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Antifungal activity of wheat root exudate extracts on *Gaeumannomyces graminis* var. *tritici* growth

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Abstract

Wheat (*Triticum aestivum* L.) is known for its ability to produce and release allelopathic compounds, which have potential for controlling weeds and diseases. Previous reports have shown the fungitoxic effects of allelochemicals present in wheat. Thus, these compounds can be exuded by roots to protect the tissues directly affected by *Gaeumannomyces graminis* var. *tritici* (*Ggt*) fungus that causes wheat take-all disease. The aim of this research was to evaluate *in vitro* the allelopathic effect of root exudate extracts from four Chilean wheat cultivars on *Ggt* growth. Root exudates were released from wheat seedlings to a sterile culture medium without nutrients. Afterward, the exudates in the culture medium were separated by liquid-liquid extraction using ethyl acetate. Eight different concentrations were tested for each cultivar. The results showed that the degree to which the extracts strongly inhibit the phytopathogen growth is highly dependent on both the concentration and the cultivar. The root extract of the Domo cultivar was significantly active against *Ggt* (MIC=0.36 mg mL^{-1}). IC_{50} and MIC values obtained for Dollinco and Domo root exudate extracts showed toxicity to *Ggt*. These findings may be considered in future studies related to the use of allelopathic potential as a selection factor in order to reduce the yield losses caused by various take-all diseases, as an alternative to chemical controls.

Key words: wheat cultivars, take-all, toxicity, allelopathy, Chilean wheat.

1. Introduction

Wheat (*Triticum aestivum* L.) is one of the most important cereal crops in the world. The main root disease of wheat (*Triticum aestivum* L.) worldwide is Wheat Take-All, which is caused by *Gaeumannomyces graminis* var. *tritici* (*Ggt*) (Freeman and Ward, 2004). In southern Chile, take-all diseases strongly affect wheat production, especially in La Araucanía and Los Lagos regions.

Although *Ggt* research has been extensive, current chemical methods to control take-all disease are not effective. Current reports show the effectiveness chemical products applied in seed disinfection in decreasing the damage caused by wheat take-all under artificial inoculation conditions (Andrade, 2004). Common methods used in the field to prevent and decrease take-all damage include the suitable management of soil fertility, weed remains, crop debris and crop rotation. Angus *et al*. (1998) evaluated both cultural and chemical methods to control wheat take-all and reported successful control methods, such as crop rotation (i.e., growing wheat after a brassica break crop) or a long fallow. Both methods increased the yield up to 72%, compared with a successive wheat crop. According to the authors, the limitation of these chemical methods is that they caused the suppression of natural antagonists of *Ggt*. This suppression is associated with reinfection at higher levels than before the use of these chemical methods. Even if an effective chemical against take-all could be developed, its long-term use would be compromised as a result of resistance/insensitivity or other factors that might reduce its effectiveness (Freeman and Ward, 2004). The absence of effective alternatives to control take-all disease and the massive use of chemicals in wheat production systems have led to several studies related to Ggt and the use of innocuous control techniques, with the aim to reduce the incidence of take-all the disease and to avoid the chemical alteration of the natural environment. These studies include biological control of take-all disease through the use of microbial antagonists (Duffy *et al*., 1996; Weller *et al*., 2007) and allelopathic compounds (Wilkes *et al*., 1999).

Plants exude a variety of substances through the surface of their roots. Some of the compounds released by certain kinds of plants seem to have inhibitory activity against certain pathogens (Agrios, 2005). Wheat as well as other cereals and wild gramineae species produce allelopathic compounds; the most abundant type of such compounds is cyclic hydroxamic acids (Niemeyer *et al*., 1992; Niemeyer and Pérez, 1995). Wu *et al*. (2000a) suggest that allelochemicals are differentially distributed in wheat seedling shoots and roots but occur in higher amounts in roots. In research reports on wheat extracts, 2.4-dihydroxy-7-methoxy-2H-1.4-benzoxazin-3(4H)-one (DIMBOA) is the most abundant aglycone, while its desmethoxy analogue 2.4-dihydroxy-2H-1.4-benzoxazin-3(4H)-one (DIBOA) is found or distributed in smaller proportions (Niemeyer, 1988a; Hashimoto and Shudo, 1996; Wu *et al*., 2000b). In the plant itself, DIMBOA and DIBOA are stabilized as (2*R*)-2-β-D-glucosides. The most toxic aglycones are produced in response to tissue damage or pathogen attack (Friebe *et al*., 1998). Wilkes *et al*. (1999) indicated that root extracts from triticale (*Triticosecale* spp.) and rye (*Secale cereale*) inhibit the growth of *Ggt*. Furthermore, they reported that similar extracts from two wheat cultivars display different inhibitory effects. Indeed, the authors showed that the addition of hydroxamic acid individually in the culture medium, DIBOA, inhibits fungal growth at concentrations as low as 0.5 mM. DIMBOA displays an inhibitory effect at double

