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RESEARCH

Effect of saponins of *Quillaja saponaria* extracts in combination with *Pseudomonas protegens* to control *Gaeumannomyces graminis* var. *tritici* in wheat

Jonathan A. González-Castillo[1], Tamara P. Quezada-D'Angelo[1], Gonzalo I. Silva-Aguayo[1], and Ernesto A. Moya-Elizondo[1*]

[1]Universidad de Concepción, Facultad de Agronomía, Av. Vicente Méndez 595, Chillán, Chile. *Corresponding author (emoya@udec.cl).

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ABSTRACT

Extracts rich in saponins from *Quillaja saponaria* Mol. and populations of rhizobacteria from the genus *Pseudomonas*, which produce antimicrobial compounds, have been associated with reduction of the fungus *Gaeumannomyces graminis* var. *tritici*, the causal agent of take-all disease, which is responsible for severe loss of wheat (*Triticum aestivum* L.) crops worldwide. However, there is a limited background on the interaction between these bacteria and natural triterpenoids. The aim of this research was to assess the effect of saponin rich extract on *Pseudomonas protegens* strains 2,4-diacetylphloroglucinol-producers under *in vitro* and *in plant* conditions and determining the synergistic effect to be used together to control *G. graminis* var. *tritici* in wheat plants. We determined that 8% and 90% of saponins rich *Q. saponaria* extracts have a differential effect on *P. protegens* according to their purity ($P \leq 0.05$). On wheat seedlings, quillaja extract with 90% of saponins did not affect the three antagonistic bacterial strain populations assessed, but affected biofilm formation at saponins concentration of 7360 mg L^{-1}. *Pseudomonas protegens* strains had a variable antagonist activity in wheat plants, and controlled the fungus when were combined with different concentrations of pure *Q. saponaria* extract, with the concentration of 1840 ppm reducing the take-all disease in 32.5% with respect to the control inoculated with *G. graminis* var. *tritici* ($P \leq 0.05$). However, no synergistic effects when the plant extract was combined with the bacterial strains were observed. These results showed the promising and complexity of combining bacterial and plant extract to develop a biopesticide, which could control this fungal disease.

Key words: Biofungicide, 2,4-DAPG, *Pseudomonas protegens*, *Quillaja saponaria*, saponins, *Triticum aestivum*.

INTRODUCTION

Quillay (*Quillaja saponaria* Mol.; Quillajaceae) is an endemic Chilean evergreen tree distributed from Valparaíso Region (30° S lat) to La Araucanía Region (38° S lat) (Schlotterbeck et al., 2015), which is rich in bidesmosidic saponins into its bark, leaves, and parts of the trunk (Donoso et al., 2011; Schlotterbeck et al., 2015). Saponins are secondary triterpenoid metabolites produced in many plant species, some with antifungal activity (Augustin et al., 2011; Ahmed et al., 2012). Various studies have shown a control effect by *Q. saponaria* on different species of *Oidium* (Apablaza et al., 2002; Moya et al., 2010), as well as on other phytopathogenic fungi such as *Botrytis cinerea* Pers.:Fr. (Ribera et al., 2008) and *Gaeumannomyces graminis* (Sacc.) Oliver and Von Arx var. *tritici* Walker (Apablaza and Moya, 2004).

Take-all disease, caused by the ascomycete fungus *G. graminis* var. *tritici* (Ggt), causes severe losses in wheat (*Triticum aestivum* L.; Poaceae) crops in Chile (Andrade et al., 2011; Moya-Elizondo et al., 2015; Durán et al., 2017) and worldwide (Paulitz et al., 2010; Yang et al., 2011; Zhang et al., 2017). This fungus triggers rotting and decomposition of the roots, crown, and basal culm of wheat plants and other cereals (Paulitz, 2010; Paulitz et al., 2010). Decreases in plant yield are associated with plant loss during crop growth and decreased size and number of spikes, or the presence of less or shrivelled grains. Ggt normally inhabits soil and infests the vascular cylinder of the culm, reducing water and nutrient absorption, and causing white spikes during flowering, premature ripening, and plant death (Paulitz et al., 2010; Yang et al., 2017).

Despite great research efforts, a successful control of take-all disease has not yet been achieved (Paulitz et al., 2010; Vera et al., 2014; Zhang et al., 2017). The majority of cultivated wheat varieties are susceptible to this disease (Vazvani et al., 2017), therefore its management is limited to selecting less susceptible varieties, balanced fertilization, crop rotation (Mathias-Ramwell et al., 2016), and the use of seed treatments with chemical fungicides (Paulitz, 2010; Yang et al., 2014; Zhang et al., 2017). Fungicides with active ingredients such as fenbuconazole, fluquinconazole, nuarimol, silthiofam, triadimefon, triadimenol, and triticonazole have been unable to completely control take-all disease, only slowing or decreasing the infection caused by Ggt (Paulitz, 2010; Andrade et al., 2011; Vera et al., 2014).

Different microbial agents with antagonist activity against Ggt have been described (Park et al., 2011; Liu et al., 2011; Kwak et al., 2012; Yang et al., 2014; 2017; Zhang et al., 2017). Among them, bacteria from the genus *Pseudomonas* spp., which inhabit the wheat rhizosphere, have been studied in depth because of their control of Ggt and other pathogenic fungi that cause root rot in wheat, such as *Rhizoctonia*, *Pythium*, and *Fusarium* (Park et al., 2011; Mavrodi et al., 2012; Yang et al., 2014). Bacteria from the genus *Pseudomonas* produce metabolites with antifungal effects such as 2,4-diacetylphloroglucinol (2,4-DAPG), hydrocyanic acid, pyoluteorin, pyrrolnitrin, and phenazine derivatives (Ramette et al., 2011; Kwak et al., 2012; Yang et al., 2017). Recently, the presence of 2,4-DAPG producing *Pseudomonas* bacteria has been reported in fields in southern Chile (Moya-Elizondo et al., 2013; Durán et al., 2017). Bacteria that produce 2,4-DAPG are a promising source for the development of seed inoculants for disease control, although this compound has high instability in the rhizosphere (Kwak et al., 2012).

