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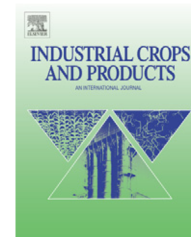

Antifungal properties of quinoa (*Chenopodium quinoa* Willd) alkali treated saponins against *Botrytis cinerea*

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ABSTRACT

Quinoa (*Chenopodium quinoa* Willd) is a Latin American food staple readily available in large quantities in Peru, Bolivia and Ecuador. The outer husk of the grain is removed prior to consumption to reduce its bitter taste. At present, quinoa husks are considered as a by-product with no commercial value, despite its high content of triterpenoid saponins (20–30%). Due to this, the present work was undertaken to test if quinoa saponins have antifungal properties against *Botrytis cinerea* and if this activity is enhanced after alkaline treatment, since recent reports indicate that alkaline treatment of quinoa saponins increase their biological activity. Six products were tested against *B. cinerea*: (1) non-purified quinoa extract, (2) purified quinoa extract, (3) alkali treated non-purified quinoa extract, (4) alkali treated purified quinoa extract, (5) non-purified quinoa extract treated with alkali but without thermal incubation and (6) purified quinoa extract treated with alkali but without thermal incubation.

Untreated quinoa extracts showed minimum activity against mycelial growth of *B. cinerea*. Also, no effects were observed against conidial germination, even at 7 mg saponins/ml. However, when the saponin extracts were treated with alkali, mycelial growth and conidial germination were significantly inhibited. At doses of 5 mg saponins/ml, 100% of conidial germination inhibition was observed, even after 96 h of incubation. Fungal membrane integrity experiments based on the uptake of the fluorogenic dye SYTOX green showed that alkali treated saponins generate membrane disruption, while non-treated saponins had no effects.

The higher antifungal activity of alkaline treated saponins is probably due to the formation of more hydrophobic saponin derivatives that may have a higher affinity with the sterols present in cell membranes.

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1. Introduction

Botrytis cinerea, the causal agent of gray mold diseases, is one of the most important diseases of table grapes, *Vitis vinifera* L., in Chile (Latorre et al., 1994). The control of the fungus is problematic because it has developed resistance to many conventional botryticides, particularly benzimidazoles and dicarboximides (Beever and Brien, 1983; Elad et al., 1992). Loss of sensitivity toward sterol biosynthesis inhibitors, e.g., phenylpyrroles and anilinopyrimidines, has also been reported (Elad, 1992; Faretra and Pollastro, 1993; Gullino et al., 1998).

To overcome resistance, an integrated pest management program, including the use of biological agents and natural products has been proposed (Elad and Shtienberg, 1995; Duke et al., 2003). Natural compounds isolated from

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plants with antifungal properties include terpenoids, aromatic compounds, nitrogen-containing compounds, aliphatic compounds, lectins and polypeptides (Grayer and Harbone, 1994; Cowan, 1999). Some of these natural compounds exhibit activity against *B. cinerea*. For example, germination of conidia of *B. cinerea* is inhibited by sakuranetin, a flavonoid isolated from the surface of *Ribes nigrum* (Grayer and Harbone, 1994). Also, resveratrol, a stilbene produced by *Vitis* spp., inhibits the spread of the *B. cinerea* infection (Langcake and McCarthy, 1979), while the natural diterpenoid 3β-hydroxy-kaurenoic acid, obtained from the resinous exudates of *Pseudognaphalium vira vira*, presents fungitoxic activity against *B. cinerea* (Cotoras et al., 2004).