the concentration of DIBOA. DIMBOA is the main hydroxamic acid present in wheat roots, and these aglycones are responsible for the inhibition of fungal growth. The differential allelopathic potential of Chilean wheat cultivars on different weeds associated with wheat production has been evaluated (Bensch *et al*., 2007; 2009). These studies showed that wheat cultivars have different allelopathic potential. In most cases, weeds were inhibited by root exudates, but in some cases, a stimulatory effect was observed. These findings motivated us to determine the effect of exudates released by wheat roots on a pathogenic plant fungus that attacks the root directly, such as take-all fungus. These exudates can be used by the plant naturally to prevent fungal infection. The objective of this research was to evaluate the *in vitro* allelopathic effect of root exudate extracts of wheat seedlings of four Chilean cultivars on *Ggt* growth.

2. Materials and methods

2.1 Fungal strain and culture conditions

G. graminis var. *tritici* was isolated from wheat roots with take-all disease and purified by hyphal isolation. The isolated fungus was maintained on potato-dextrose-agar (PDA, Difco) medium at 25 ± 1ºC in the dark and kept on PDA slants stored at 4 ºC.

2.2 Wheat seedling growth

On the basis of previous experiments, four wheat cultivars were selected in order to study the inhibitory effect of wheat root exudates on *Lolium rigidum* root growth (Bensch *et al*., 2007). Three ranges of inhibition were used for reference: low (Domo, 24%), intermediate (Otto, 48% and Dollinco, 49%) and high (Tukán, 80%). Wheat seeds were obtained from the Instituto de Investigaciones Agropecuarias (INIA-Carillanca). Seeds of 4 wheat cultivars (500 g) were disinfected with an aqueous solution of 1.8% benomyl + 2.7% captan (Benlate 50 PM + Captan 80 WP) and then rinsed 4 times with sterile distilled water. Afterward, the seeds were immersed in ethanol (70% v/v) for a few minutes and then rinsed 4 times with sterile distilled water. Finally, the seeds were immersed in sodium hypochlorite (2.5% v/v) solution and then rinsed 5 times with sterile distilled water. Disinfected seeds were pre-germinated during 48 h at 25 ºC in the dark.

The methodology reported by Wu *et al.* (2000a) was modified to sow pre-germinated wheat seeds. Eight thousand pre-germinated wheat seeds (surface-sterilized) of each cultivar were uniformly selected and aseptically sown (with the embryo up) on 200 glass bottles (500 mL) with 60 mL of 3% water agar autoclaved. The bottles were wrapped with Parafilm and placed in a growth chamber with a daily photoperiod 16:8 (L:D) for 14 days at 20 ºC ± 1 in order to allow the release of exudates by wheat seedlings into the culture medium (without nutrients). After the 14-day-old wheat seedlings were removed manually from the nutrient-free agar medium, the agar solution was collected and conserved in an airtight container at 4 ºC until the extraction procedure.

2.3 Extraction of total root exudates from agar medium

Root exudates released in 200 replicates of agar cultures (6 L approx.) were extracted and disposed into a glass separator funnel in portions of 2 L with 1.8 L of ethyl acetate (added in 3 portions of 600 mL each). The organic phase (exudates and ethyl acetate) was concentrated to dryness under reduced pressure in a rotary evaporator at 45 °C. The obtained extracts were transferred to amber glass vials and stored at 4 °C.

2.4 Wheat allelopathic effect evaluation

Wheat exudate extracts were dissolved in ethanol (Merck) to a final volume of 50 mL. Extracts were applied in eight different concentrations on sterilized Petri dishes (50 x 15 mm) under sterile conditions. After removing the solvent (ethanol) for 1 h at room temperature in the laminar chamber, 2 mL of 3.9% potato-dextrose agar (PDA) medium with streptomycin (1 mL L^{-1}) was deposited in the Petri dish with the extract. Exudates and the PDA medium were left for 48 h at 5 ° C to allow the diffusion of extract into the medium. Then, mycelia disks taken out from *Ggt* fresh cultures (2 mm diameter) were placed in the center of Petri dishes prepared with the medium and extract. Fungi were incubated at 25 ºC until the control reached the edge of the plate.