Commercial use of these strains requires exhaustive research on its formulation as a biopesticide, which could require natural compounds that affect the pathogen, but also favor the growth of beneficial bacteria in the rhizosphere. Although the potential impact of *Q. saponaria* extracts on fungi such as Ggt is valuable, there is limited research evaluating the direct effect of these components on pseudomonads populations and their combined effect on the decrease of pathogenic fungi in wheat. This is important because the combined use of *Pseudomonas* spp. bacteria with saponinic extracts could lead to the development of a biofungicide that diminishes Ggt. The present study evaluated the bioactive effects of saponin-rich *Q. saponaria* extracts combined with strains of *P. protegens* on the fungi Ggt in wheat plants. We determined the *in vitro* effect that triterpenic extracts from *Q. saponaria* exhibit on populations of *P. protegens*. Furthermore, we compare growth of *P. protegens* populations on wheat roots treated and untreated with *Q. saponaria* extracts high in saponins, and the effect of these mixtures on the development of the take-all disease in wheat plants.

MATERIALS AND METHODS

Biological material

Two saponin-rich *Q. saponaria* extracts were used: a crude extract with a concentration of 8% (w/w) of saponins (QL1000, Desert King Chile, Quilpué, Chile), and a pure extract with 90% (w/w) of saponins (Vet Sap, Desert King Chile Ltda., Quilpué, Chile). Saponins concentrations of these extracts were determined by HPLC (San Martín and Briones, 2000).

Three strains of *Pseudomonas protegens* 2,4-diacetylphloroglucinol (2,4-DAPG)-producers were used: strain Pf-5 (Ramette et al., 2011) and the Chilean strains Ch-b7 and Ca-10, which had the gene *phlD+* associated with production of this compound. The strain Pf-5 was gently facilitated by Dr. Brian McSpadden from Ohio State University, USA. Strains Ch-B7 and Ca-10 were isolated from wheat roots in a survey conducted in Chile between the Biobío and Los Lagos Regions in 2012 (Moya-Elizondo et al., 2013). The pathogenic isolate Oso1 of the fungus *G. graminis* var. *tritici* was obtained from English ryegrass plants (*Lolium perenne* L.; Poaceae) from Osorno, Chile. This fungal isolate presents high aggressiveness in wheat plants.

Bioactivity of quillay extracts on beneficial strains of *Pseudomonas protegens*

Pure isolations of the three bacterial strains were seeded on Petri dishes with King's B medium, and pure colonies were obtained, which were then incubated in individual tubes with 10 mL of King's B medium at 24 °C under continuous agitation at 150 rpm for 24 to 72 h. At the end of incubation, 50 µL were taken from the bacterial colonies, which were put onto ELISA microplates that contained 200 µL of King's B medium at concentrations of 0, 200, 400, 800, and 8000 mg L^{-1} of each *Q. saponaria* extract. Optical density was measured at 600 nm with a microplate reader (RT-2100C; Rayto, Shenzhen, P.R. China). Spectrophotometric correction controls were wells with the different concentrations of *Q. saponaria* extract plus 50 µL sterile King B media without bacteria. Each concentration and control was repeated three times for each treatment, using one microplate for each extract of *Q. saponaria*. At the same time, the content of the tubes was diluted in ELISA microplates, obtaining dilutions of 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} of the bacterial suspensions in King's B medium. From these dilutions, three microdrops of 10 µL were placed on a Petri dish with agar King's B medium, and these plates were incubated at 24 °C during 24 h to determine the initial bacteria concentration by quantifying the number of colony-forming units (CFU). Additionally, after obtaining the 24 h-spectrophotometric reading from the bacterial populations grown in the different concentrations of extracts described above, the number of CFU was determined following the methodology previously described. This experiment was carried out in triplicate.

The direct effects of *Q. saponaria* saponins on the growth of *P. protegens* and on the antagonistic activity of the bacteria against Ggt were assessed. Three bacterial strains were cultivated (10^5 initial CFU) in 1.5 mL tubes with different *Q. saponaria* saponin concentrations of the pure extract (0, 1840, 4600, and 9200 mg L^{-1}) diluted in water. One control of each strain and concentration was grown in King's B medium. All concentrations were incubated separately for 24, 48, and 72 h. For each experimental time, and for each saponin concentration, a 10 µL aliquot was taken from each bacteria population and placed on a Petri dish with potato dextrose agar (PDA) plus King's B medium, which also contained a 5 mm diameter plug of Ggt isolate Oso1. The fungus had been previously grown in PDA for 7 d at 25 ± 1 °C in an incubation chamber. The fungus, together with the bacteria treated with different concentrations of saponins, was grown for 4 d. Mycelial growth was measured with a ruler from the border of the slice of mycelium, to the point inoculated with bacteria. After 24 h, the bacteria grown in different concentrations of saponins were centrifuged for 2 min at 3000 rpm, and the supernatant was extracted. Bacteria were then re-suspended twice in sterile distilled water (SDW) in order to subsequently quantify CFU as previously described.

***In vitro* activity exhibited by saponinic concentration of *Q. saponaria* extracts on antagonistic bacteria of *G. graminis* var. *tritici* in wheat seedlings**

Wheat (*Triticum aestivum* L.) 'Pandora-INIA' (Instituto de Investigaciones Agropecuarias INIA, Chile) seeds were sterilized for 1.5 min in 0.5% sodium hypochlorite, constantly agitated, and washed six times with SDW (3 min per wash) in a laminar flow cabinet. Seeds were placed in Petri dishes on wet paper towels until they germinated. Then, seedlings were then placed in a 15 cm Petri dish with water-agar medium. Seedlings were grown until their roots reached 1-2 cm. The three strains of *P. protegens* were previously grown in a tube with 10 mL of King's B medium at 24 °C at 48 h. After inoculation, bacteria samples were diluted in series in Eppendorf tubes with 900 µL SDW to determine the initial CFU by using the three-microdrop method described previously. The concentrations of each bacterium in a dilution of 10^{-4} were centrifuged at 3000 rpm for 10 min to eliminate the supernatant and were resuspended in SDW. From each suspension, 200 µL were taken and mixed with 800 µL of the pure 90% saponins extract, at concentrations of 0, 1840, 4600, and 9200 mg L^{-1} of *Q. saponaria* saponin extract. The resulting mixtures of 0, 1472, 3680, and 7360 mg L^{-1} of saponins were separately placed on the roots of each wheat seedling. Three roots of each seedling were inoculated with 20 µL of the mixture, and the samples were incubated at 24 °C for 24, 48, and 72 h, respectively. After each incubation time, three 1 mm slices were cut from each root from the sections previously inoculated with the *Pseudomonas* and saponin mix. These slices were placed onto individual wells of an ELISA microplate with 100 µL of King's B medium, to be incubated for 24 h at 24 °C. Following incubation, the samples were placed on a Petri dish with agar King's B medium to obtain the final CFU count using the three-microdrop method. Each treatment was applied three times, and each experiment was carried out in triplicate. All the *in vitro* trials were conducted in a completely random design.