One important class of antifungal compounds are saponins, since they are often present in relatively high levels in healthy plants and have been implicated as a determinate of plant resistance to fungal attack (Morrissey and Osbourn, 1999). Chemically, saponins consist of a hydrophobic nucleus (sapogenin), to which sugar chains of a hydrophilic nature are bound. There are two main types of saponins depending on the chemical structure of sapogenin: triterpenic and steroidal saponins, where the sapogenin is a triterpene and a steroid, respectively. Another important structural feature refers to the number and nature of sugar moieties attached to the sapogenin: monodesmosidic saponins contain one sugar moiety, while bidesmosidic saponins contain two sugar moieties. Some examples of antifungal saponins are avenacin A-1 from oat roots (Crombie et al., 1987) and CAY-1 isolated from the dried fruit of *Capsicum frutescens* L. (De Lucca et al., 2002). The major mechanism of antifungal activity of saponins is associated with their ability to complex with sterols present in fungal membranes and to cause loss of membrane integrity with formation of transmembrane pores (Keukens et al., 1995; Armah et al., 1999). However, not all saponins exhibit antifungal activity, since this depends on their chemical structure. Maximum activity is shown by monodesmosides with four or five monosaccharides (Hostettmann and Marston, 1995). Shorter carbohydrate chains lead to lower water solubility and weaker antifungal activity (Anisimov and Chirva, 1980).

In this work we concentrate on the saponins present in the husks of the pseudocereal quinoa, *Chenopodium quinoa* Willd, since this resource is readily available in large quantities in countries such as Peru, Bolivia and Ecuador, where quinoa has been consumed for centuries as a staple food. The outer husk of quinoa contains saponins, and is removed prior to consumption to reduce the bitter taste of the grain (Villacorta and Talavera, 1976). Quinoa husks represent about 8–12% (w/w) of the grain and are considered a by-product with no commercial value. The saponin content depends on the quinoa variety: so called "sweet" quinoas contain lower amounts of saponins than "bitter" quinoas. Previous studies (Dini et al., 2001a,b; Woldemichael and Wink, 2001; Zhu et al., 2002), determined the existence of 4 monodesmosidic and 22 bidesmosidic triterpene quinoa saponins based on 4 different aglycones (e.g., oleanolic acid, hederagenin, phytolaccagenic acid and serjanic acid). However, a recent analysis based on nano-HPLC electrospray ionization multi-stage mass spectrometry revealed the existence of 87 triterpene saponins, comprising 19 reported and 68 novel components. The study also showed the existence of 5 novel triterpene aglycones (Madl et al., 2006).

Few reports exist on the use of quinoa saponins against agricultural pests. Particularly, against fungi, the total saponin fraction of *C. quinoa* was found to slightly inhibit the growth of *Candida albicans* (Woldemichael and Wink, 2001). However, it has recently been reported that the biological activity of quinoa saponins can be increased if they are treated with alkali (San Martín et al., in press). Alkaline treatment results in the formation of more hydrophobic saponin derivatives that may have higher affinity with the sterols present in cell membranes. Based on this, the present work was undertaken to test if saponins obtained from quinoa husks are active against *B. cinerea* mycelia and conidial germination, and if this activity is enhanced after alkaline treatment.

2. Materials and methods

2.1. Plant material

Quinoa real husks (bitter quinoa) were obtained directly from Bolivian producers located in the Salar de Uyuni. Quinoa husks consist of a fine white powder with a moisture and protein content of 8% (w/w) and 6.0% (w/w), respectively, determined by standard AOAC methods. The husks contained approximately 33% (w/w) saponins as determined by reverse phase HPLC (RP-HPLC) (Joshi et al., in press).

2.2. Extraction and purification procedure

Quinoa husks were extracted using 1 part by weight and 10 parts of distilled water during 30 min at room temperature and agitation, followed by filtration with Whatman #2. The filtrate (pH 5.7) was acidulated with HCl 37% to pH 3.5, to obtain the isoelectric precipitation of quinoa proteins (Lindeboom, 2005), and filtered with the aid of 5 g/l of diatomaceous earth and Whatman #2. This non-purified extract was then evaporated to a final concentration of 100 g soluble solids/l. To remove low molecular weight non-saponins impurities, purified extracts were prepared using dialysis-ultrafiltration with 5 volumes of distilled water, with 10 kDa ultrafiltration membranes (Amicon, USA). Saponins are retained by the ultrafiltration membranes due to their capacity to form micelles at concentrations above the critical micelle concentration. The final purified product contained 60 g soluble solids/l. The saponin content of the non-purified and purified extracts was 50 g saponins/l, determined by RP-HPLC (San Martín et al., in press). Proximal analysis of non-purified quinoa extracts revealed the presence of 50% (w/w) non-saponins compounds, while purified quinoa extracts contained only 20% (w/w) non-saponins compounds. These non-saponin compounds are proteins, fat, fiber and ash.