The treatments comprised the exudate extracts of four cultivars (Otto, Domo, Tukan and Dollinco) evaluated in eight concentrations, expressed in mg of extract per mL of culture medium (0.05, 0.15, 0.25, 0.3, 0.35, 0.5, 0.75 and 1.00 mg mL^{-1}). The evaluated concentrations were established by means of a preliminary test based on the maximum concentration of total extracts used in tests to evaluate biological properties reported by Miller *et al*. (1988). Two control plates were made to compare the growth in bioassays. Control 1 consisted of Petri dishes containing the PDA medium + ethanol (in the same quantity of the extract and removed in the chamber), and control 2 consisted of Petri dishes containing the PDA medium + sterile distilled water (in the same extract quantity and removed in the chamber); both controls did not contain extract and were inoculated with *Ggt*. Mycelial growth was measured using an optical microscope (Japan Optical), and data were expressed as the inhibition percentage in relation to control 1.

2.5 IC_{50} and MIC determination

To compare the allelopathic potential of the wheat cultivar extracts, the half maximal inhibitory concentration (IC_{50}) and minimum inhibitory concentration (MIC) values of the total extracts obtained from the root exudates of wheat against *Ggt* were calculated by means of an analysis of the dose-response relationship.

2.6 Experimental design and data analysis

The Petri dishes were placed or distributed following a completely random design, with three replications for each treatment. The treatments were eight different concentrations of root exudate extracts for each cultivar. The percentage values of mycelial growth in relation to control plates treated by different concentrations of exudate extracts and the IC_{50} and MIC values were compared using a Kruskal-Wallis test (a nonparametric one-way analysis) (Sokal and Rohlf, 1995), and the groups were separated using the Conover-Inman test ($P < 0.05$) (Conover 1999). IC_{50} and MIC values were calculated by a regression analysis followed by an ANOVA to establish the relationship between the variables (Quinn and Keough, 2002). The contribution of each coefficient to the regression model was tested using the t-student test ($P < 0.05$) (Sokal and Rohlf, 1995).

3. Results and discussion

Wheat cultivars released different amounts of root exudate extracts (Table 1). Dollinco released the highest amount of exudates. However, this value may not be representative of the compound amount actually synthesized by the plants and/or stored in the root. Wu *et al.* (2000b) reported that the amounts of compounds exuded by wheat seedlings into the agar growth me-

dium are not proportional to the amounts of allelochemicals in the roots, because a plant can retain them and not exude them into the agar medium. These compounds also have been detected in whole grain wheat samples (Hanhineva *et al*., 2011). These results could indicate that the wheat seeds not only stored nutrients used in the germination process but also stored compounds of defense against pathogens.

Table 1. Yield of total root exudates extract of wheat, obtained from 8,000 seedlings.

Cultivar	Yield (mg)
Dollinco	187
Tukán	180
Otto	168
Domo	120

Although the amount of extract exuded by the wheat cultivars tested can be considered an important factor in evaluating the allelopathic potential, it is not the only factor influencing the effect. The allelopathic sensitivity of receptor species to allelochemicals is also related to the type and concentration of allelochemicals in root exudates or their combination. This sensitivity would be correlated with the genotype of each cultivar (Hashimoto and Shudo, 1996; Wu *et al*., 2000a; 2000b; Einhellig, 1996).

The effect of four extracts of wheat root exudates on the mycelial growth of *Ggt* evaluated at a standard concentration (0.25 mg mL^{-1}) was significantly different between the extracts ($P < 0.05$). The highest activity was found in Dollinco (64%), followed by Tukán (53%), Domo (43%) and Otto (17%) (Figure 1).