Bacterial growth on the roots of wheat 'Pandora-INIA' was observed under scanning electron microscopy. Seeds were sterilized and germinated according to the protocol formerly mentioned. Individual seedlings were placed on Petri dishes with agar-water medium. At the same time, 600 µL bacterial suspension were diluted 10^{-4} and mixed with 2400 µL saponin 90%-rich *Q. saponaria* extract, arriving at a saponin concentration of 7360 mg L^{-1}. The roots were individually inoculated, with 3 mL mixes of *Pseudomonas* and *Q. saponaria*. Additionally, one treatment was prepared with only bacteria, as well as a control with only SDW. Petri plates were kept in constant agitation for 24 h and later left to incubate at 24 °C for 24 h. Tubes containing the slices of treated roots were prepared in triplicate and incubated for 5 d. They were then fixed in 2.5% 0.1 M glutaraldehyde phosphate buffer (pH 7.2) for 1 h at 4 °C, and then fixed in 1% osmium tetroxide buffer for 1 h. Samples were dehydrated in a series of ethyl alcohol washings, followed by two changes of propylene oxide until reaching a critical drying point and then covered with gold particles. Sections were examined under a scanning electron microscope (SEM) (JEOL JSM-6380 LV, JEOL USA, Peabody, Massachusetts, USA) in the Advanced Microscopy Centre at the Universidad de Concepción, Chile.

Control activity by mix of saponinic concentration of *Q. saponaria* extracts and antagonist bacteria on *G. graminis* var. *tritici* in wheat plants.

Individual PVC pots of 28 mm diameter and 200 mm depth were filled with 180 g of an Andisol soil (Humic Haploxerands; Soil Survey Staff, 2014) that had been sized (2 mm grain size), sterilized, and inoculated. Two oat grains infested with Ggt isolate Oso1 were placed at 10 cm and 5 cm under the upper surface of the pot into the soil substrate. Each pot was seeded with one previously germinated 'Pandora-INIA' seed at 1.5 cm depth. The pre-germinated seeds were inoculated separately with the three bacterial strains diluted at 0, 1840, 4600, and 9600 mg L^{-1} saponins from the 90% saponins extract. These treatments were applied to the pre-germinated seeds, being inoculated with 500 µL of an individual suspension of 10^{-8} CFU per mL of each strain (grown in the conditions previously reported) plus 100 µL of the different triterpenic extract concentrations from *Q. saponaria* saponins. Ggt inoculated and non-inoculated controls were considered, and they were treated with 600 µL SDW. An additional control inoculated with Ggt was treated only with a saponin concentration of 9200 mg L^{-1} from de pure extract of *Q. saponaria* with 90% saponins, receiving 500 µL SDW in replacement of the bacteria. All treated pots were kept in a plant growing room under controlled temperature conditions of 22 ± 2 ºC and a 14:10 h photoperiod (68.0 to 88.3 µmol m^{-2} s^{-1}) with LED lights: red 630-660 nm, yellow 615 nm, and blue 460-490 nm. In total, each treatment was repeated six times, considering one pot as an experimental unit. Pots were watered three times per week and fertilized every 2 wk with 5 mL of commercial nutrient solution (12% N; 4% P; 7% K, Best Garden, Comercial VOS S.A., Colina, Chile) for each pot. This experiment was carried out in triplicate and all the trials were conducted in a completely random design.

Plants were grown for 45 d, and subsequently, roots of each plant were evaluated for level of infection, according to a Ggt damage index proposed by Freeman et al. (2005), in which 0 = no infected roots; 1 = up to 10% infected roots, 2 = 11%-25% infected roots, 3 = 26%-50% infected roots, 4 = 51%-75% infected roots, and 5 = 76%-100% infected roots. Additionally, the average of the values obtained from the damage index for the three bacterial strains assessed were used to calculate a the take-all index (TAI) for each treatment, by using the following formula: TAI = [(0 × nr plants value 0) + (10 × nr plants value 1) + (25 × nr plants value 2) + (50 × nr plants value 3) + (75 × nr plants value 4) + (100 × nr plants value 5)]/Total number of plants. These data were analyzed to determine average differences among treatments and considered a randomized block design, where the bacterial strain was a block.

Data analysis

Data were analyzed in the normality of their distributions by the Shapiro-Wilk test. Data that did not present normal distribution were logarithmically transformed. The F test was used to determine the differences between each of the experiment replicates. If no differences were obtained ($p \leq 0.05$), data obtained from each experiment replicate were averaged and analyzed; if differences were observed between experiments, they were separately analyzed. In the *in vitro* and *in vivo* experiments, ANOVA was used to analyze effects of the saponinic extracts on *Pseudomonas* and control the activity that the mix of the bacteria with the concentration of the extract had on Ggt and the disease expression. Tukey multiple comparison test was used to determine differences among means of the treatments ($p \leq 0.05$). Qualitative and transformed data that did not show normality in their distributions were analyzed with the non-parametric Kruskal-Wallis test. All statistical analyses were performed with the program InfoStat version 2015 (Grupo InfoStat, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentina).

