevent this

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phytopathological problem (Cook, 2003; Cunfer *et al*., 2006). This method is used in Chile, but its effectiveness varies according to climate and soil conditions; it is not necessarily suitable for all technological and economic purposes (Campillo *et al*., 2001). Since there are few non-susceptible alternative crops to rotate with Chilean wheat crops, it is very likely that phytopathological problems will persist and increase (Andrade, 2004). Thus, it is imperative to develop economically viable methods to control this serious disease (Cook, 2003).

In this context, an interesting alternative to explore can be plants as a source of natural fungicides to control pathogens affecting crops. This is based on the fact that synthesis and production of secondary metabolites by plants are a natural plant defense mechanism against pathogen infection (Wink, 2003; Kliebenstein, 2004). Plants possess extensive qualitative and quantitative bioactive molecule variations that are potentially therapeutic (Oksman-Caldentey and Inzé, 2004; Gershenzon and Dudareva, 2007). Research into the biological activity of crude extracts, essential oils, and metabolites obtained from plants has demonstrated a capacity to control various fungal phytopathogens (Isman, 2000; Lazzeri and Manici, 2000; Wedge *et al*., 2000; Apablaza *et al*., 2002; Park *et al*., 2008).

Drimys winteri J.R Forst. & G. Forst. is an arboreal species of the Winteraceae family, and its metabolites have demonstrated important biological activity against microorganisms and insects (Muñoz *et al*., 2001; Zapata *et al*., 2009). Its bark contains drimane-type sesquiterpenes (Rodríguez *et al*., 2005), as well as abundant essential oils (Barrero *et al*., 2000).

This research study sought to evaluate the *in vitro* activity of *D. winteri* bark and its extracts, sequentially extracted with n-hexane, acetone, and methanol, on the growth of Ggt mycelium to establish the groundwork for alternatives to control phytopathogens affecting wheat crops.

MATERIALS AND METHODS

Plant material

Stem bark of adult *D. winteri* trees was collected during March 2007 (Ñuble Province, Chile). A voucher specimen has been placed in the Herbarium of the Faculty of Agronomy, Universidad de Concepción, Chile. Fresh bark was carefully and extensively washed with distilled water to remove any debris. It was then oven-dried (FD 115 Binder, Tuttlingen, Germany) at 35 °C. Once dried, stem bark was ground to less than 2.0 mm (MF10, IKA, Staufen, Germany), and stored at 4 °C in hermetic plastic containers.

Extract preparation

Dried ground *D. winteri* bark (20 g) was sequentially extracted (from low to high polarity) at room temperature for 48 h with n-hexane (400 mL), 24 h with acetone (400 mL), and 48 h with methanol (400 mL). Each extract was dried in a rotary evaporator at 35° (R-210A, Buchi, Flawil, Switzerland). Extracts were then stored at 4 °C until used in experiments. Extraction yields were: n-hexane = 16.4%, acetone = 10.9% and methanol = 3.6%.

Pathogen

Gaeumannomyces graminis var. *tritici* was provided by Dr. Orlando Andrade from the Instituto de Investigaciones Agropecuarias INIA Carillanca, Chile. The pathogen grew in potato dextrose agar (PDA) medium and maintained in a growth chamber at 24 ± 1 °C during the experiments.

Experiment with ground bark

In order to determine whether *D. winteri* ground bark would have an inhibitory effect on Ggt growth *in vitro*, growth media were prepared by mixing crushed bark with PDA at concentrations of 250, 500, 1000, 2000, and 4000 mg L^{-1}. The mixture was sterilized in an autoclave for 15 min at 121 °C. Medium growths were poured into sterile 100 mm diameter Petri plates, subsequently were inoculated in the center with a 5 mm diameter disk of actively growing Ggt mycelium. Plates were incubated at 24 ± 1 °C in all the experiments. Five replicates were established for treatment, including a control (PDA alone).

Experiments with extracts

Experiments were conducted with each of the extracts (n-hexane, acetone, and methanol). In each experiment, extract was added to the growth medium (PDA) at concentrations of 100, 200, 400, and 800 mg L^{-1}. Bioassays were developed with 100 mm diameter Petri plates. To ensure homogeneous dispersion in the growth medium, extracts were resuspended in ethanol at 1% of total PDA volume before the experiments. Petri plates remained in the incubation chamber for 24 h to facilitate ethanol evaporation from the medium and avoid subsequent potential toxic effects on Ggt. Plates were inoculated in the center with a 5 mm diameter disk of actively growing Ggt mycelium and incubated at 24 ± 1 °C. Five replicates were established for each treatment, including an absolute control (PDA alone) and a partial control (PDA + 1% ethanol).