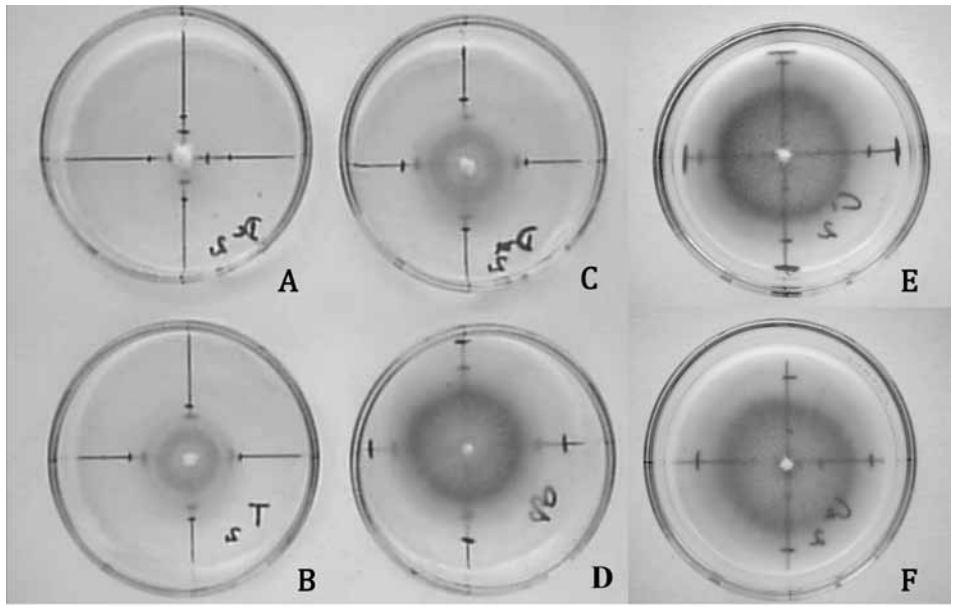

Figure 1. Mycelial growth of *Gaeumannomyces graminis* var. *tritici* in potato-dextrose-agar (PDA) enriched with 0.25 mg mL^{-1} of total extract from root exudates of wheat (*Triticum aestivum* L.). Cultivars: Dollinco (**A**), Tukán (**B**), Domo (**C**) y Otto (**D**). Controls: control 1 (**E**) and control 2 (**F**).

The effect of the concentration of the root exudates of the four wheat cultivars on *Ggt* growth is shown in the Figure 2. In the range of concentrations tested, the extracts of cultivars Tukán and Dollinco showed no significant difference ($P > 0.05$) in *Ggt* growth, except at 0.25 mg mL^{-1}. The inhibition of *Ggt* mycelial growth was highly dependent on the extract concentration and wheat cultivar. These results are consistent with reports indicating that the concentration and the compound nature strongly influence the allelopathic inhibition (Hashimoto and Shudo, 1996; Wu *et al*., 2000a, 2000b; Einhelling, 1995).

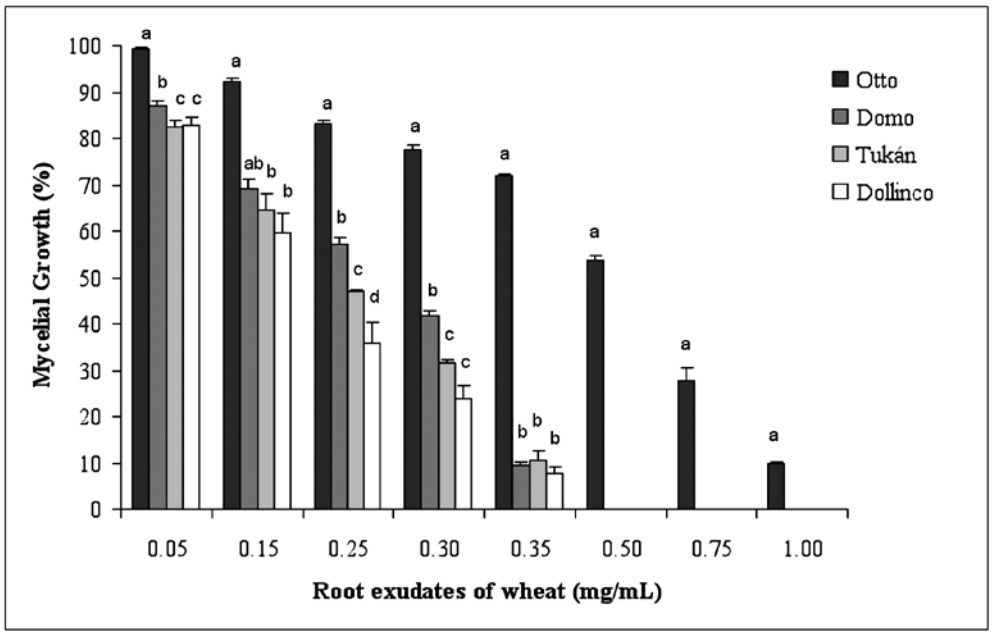

Figure 2. Effect of root exudates concentration of four cultivars of wheat (*Triticum aestivum* L.) on mycelial growth of *Gaeumannomyces graminis* var. *tritici* (% ± SE), grown on potato dextrose agar (PDA) at 25ºC ±1 (N = 3). Growth was expressed as % of growth compared to the control 1. Bars within a strain with the same letter are not significantly different according to the Kruskal-Wallis test ($p > 0.05$) followed by Conover-Inman test.