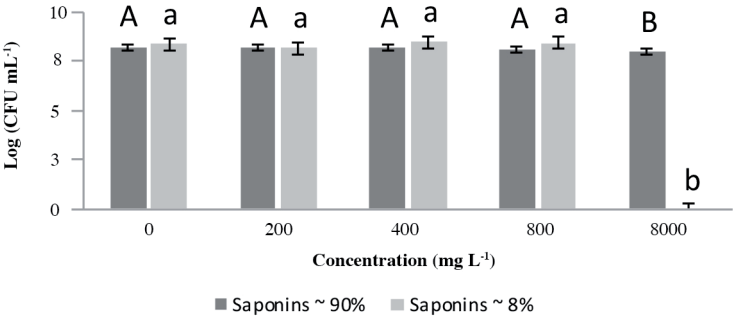
RESULTS

Bioactivity of *Q. saponaria* extracts on beneficial strains of *Pseudomonas protegens*

The average results of bacterial count after 24 h of growth showed significant differences between the concentrations for both *Q. saponaria* extracts ($P \leq 0.05$), but not between the bacterial strains (Figure 1). Bacterial growth of the strains was similar among concentrations of 0 and 800 mg L^{-1} of saponins, while the 8000 mg L^{-1} saponins from the extract with 90% of saponins reduced CFUs and absorbance values in 36% and 16.2%, respectively. At 8000 mg L^{-1} saponins no live colonies were isolated from the crude extract with 8% of saponins (Figure 1). It is important to note that at 8000 mg L^{-1} saponin of the 8% saponins extract, precipitate was produced in the bottom of the microplate wells, meaning that absorbance readings were invalid.

On average, the pure 90% saponins extract from *Q. saponaria* significantly reduced bacterial growth of *P. protegens* at 63% when grown at 1840 mg L^{-1} saponins during 24 h with respect to the control (King's B medium). Nevertheless, this decrease in bacterial growth was lower in comparison to the treatment with bacteria grown only in SDW (0 mg L^{-1}), in which the bacteria population decreased 78.3% (Table 1). However, when the strains were analyzed individually, this decreasing behavior was only observed in the strain Ch-B7, given that the other two showed that the addition of saponins from *Q. saponaria* did not affect the population growth of the antagonist bacteria (77.8% for 0 mg L^{-1}; 57.3% for 1840 mg L^{-1}; 49.7% for 4600 mg L^{-1}; 27.9% for 9200 mg L^{-1}) (Table 1). When comparing bacterial growth in the

Figure 1. Average *Pseudomonas protegens* colony-forming units (CFUs) expressed in log base after 24 h incubation in King's B medium supplemented with different saponin concentrations from two *Quillaja saponaria* extracts: a pure extract with 90% saponins and a crude extract with 8% of saponins.



Different letters on the bars of each extract show significant differences among doses, according to the Tukey multiple comparison test ($P \leq 0.05$). Data are expressed as mean ± MSD of three experiments.

Table 1. Bacterial colony-forming units (Log CFU mL^{-1}) of the three *Pseudomonas protegens* strains grown in different concentrations of *Quillaja saponaria* extract with 90% saponins.

	Pseudomonas protegens strains				
Treatments[1]	Pf-5	Ch-B7	Ca-10	Mean	Decrease percentage
mg L^{-1}					%
0	6.2a	5.9b	6.1a	6.1b	78.3%
1840	6.7a	6.2b	6.2a	6.4b	63.0%
4600	6.7a	6.5ab	6.3a	6.5ab	48.1%
9200	6.5a	6.6ab	6.1a	6.4ab	30.2%
Control[2]	6.9a	6.8a	6.8a	6.8a	-
P-Value	0.397	0.016	0.274	0.003	

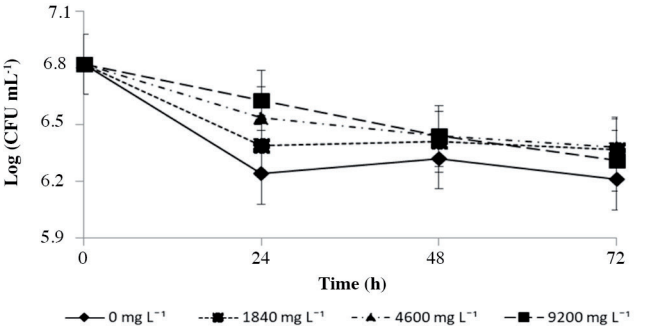
Means of each treatment were compared with the control grown in King's B medium to obtain the reduction percentage in CFU.
Different letters in columns show significant differences between treatments for each bacterial strain, according to the Tukey multiple comparison test ($P \leq 0.05$).
[1]Values expressed in mg L^{-1} saponins obtained from the pure extract of *Quillaja saponaria* with 90% w/w of saponins.
[2]Control considering bacterial growth obtained in King' B medium.

different concentrations of saponin extracts (0, 1840, 4600, and 9600 mg L^{-1}) in the different evaluation moments (24, 48, and 72 h), a decrease was observed in bacterial count for all concentrations with respect to the initial CFU count of 1.7×10^{-6} (Figure 2). This decrease was marked at 24 h, stabilized at 48 h, and maintained constant at 72 h, without observing significant differences among times or concentrations of *Q. saponaria* saponins ($P > 0.05$). Although a more linear decrease was observed at 4600 and 9200 mg L^{-1} saponin, the treatment without saponins and also with the lowest concentration (1840 mg L^{-1}) presented a more marked decline in bacterial growth (Figure 2). Moreover, decreasing control activity by *P. protegens* on Ggt was not observed in the assays carried out *in vitro* after the bacteria was grown during 24 h in different saponin concentrations of the *Q. saponaria* extract with 90% of saponins (Figure 3). The bacteria maintained their antagonistic activity as collected bacteria obtained from the tubes with different concentrations of saponins were able to detain the mycelial growth of the fungus around 5 to 7 mm before entering in contact with the bacterial colony.