Evaluation parameters

The four axes of mycelial radial growth in all the experiments were measured daily until the control reached the edge of the plate. Growth rates for each treatment were determined from these measurements.

Mycelial growth inhibition (MGI) index was determined in each experiment on the eighth day of assessment as follows: MGI = [(MGC-MGT)/MGC] x 100, where MGC is mycelial growth (cm) in the control, and MGT is growth of mycelium treated with ground bark or extracts. In experiments with extracts, MGI was calculated with the partial control. We also determined the ground bark or extract concentration needed to inhibit Ggt mycelial growth at 50% (MGI_{50}) and 90% (MGI_{90}).

Data analysis

The experimental design for all experiments was completely randomized. Growth rates (simple regression slopes) were compared by the Tukey test. MGI data were subjected to ANOVA, and mean comparison was performed by the Tukey test ($P \leq 0.05$) to determine significant differences among concentrations. MGI_{50} and MGI_{90} were determined by linear regression analysis with percentage MGI to the logarithm of the applied concentration (Jin *et al*., 2004). We used Statgraphics Plus Version 5.0 software (STSC, 1987) for data analysis.

RESULTS

Effects of ground bark on Ggt growth

According to the results obtained in this study, *D. winteri* bark exhibited a high inhibitive capacity on Ggt mycelial growth because bark concentration in PDA increased (Tables 1 and 2). The growth rate was significantly lower than the control when growth medium was amended with 1000 mg L^{-1} ground bark; similarly, growth rate decreased significantly from 1000 to 2000 mg L^{-1} bark. The growth rate did not vary significantly when concentrations of 2000 and 4000 mg L^{-1} ground bark were applied (Table 1).

There was virtually no Ggt growth in PDA media treated with 2000 and 4000 mg L^{-1} ground bark. Cumulative 8 d growth in the medium treated with 2000 mg L^{-1} bark barely reached 0.14 cm, and there was no growth with 4000 mg L^{-1}. Growth (only PDA) during this same time period totaled 3.8 cm, indicating a 96.26% growth inhibition for the 2000 mg L^{-1} concentration. Inhibiting Ggt growth by 50% required a 978 mg L^{-1} bark concentration, whereas 90% required a 2760 mg L^{-1} concentration (Table 2).

Effects of extracts on Ggt growth

Extracts, sequentially obtained with n-hexane, acetone, and methanol, significantly inhibited Ggt mycelial growth. Moreover, extracts with n-hexane and acetone exhibited higher suppressive activity. When extract concentration in PDA medium was increased, Ggt growth decreased (Tables 3 and 4). When PDA growth medium was treated with extracts obtained with n-hexane or acetone, the

Table 1. Linear regressions and growth rates of *Gaeumannomyces graminis* var. *tritici* growing in PDA medium amended with *Drimys winteri* ground bark.

Concentrations mg L^{-1}	Linear regression equation[1]	Coefficient of determination	Growth rate cm d^{-1}
Control	y = -0.27 + 0.53x	0.99	0.53a
250	y = -0.41 + 0.51x	0.98	0.51a
500	y = -0.47 + 0.50x	0.98	0.50a
1000	y = -0.58 + 0.38x	0.97	0.38b
2000	y = -0.04 + 0.02x	0.60	0.02c
4000	y = 0	0.00	0.00c

Different letters in column denote differences between treatment slopes according to Tukey test ($P \leq 0.05$).
[1]Linear adjustment, where y: mycelial growth (cm), x: days of evaluation; PDA: potato dextrose agar.

Table 2. Mycelial growth inhibition (MGI) index of *Gaeumannomyces graminis* var. *tritici* growing in PDA medium amended with *Drimys winteri* ground bark.

Concentrations mg L^{-1}	MGI (%)	MGI_{50} CL (95%)	MGI_{90} CL (95%)
250	8.45 ± 0.89a		
500	12.05 ± 1.97a		
1000	37.40 ± 1.60b	978 (843 - 1134)	2760 (2229 - 3418)
2000	96.26 ± 2.95c		
4000	100.00 ± 0.0c		

Data expressed as mean ± standard error of five replicates. Different letters in column indicate that values differ according to Tukey test ($P \leq 0.05$).
MGI_{50} and MGI_{90} values expressed in mg L^{-1}. CL: confidence limits; PDA: potato dextrose agar.

Table 3. Linear regressions and growth rates of *Gaeumannomyces graminis* var. *tritici* growing in PDA medium amended with extracts of *Drimys winteri* bark.