The chemical nature of substances present in the extracts was not investigated. However, the literature reported a variety of chemical compounds (Mathiasen, 2004) and proteins (Nóbrega *et al.*, 2005) that can be exuded by plant roots and that may function to repress the growth of root pathogenic fungi. For example, Mathiasen (2004) identifies DIMBOA as the most active compound in wheat exudates. The exudation of this compound has been highly dependent on the variety, which indicates that the DIMBOA exudation process is determined by genetic factors (Wu, 2005). This could explain the differences found in the four wheat cultivars evaluated in this study. According to Wu *et al.* (2003), the linkage analysis of genetic markers and the quantitative trait loci may improve genetic input gains for the allelopathic activity through marker-assisted selection in wheat breeding. Other acids reported by Wu (2005) and considered to be allelopathic

compounds have been found in root exudates but with less potential for inhibition in relation to DIMBOA. These compounds include *p*-hydroxybenzoic, vanillic, *p*-coumaric, cis-ferulic, trans-coumaric, trans-ferulic and syringic acids. Martyniuk *et al*. (2006) evaluated *in vitro* antifungal activity of the benzoxazolinones benzoxazolin-2(3H)-one (BOA) and 6-methoxybenzoxazolin-2(3H)-one (MBOA) against *Cephalosporium gramineum*, *Ggt*, and *Fusarium culmorum*. *Ggt* displayed the highest sensitivity to both compounds, and *F. culmorum* was the most resistant strain. Stochmal *et al.* (2006) reported significant differences between old and new varieties of wheat. These authors showed that the concentrations of DIBOA and its derivatives were significantly lower in old accessions. Considering the differences found among different wheat cultivars, the use of allelopathy or allelochemicals released by wheat plants could be used as a selection factor for cultivars that are resistant to take-all disease. However, Niemeyer (2009) explained that the efficient use of benzoxazinoid hydroxamic acids as a resistance factor is limited by the inability to selectively increase their levels at the plant growth stage and in the plant tissues where they are mostly needed for a given pest.

Table 2. Half maximal inhibitory concentration (IC_{50}) and minimum inhibitory concentration (MIC) values of total extracts obtained from root exudates of wheat against the plant pathogenic fungus *Gaeumannomyces graminis* var. *tritici.*

Cultivar	IC_{50}	MIC
	mg mL^{-1}	
Otto	0.56 ± 0.01a	1.07 ± 0.02a
Domo	0.28 ± 0.01b	0.36 ± 0.01c
Tukán	0.24 ± 0.02c	0.39 ± 0.02b
Dollinco	0.19 ± 0.02d	0.39 ± 0.01b

Letters indicate statistical differences ($p \leq 0.05$) between treatments in the same column according to the Kruskal-Wallis test followed by the Conover-Inman test.

IC_{50} and MIC values of total extracts from wheat exudates against the plant pathogenic fungus are shown in Table 2. Values above IC_{50} in Domo and Tukán cultivars showed a marked inhibition effect, with small increases in the concentration of extracts. The results indicated that the effect of the concentration of the extracts on mycelial growth of the fungus was not linear. As indicated by the IC_{50} values, Dollinco cultivar was the most active of the evaluated cultivars. These results, together with the higher amount of extract obtained from the Dollinco cultivar, indicate that the Dollinco cultivar has the highest allelopathic potential. The obtained MIC values show that Domo exudates are the most active on the *Ggt* fungal growth. In contrast, Otto exudates had a much lower inhibitory effect than the other cultivars. In fact, their MIC was at a value close to the MIC sum of all the other cultivars.

4. Conclusions

Extracts of wheat root exudates released by Otto, Domo, Dollinco and Tukán cultivars into an agar medium without nutrients had an inhibitory effect on *Gaeumannomyces graminis* var. *tritici* growth. This allelopathic effect was highly dependent on the extract concentration and the wheat cultivar.

According to half maximal inhibitory concentration values, Dollinco and Otto were the cultivars with the highest and the lowest allelopathic potential, respectively, for the phytopathogenic fungus growth. Nevertheless, Domo was the cultivar with the highest toxicity according to the minimum inhibitory concentration. In contrast, the Otto exudate extract was the least inhibitory to the fungal growth. The toxicity shown by the total extracts of the root exudates of Domo, Tukán and especially Dollinco wheat cultivars on *G. graminis* var. *tritici* growth could be considered for future studies focused on the use of allelopathic

potential as a selection factor. The use of allelopathic potential may lead to a decrease in yield losses caused by take-all disease without chemically altering the environment.

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