In vitro activity of saponins from *Q. saponaria* on beneficial bacteria in seedling wheat roots

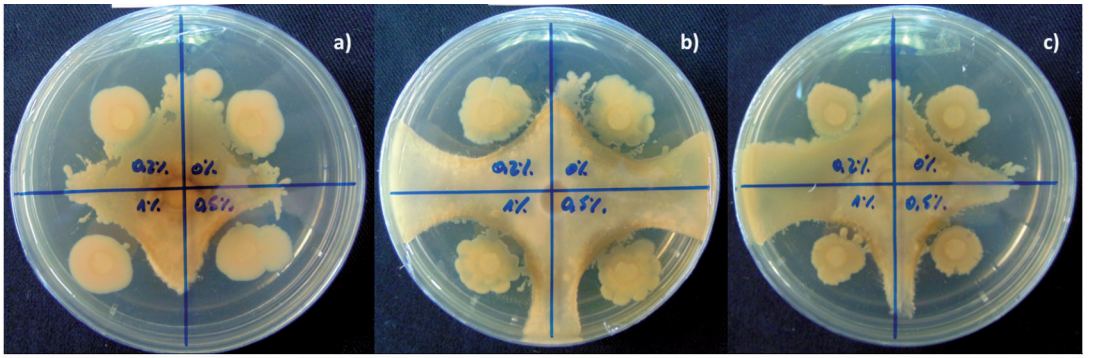
The time elapsed between the three replicates of the experiments did not influence treatment response (P-value > 0.05). For this reason, the three experiments were used as the same dataset for analysis of each evaluation period (24, 48, and 72 h) and bacterial strain (Pf-5, Ca-10, and Ch-B7). The 90% saponins extract applied to the root did not present antagonistic activity on the growth of *P. protegens* populations in any of the four concentrations assessed. However, the bacterial count was variable between the strains at different concentrations and evaluation times, when the bacterial strains were inoculated on the wheat roots ($p < 0.05$). The roots that were not treated with the saponin-rich extract did not present

Figure 2. Average colony-forming units (CFUs) expressed in log base for different *Pseudomonas protegens* strains grown in different saponins concentrations obtained from *Quillaja saponaria* extract with 90% saponins at three different inoculation times.



Data are expressed as mean ± standard error.

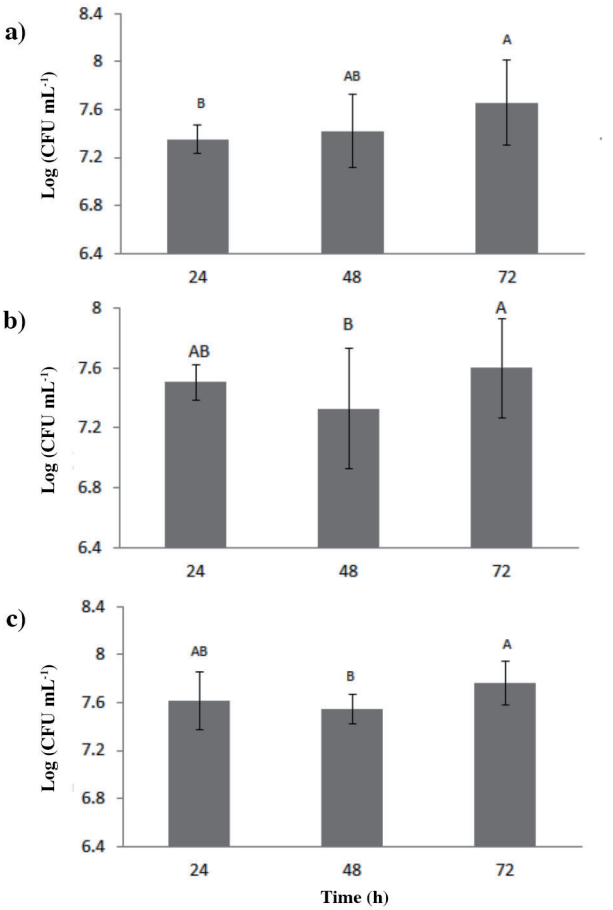
Figure 3. Inhibition of *Pseudomonas protegens* on *Gaeumannomyces graminis* var. *tritici* under *in vitro* conditions for the strains Pf-5 (a), Ch B-7 (b), and Ca-10 (c) after 4 d incubation on a PDA-King's B medium observed for bacterial population previously grown during 24 h in different saponins concentrations from a pure *Quillaja saponaria* extracts with 90% saponins, where 0% = 0 mg L^{-1}, 0.2% = 1472 mg L^{-1}, 0.5% = 3680 mg L^{-1}, and 1% = 7360 mg L^{-1} saponins.



significant differences in CFU for any of the three bacteria during the evaluation periods. *Pseudomonas protegens* strain Pf-5 at a 7360 mg L^{-1} saponin showed a significantly lower CFU population at 24 h than at 72 h, while the bacterial population observed at 48 h was not different in comparison to those observed at 24 and 72 h (Figure 4a). The reduction of CFU observed at 24 h was 35.4% less than the initial measurement (0 h), and at the same time was surpassed up to 102% at 72 h. Strain Pf-5, at 3680 mg L^{-1} saponins, slightly decreased at 24 h (8.7%) and arrived at a reduction of 39.5% in the bacterial population observed at 48 h in comparison to the initial measurement at 0 h. Nonetheless, the bacterial growth was recovered at 72 h after inoculation on the root (Figure 4b).

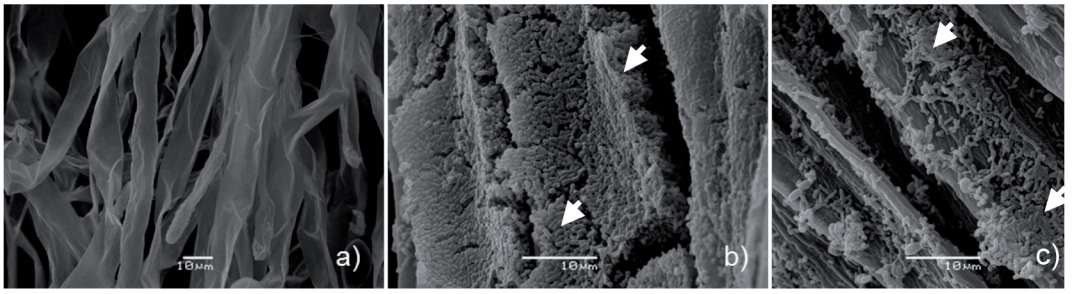
Bacterial strain Ca-10 showed an increase in bacterial counts at 72 h in a 1472 mg L^{-1} semi-pure *Q. saponaria* saponin extract concentration, reaching 64.7% more than CFU with respect to measurements made at 24 and 48 h (Figure 4c), which were similar to initial CFU measurements used to inoculate roots (2.14×10^{-7} CFU). Meanwhile, the strain Ch-B7 showed no differences between the three time periods in any of the *Q. saponaria* concentrations evaluated. Scanning electron microscopy showed that *P. protegens* strains settled when inoculated on root surfaces of wheat for 24 h (Figure 5). However, when these bacteria were applied without *Q. saponaria* extracts, a more compact and dense bacteria population was observed, forming biofilm that grouped them together (Figure 5b). On the other hand, combining bacteria with saponin rich extracts from the *Q. saponaria* strain resulted in a decline in bacteria populations, with lesser compaction on root surfaces, which is associated with less aggregation of bacteria under a biofilm (Figure 5c).