Concentrations mg L^{-1}	Linear regression equation[1]	Coefficient of determination	Growth rate cm d^{-1}
n-Hexane extract			
Control PDA	y = -0.01 + 0.50x	0.98	0.50a
Control ethanol	y = -0.19 + 0.47x	0.99	0.47a
100	y = -0.58 + 0.37x	0.96	0.37b
200	y = -0.49 + 0.28x	0.94	0.28bc
400	y = -0.23 + 0.14x	0.92	0.14c
800	y = -0.03 + 0.01x	0.87	0.01d
Acetone extract			
Control PDA	y = -0.39 + 0.52x	0.99	0.52a
Control ethanol	y = -0.33 + 0.51x	0.99	0.51a
100	y = -0.42 + 0.40x	0.99	0.40b
200	y = -0.35 + 0.34x	0.99	0.34b
400	y = -0.26 + 0.14x	0.90	0.14c
800	y = -0.14 + 0.06x	0.85	0.06c
Methanol extract			
Control PDA	y = -0.39 + 0.53x	0.99	0.53a
Control ethanol	y = -0.33 + 0.51x	0.99	0.51a
100	y = -0.23 + 0.49x	0.99	0.49a
200	y = -0.15 + 0.47x	0.99	0.47a
400	y = -0.32 + 0.42x	0.99	0.42ab
800	y = -0.39 + 0.35x	0.88	0.35b

Different letters in column denote differences between treatment slopes according to Tukey test ($P \leq 0.05$).
[1]Linear adjustment, where y: mycelial growth (cm), x: days of evaluation; PDA: potato dextrose agar.

growth rate was significantly lower than the controls, even at the lowest evaluated concentration (100 mg L^{-1}). The methanol extract only significantly reduced Ggt growth rate when applied at 800 mg L^{-1}. Control (PDA or ethanol) growth rates were not significantly different (Table 3).

N-hexane extract caused an inhibition halo of 30.19% when applied at a 100 mg L^{-1} concentration in PDA medium, but inhibition increased to 97.84% at 800 mg L^{-1}. Similar rates were observed with the acetone extract which inhibited growth by 25.19% at a 100 mg L^{-1} concentration,

Table 4. Mycelial growth inhibition (MGI) index of *Gaeumannomyces graminis* var. *tritici* growing in PDA medium amended with extracts of *Drimys winteri* bark.

Concentrations mg L^{-1}	Growth inhibition index (%)		
	n-Hexane	Acetone	Methanol
100	30.19 ± 2.51a	25.19 ± 2.50a	3.20 ± 3.20a
200	47.03 ± 3.35b	37.21 ± 2.22b	3.13 ± 1.32a
400	71.43 ± 3.12c	72.87 ± 2.43c	18.21 ± 1.75b
800	97.84 ± 2.15d	89.28 ± 2.88d	33.46 ± 1.66c
MGI_{50}	198	234	nc
CL (95%)	(178-219)	(209-261)	
MGI_{90}	672	792	nc
CL (95%)	(584-772)	(663-944)	

Data expressed as mean ± standard error of five replicates. Different letters in column indicate significant differences according to Tukey test ($P \leq 0.05$).
MGI_{50}, MGI_{90}: values expressed in mg L^{-1}. CL: confidence limits. nc: not calculated.
PDA: potato dextrose agar.

and 89.28% at 800 mg L^{-1}. However, this trend was not observed with methanol extract which only achieved a growth inhibition of 3.20% when applied at 100 mg L^{-1} and 33.46% at 800 mg L^{-1}. According to our data, a 198 mg L^{-1} concentration of n-hexane extract was necessary to inhibit Ggt growth by 50% and 672 mg L^{-1} to reach 90%. To achieve similar results with the acetone extract, concentrations of 234 and 792 mg L^{-1}, respectively, were required (Table 4).

DISCUSSION

The suppression of Ggt growth is quite possible with plant compounds, such as flavonoids (Weidenbörner and Jha, 1997), saponins from the *Quillaja saponaria* Molina tree (Vissuetti, 2004), and saponins (avenacin) in oat roots (Crombie, 1999). These plant compounds, as well as sesquiterpenes from *Artemisia sieversiana* Ehrh. (Tang *et al*., 2000), have demonstrated suppressive Ggt growth activity.

Promising results from the study of *D. winteri* bark on Ggt indicate that the tree's compounds have high fungicide activity (Monsálvez *et al*., 2010). Without extraction, *D. winteri* ground bark has growth inhibitory effects on Ggt at very low concentrations (< 0.4%). These results are consistent with other studies in which the incidence of soil pathogens with was reduced tree bark, although those concentrations were higher than in this study (Spencer and Benson, 1982; Hoitink and Fahy, 1986; Huang and Kuhlman, 1991; Yu and Komada, 1999). It is important to consider the possible phytotoxic effect on cultivation when crushed bark is added to growth substrates (Hoitink and Fahy, 1986). The low bark concentration used to stop Ggt growth in this study should not be a serious problem. Nonetheless, phytotoxic activity and its actual *in vivo* effect should be considered in future trials.