Figure 4. Average colony-forming units (CFUs) expressed in log base for various strains of *Pseudomonas protegens* inoculated on wheat roots mixed with pure extracts of *Quillaja saponaria* with 90% saponins at three different times from inoculation. Strain Pf-5 populations at *Q. saponaria* saponin concentrations of 7360 mg L^{-1} (a), 3680 mg L^{-1} (b), and strain Ca-10 at 1472 mg L^{-1} (c).



Different letters on the bars show significant differences according to the Tukey multiple comparison test ($P \leq 0.05$).
Data are expressed as mean ± standard deviation.

Figure 5. Observations through scanning electron microscopy for wheat root surfaces without inoculation (a), inoculated only with *Pseudomonas protegens* (b) and wheat roots treated with bacteria in combination with 7360 mg L^{-1} saponins from *Quillaja saponaria* (c), after 24 h.



Arrows indicate aggregation of bacteria and biofilm formation.

***In vivo* effect of saponin extract in combination with antagonistic bacteria on the disease caused by *G. graminis* var. *tritici* in wheat plants.**

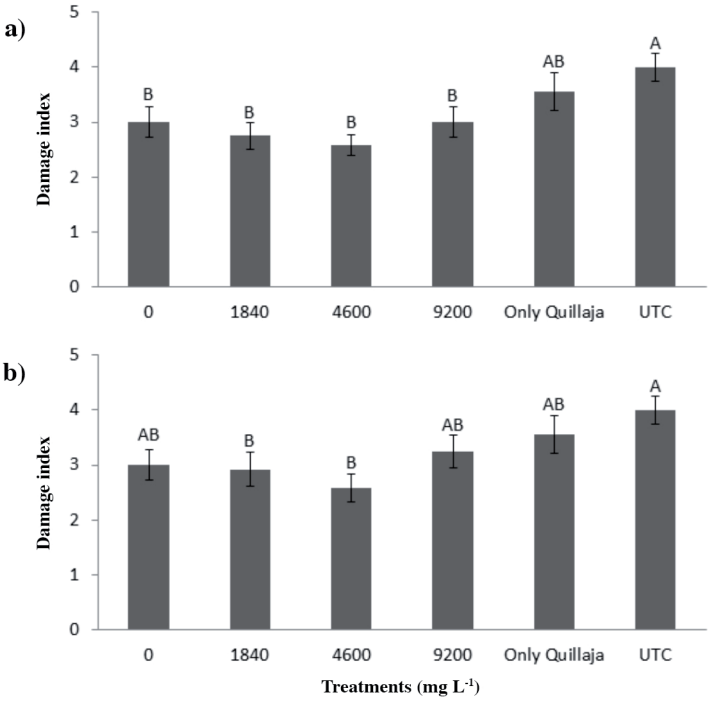
When analyzing the damage index of Ggt on wheat plants inoculated with different *P. protegens* strains mixed with different *Q. saponaria* extract concentrations (0, 1840, 4600, and 9200 mg L^{-1}), significant differences were observed for each bacterial strain ($P < 0.05$). Moreover, all inoculated treatments were infested by the fungus and presented damages significantly different than the treatment non-inoculated with the pathogenic fungus. The bacterial strain Pf-5 did not show differences on damage caused by *G. graminis* var. *tritici* when this bacterium was combined with the different saponin concentrations. The same occurred for treatment where wheat plants were only inoculated with a 9200 mg L^{-1} saponin concentration. The strain Ca-10 presented a reduction of the damage index with respect to treatment inoculated only with the fungus, when this bacterium was used alone or in combination with the three different saponins concentrations derived from *Q. saponaria* (1840, 4600, and 9200 mg L^{-1}) ($P < 0.05$) (Figure 6a). Strain Ch-B7 had a significant decrease in damage caused by fungus on the wheat plants when this bacterium was combined with 1840 and 4600 mg L^{-1} *Q. saponaria* saponin. The strain Ch-B7 reduced the damage index from an average value of 4 for the control inoculated with *G. graminis* var. *tritici* to 2.7 for the 1840 mg L^{-1} concentration (32.5% of disease reduction), which was the maximum decrease in damage achieved in this experiment (Figure 6b).

Values of the damage index obtained from the three strains were used to calculate TAI, determining a significant decrease of the disease with respect to the inoculated control, from treating pre-germinated seeds with only *P. protegens*, as well as by mixing them with the three saponin concentrations (1840, 4600, and 9200 mg L^{-1}) assessed (Figure 7). Use of 9200 mg L^{-1} *Q. saponaria* saponin without bacteria to control take-all disease showed no significant differences in the TAI from the untreated control inoculated with the fungus.

DISCUSSION

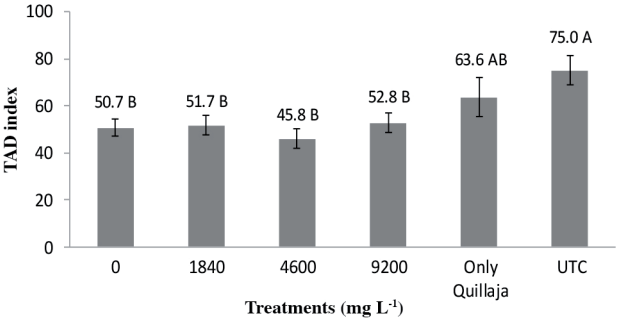
Many saponins are considered preformed antimicrobial compounds that are part of the defense system of certain plants (Sherif et al., 2009; Augustin et al., 2011). Therefore, it is to be expected that saponins exert this type of activity on bacteria that inhabit the rhizosphere and phyllosphere of plants. Moreover, several species of plants have symbiotic associations with bacteria that contribute to their defenses against diseases and improve their physiological functions (Sivasakthi et al., 2014). In this study, the use of a crude *Q. saponaria* extracts with 8% of saponins showed a higher bactericidal effect when concentrations of *Q. saponaria* were increased. This bactericidal activity could be attributed to the lower purity of saponins of this extract, noting that 8000 mg L^{-1} saponin produced a precipitate at the bottom of micro-plate cells, possibly due to the presence of sodium benzoate preservative (1 g L^{-1}) and other solids existing in the composition of this crude extract of quillaja. The pure saponin extract with 90% saponins did not exhibit this marked effect on bacterial growth, and this was why it was considered for studies on wheat roots. *Pseudomonas protegens* strains were slightly affected by concentrations between 0 and 8000 mg L^{-1} pure saponins, under *in vitro* conditions, maintaining antagonistic activity on the fungus after having been in contact with *Q. saponaria* extracts with 90% of saponins. At the same time, this low effect