The advantage of using *D. winteri* ground bark to reduce Ggt incidence in wheat crops and other phytopathogenic soil is the low cost of processing the ground bark, thus making it a sustainable agricultural production system. The bark's protective effects may be lasting, since the release of active compounds into the growth substrate depends on decomposition (Anaya, 2006).

The low polarity compounds present in *D. winteri* bark could be responsible for high growth inhibition activity against Ggt. Since bark was extracted sequentially with n-hexane, acetone, and methanol (from low to high polarity), it showed decreasing activity. Activity against the fungus observed in this study using extracts of low polarity is not unique to this species of the *Drimys* genus; extracts of low polarity obtained from *Drimys brasiliensis* Miers bark also have high antifungal activity (Malheiros *et al*., 2005).

Sesquiterpenes are the low polarity compounds abundant in *D. winteri* bark (Jansen and Groot, 2004). These compounds represent a large group possessing a broad spectrum of biological activity, and they apparently play an important role in the plant defense mechanism (Fraga, 2002). The main sesquiterpenes identified in *D. winteri* extracts obtained with n-hexane are of the drimane type (Rodríguez *et al*., 2005; Zapata *et al*., 2009). Among these drimanes, polygodial has the highest concentration in *D. winteri* dried bark (8.15%) (Rodríguez *et al*., 2005).

However, the role of polygodial, or other sesquiterpenes of the drimane type present in *D. winteri* bark in the observed activity on Ggt must still be determined. Given the abundant research on antifungal activity, we believe that polygodial or other sesquiterpenes of the drimane type are involved. Polygodial has shown potent antifungal activity against yeast fungi: *Candida albicans*, *C. utilis*, *C. krusei*, *Cryptococcus neoformans*, *Saccharomyces cerevisiae*, and *Pityrosporum ovale* (Kubo and Taniguchi, 1988; Himejima and Kubo, 1993; Kubo 1993; Lee *et al*., 1999). Polygodial is also capable of inhibiting growth of the filamentous fungi *Epidermophyton floccosum*, *Trichophyton mentagrophytes*, *T. rubrum*, and *Penicillium marneffei* (Lee *et al*., 1999; Malheiros *et al*., 2005).

In light of the results obtained in this preliminary study, we believe it is very important to further investigate compounds with antifungal activity, as well as to determine their effectiveness both *in vivo* and in the field. Possible strategies for extract formulation and implementation in the field must be further explored.

CONCLUSIONS

Ground bark and its crude extracts obtained sequentially with n-hexane, acetone, and methanol from *D. winteri* bark,inhibit the growth of wheat take-all (*Gaeumannomyces graminis* var. *tritici*) when applied *in vitro*. The n-hexane extract showed the highest inhibitory growth activity.

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RESUMEN

Inhibición del crecimiento de *Gaeumannomyces graminis* var. *tritici* empleando extractos obtenidos de corteza de *Drimys winteri.* El objetivo de esta investigación fue evaluar la actividad de la corteza de *Drimys winteri* J.R Forst. & G. Forst. y sus extractos obtenidos secuencialmente con n-hexano, acetona y metanol sobre el crecimiento *in vitro* de *Gaeumannomyces graminis* var. *tritici* (Ggt). Con este propósito se mezclaron en placas Petri, corteza molida de *D. winteri* con medio de crecimiento agar papa dextrosa a concentraciones de 250, 500, 1000, 2000 y 4000 mg L^{-1}. Cada extracto fue mezclado a concentraciones de 100, 200, 400 y 800 mg L^{-1}. Las placas se inocularon en su centro con un disco de 5 mm de micelio de Ggt y se incubaron a 24 ± 1 ºC. Diariamente se midió el crecimiento radial del micelio, se determinó velocidad de crecimiento e índice de inhibición del crecimiento (%). Para inhibir el crecimiento de Ggt en un 50% fue necesaria una concentración de 978 mg L^{-1} de corteza molida. En cambio, cuando se emplearon extractos obtenidos con n-hexano y acetona sólo fueron necesarios 198 y 234 mg L^{-1}, respectivamente. El extracto obtenido con metanol sólo alcanzó a inhibir el crecimiento de Ggt en un 33% a la máxima concentración estudiada. En conclusión, la corteza triturada de *D. winteri* y sus extractos crudos obtenidos secuencialmente con n-hexano, acetona y metanol inhiben el crecimiento *in vitro* de Ggt. El extracto obtenido con n-hexano demostró la mayor actividad inhibitoria del crecimiento.

Palabras clave: mal del pie, hongos del suelo, extractos de plantas, Ggt.

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