Figure 6. Damage index of wheat plants infected with *Gaeumannomyces graminis* var. *tritici*, which were treated with different *Pseudomonas protegens* strains combined with different concentrations of a pure *Quillaja saponaria* extract with 90% saponins. Results are for strains Ca-10 (a) and Ch-B7 (b). Graphs include treatments only inoculated with bacteria (0), only treated with a concentration of 9200 mg L^{-1} *Q. saponaria* extracts, and an untreated control (UTC), which was only inoculated with *G. graminis* var. *tritici*.



Bars represent the means of each treatment (n = 6). Data are expressed as mean ± experimental error. Different letters above bars indicate significant differences between treatments by the non-parametric Kruskal-Wallis test ($P \leq 0.05$).

Figure 7. Take-all disease (TAD) damage index, caused by *Gaeumannomyces graminis* var. *tritici* in wheat plants treated with *Pseudomonas protegens* mixed with different concentrations of a pure *Quillaja saponaria* extracts with 90% saponins. Results are the average of the three strains used (Pf-5, Ch B-7, and Ca-10). Graph include treatments only inoculated with the bacteria (0), only treated with a concentration of 9200 mg L^{-1} *Q. saponaria* extracts, and an untreated control (UTC), which was only inoculated with the fungus.



Data are expressed as mean ± experimental error. Different letters above bars indicate significant differences between treatments by the non-parametric Kruskal-Wallis test ($P \leq 0.05$).

on the bacterial growth of pure saponin extract was reinforced by the fact that *P. protegens* bacteria, cultured at 24, 48, and 72 h with *Q. saponaria* saponin concentrations of 1840, 4600, and 9200 mg L^{-1}, did not lose their inhibitory effect on the fungus grown in the tests performed under *in vitro* conditions.

Antagonistic bacteria were slightly affected by *Q. saponaria* saponin concentrations between 1472 and 7360 mg L^{-1} when they were placed on the roots of wheat plants and evaluated at three different sampling times (24, 48, and 72 h). In general, *P. protegens* populations, when inoculated without saponin extracts, formed biofilm containing high bacteria concentrations on the roots of wheat. This effect was not observed when combined with different concentrations of pure extracts rich in saponins 24 h of culture. In the presence of saponins from *Q. saponaria*, a decrease was observed in the number of *P. protegens* bacteria, which was consistent with the number of CFU observed after 24 h from the inoculation with the strain Pf-5 on wheat seedling roots, containing equal concentrations of saponins (7360 mg L^{-1}).

Assessment of bacterial growth in combination with diverse *Q. saponaria* saponins concentration, fluctuating between 200 and 9200 mg L^{-1}, was associated with detecting a wide range of concentrations where these molecules affect *P. protegens*. Assessing high concentrations of *Q. saponaria* in combination with the antagonistic bacteria is required when a biopesticide is developed. Bacteria and saponins extract will need to be applied in concentrated form on the seed's surface before seeding, which is the more common commercial way to apply a fungicide seed treatment in order to control take-all disease. Antifungal effects of commercial *Q. saponaria* extracts against *G. graminis* var. *tritici* have an *in vitro* effect at concentrations of 50 to 500 mg L^{-1} of saponins (Apablaza et al., 2002; 2008). This concentration range is associated with hyphae deformation, mycelium thinning, and breakage of mycelia strands between fungus hyphae, as reported by Apablaza et al. (2008). This effect is attributed to the presence of triterpenic saponins, which show fungicidal activity associated with the membranolytic actions on the sterols present in the fungal cell membranes (Apablaza et al., 2002; Ribera et al., 2008; Augustin et al., 2011). Antifungal properties of saponin are attributed to aglycones present in these molecules and their ability to form a complex with sterols of the cell membranes of fungi, causing pore formation and loss of membrane integrity (Osbourn, 2003; Augustin et al., 2011). However, by placing this compound on seeds, this imply that saponins concentrations should dilute during the process of root development to protect these plant tissues. On the other hand, saponins can be lost during this process due to irrigation or rainfall. Therefore, high extract concentrations are necessary to treat wheat plants, and this was the reason for assessing between 1840 and 9200 mg L^{-1} saponins in pot experiments with wheat plants.

In vitro results with *P. protegens* suggest that crude or less-refined extracts present varied antimicrobial activities, as reported by Sen et al. (1998) and Sherif et al. (2009). This difference between the two extracts could be due to the fact that the crude quillaja extract has a lower purity level in composition (8% w/w of saponins) and contains other substances that may affect growth of *Pseudomonas*, such as polyphenols, salts, and simple sugars (San Martín and Briones, 2000), which may present antimicrobial activity (Negri et al., 2014).

Antibacterial properties present in saponins depend on factors such as concentration and saponins sources (Sen et al., 1998; Sherif et al., 2009; Sewlikar and D'Souza, 2017). For example, various commercial extracts that are rich in saponins have different antibacterial activity, and in some cases, have low antibacterial activity against Gram-negative bacteria such as *Escherichia coli* (Sen et al., 1998). This is consistent with results obtained in our study, where extracts rich in saponins from *Q. saponaria* did not reduce populations of *P. protegens*, a Gram-negative proteobacteria. It is possible that these saponin concentrations or aglycone remnants, which are responsible for the appearance of pores in cell membranes, may cause an increase in cell permeability, leading to improved nutrient transport in bacterial cells (Sen et al., 1998). However, an increasing growth in *P. protegens* was not observed in the assessed concentrations (0, 1472, 3680, and 7360 mg L^{-1}) nor when each time assessed was considered separately. Nevertheless, bacterial populations of the strain Pf-5 increased after 72 h of incubation at 3680 and 7360 mg L^{-1} saponins from the pure *Q. saponaria* extract with 90% of saponins, while strain Ca-10 increased the CFU at 1472 mg L^{-1}. This situation suggested that measurement times could not be enough to determine changes in bacterial populations or imply that *P. protegens* bacteria require some period of time previously to activate the mechanism associated with degradation of the sugar present in saponins molecules by the action of non-specific glycosidases (Sen et al., 1998). For the bacterial growth with different saponin concentrations, glycosidases seemed to be more active between 4600 and 9200 mg L^{-1}, considering that CFU were not different from the control (King B medium) in the first 24 h and achieved higher bacterial populations. This situation suggests that saponin molecules were used by the bacteria (Sen et al., 1998). This *P. protegens* degrading activity on saponin molecules used

to increase bacterial populations seems to stabilize over time, considering that nonsignificant differences were found between 24, 48, and 72 h. However, this result could be partially explained by the high level of variability observed in the observations. Studies conducted by Sen et al. (1998) demonstrated regulatory effects of different extract concentrations from two saponin sources *Q. saponaria* and *Yucca schidigera* (Roezel ex Ortigies; Agavaceae) on bacteria, as these could linearly increase or decrease bacterial populations.

Various studies have shown the fungicidal effect of *P. protegens* strains that produce 2,4-DAPG (Kwak et al., 2012; Mavrodi et al., 2012), and of extracts rich in saponins from *Q. saponaria* (Apablaza and Moya, 2004; Apablaza et al., 2008). In this study, the combination of these two sources of Ggt control showed no clear synergism on the reduction of the disease caused by this fungus. *Quillaja saponaria* saponins at 0, 1840, 4600, and 9200 mg L^{-1} in combination with strain Ca-10 and at 1840 and 4600 mg L^{-1} for the strain Ch-B7 of *P. protegens* significant controlled the disease. Similarly, under *in vitro* conditions, the inhibition activity of the bacteria on the fungus was maintained despite the bacteria being grown for 72 h in a medium with 9200 mg L^{-1} saponin derived from the pure quillaja extracts. This result discarded possible effects of the *Q. saponaria* saponins on the fungal control effect associated with the *P. protegens* bacteria. However, control of Ggt in plants treated only with bacteria or with mixtures of *P. protegens* and different *Q. saponaria* extract concentrations was relatively low, considering that both treatment sets averaged between 29.2% and 22.2% less root damage. Low control levels could possibly be due to the highly aggressive nature of the isolate Oso1, considering that this fungal isolate reached the maximum values of the damage index when it was inoculated in plants that did not receive treatments with the bacteria alone or in combination with the saponinic extract.

Suppressiveness of *G. graminis* var. *tritici* in certain soils is produced by the action of the different members of the microbial biomass present in these soils (Yang et al., 2011; Durán et al., 2017; Yin et al., 2017), which could be reduced by soil sterilization conducted prior to establishing the experiments. Although the specific suppression by strains that produce 2,4-DAPG tends to be higher than the rest of the microbial rhizosphere biomass, variability in the control of Ggt by beneficial bacteria is a normal phenomenon (Kwak et al., 2012). Considering the results of this study, where pure *Q. saponaria* saponin extracts showed no antagonistic effect on populations of *P. protegens*, it is not possible to attribute some kind of effect of this extract on the control properties of *P. protegens* on Ggt. Nevertheless, certain concentrations of saponins extracts favor bacterial growth (Sen et al., 1998); thus, it is possible that saponins extract affected the amount of antimicrobial compounds produced by *P. protegens* or affected the compound stability. For example, 2,4-DAPG has high instability in the rhizosphere of wheat plant, with an average life of 6 h (Kwak et al., 2012). Moreover, high soil moisture, associated with rainfall or irrigation, is one of the factors that increase the aggressiveness of Ggt in wheat plants (Paulitz, 2010; Yin et al., 2017). Irrigation performed three times a week during the experiments maintained constant moisture in the soil, which could have activated the inoculum and helped in infesting wheat plants at early stages of development. This high soil moisture could increase root damage, reducing effectiveness of triterpene compounds and antagonistic bacteria. Additionally, frequent irrigation could reduce concentrations of 2,4-DAPG and saponins, considering that bacteria and plant extracts could lixiviate or percolate bacterial cells, saponins, or antimicrobial compounds from the wheat plant roots. On the other hand, the disappearance of biofilm produced by *P. protegens* and observed in roots treated with *Q. saponaria* after 24 h suggests that bacteria loss could occur in early stages of inoculation, affecting its efficacy of control.

Although results obtained from the pots experiment with wheat plants were variable, some bacterial strains of *P. protegens* reduced the damage of *G. graminis* var. *tritici* when bacteria were mixed with certain concentrations of pure *Q. saponaria* saponin extract. This situation suggests that both concentrations of saponin extracts and bacterial strains used could influence Ggt control. Therefore, this research provides insight into the use of both compounds to formulate a bio-fungicide that could constitute an alternative to control this pathogenic fungus in both traditional agriculture as well as organic agriculture.

CONCLUSIONS

Pure extracts rich in triterpene saponins from *Quillaja saponaria* (90% saponins) did not affect population development of the antagonistic *Pseudomonas protegens* bacteria, as observed for high concentrations of crude extracts with 8% saponins under *in vitro* conditions. *Pseudomonas protegens* strains producing 2,4-diacetylphloroglucinol mixed with certain concentrations of pure *Q. saponaria* saponin extracts did not affect the fungal antimicrobial activity of the bacterium,

while they showed an initial decrease of the bacterial population after 24 h and increase of the populations after 72 h on wheat root seedlings. The initial decrease after 24 h was associated with biofilm formation loss in those roots treated with pure saponin extracts. Take-all disease control varied in the pots experiment with wheat plants; however, some bacterial strains of *P. protegens* generated a decrease in *Gaeumannomyces graminis* var. *tritici* damage when they were mixed with concentrations between 1840 and 9200 mg L^{-1} of pure *Q. saponaria* saponin extracts. These results suggest that mix of saponinic extracts with bacterial strains with antimicrobial activity on *G. graminis* var. *tritici* will be no affected and they could be considered in the formulation of an alternative bio-fungicide.

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