2.3. Alkaline treatment

The aqueous extracts were treated with alkali to obtain more hydrophobic saponin derivatives. For this purpose solutions of both non-purified and purified extracts containing 50 g saponins/l were contacted with 1 N NaOH, at 93–95 °C for 2.5 h with agitation. These conditions maximized the formation of hydrophobic saponin derivatives as determined by RP-HPLC

(San Martín et al., in press). Once the alkali treatment was completed, the mixture was cooled at room temperature and HCl 37% was added to bring the extract to pH 7.

Since, high amounts of salt form due to the reaction of NaOH with HCl used for neutralization during the alkaline treatment, we prepared products following the alkali treatment protocol, but without thermal incubation. The objective was to determine if quinoa saponins in the presence of salts had any antifungal activity. Previous experiments had determined that without incubation at 93 °C, the structure of quinoa saponins is not affected by the alkaline treatment.

2.4. Determination of antifungal activity

Six products were tested against *B. cinerea*: (1) non-purified extract, (2) purified extract, (3) alkali treated non-purified extract, (4) alkali treated purified extract, (5) non-purified extract treated with alkali but without thermal incubation and (6) purified extract treated with alkali but without thermal incubation.

B. cinerea strain ToL was obtained from the Department of Phytopathology of the Pontificia Universidad Catolica de Chile. Antifungal activity of the extracts was assessed using the radial growth test on PDA (Potato Dextrose Agar, Meck). The extracts were added at different saponin concentrations (1, 3, 5 and 7 mg/ml) to Petri dishes containing 20 ml of PDA. For each treatment and for each dose tested, three replicate Petri dishes were used. The culture medium was inoculated with 7 mm agar disks from an actively growing culture of *B. cinerea*. Cultures were incubated in the dark at 20 °C. After 8 days of incubation, the diameter of the colonies was recorded. The experiments were repeated twice.

Conidial germination assays were carried out on Petri dishes containing PDA amended with different final saponin concentrations (1, 3, 5 and 7 mg/ml). For each treatment and for each dose tested, three replicate Petri dishes were used. Petri dishes were inoculated covering the entire surface with a suspension of 200 μl of *B. cinerea* conidia (10^6 ml^{-1}), obtained from sporulated mycelia of 10-day-old cultures, and incubated in the dark at 20 °C. The germinated conidia were observed and recorded at 6, 12, 24, 48, 72 and 96 h. Percentages of germinated conidia were determined by microscopic examination of 5 microscopic fields per dish. Conidia were considered germinated when the germ tube length was equal or longer than the diameter of the conidia. The experiments were repeated twice.

2.5. Hyphal membrane permeabilization assay

Membrane permeabilization was measured by the uptake of SYTOX Green (Molecular Probes, Eugene, OR) a high affinity nuclear stain that penetrates cells with compromised membranes and fluoresces upon binding to nucleic acids. The assay was carried out by a modification of the method of Thevissen et al. (1996). In brief, *B. cinerea* pre-germinated conidia (1×10^6 ml^{-1}) were treated during 4 h at 20 °C with the 6 different products at a concentration of 5 mg saponins/ml. SYTOX Green was added to the conidial suspensions at a final concentration of 0.2 μM. After incubation for 10 min at 20 °C, germinated conidia were analyzed with a Carl Zeiss LSM 510 microscope equipped with an Ar laser that transmitted at 488 nm and received at 505–550 nm. Compromised membranes under control conditions were obtained with pre-germinated conidia incubated in 1% (w/v) of Triton X-100 at room temperature and then stained with SYTOX Green.

2.6. Statistical analysis

One-way ANOVA was performed for mycelial growth assay and Tukey HSD test was used to compare means (Table 1). Probit analysis was used to determine the 95 and 50% inhibitory doses (ID_{50} and ID_{95}, respectively) of conidial germination assays. These analyses were performed using the statistical package Statgraphics Plus v 5.1 (StatPoint, Inc.).

3. Results and discussion

3.1. Analysis of quinoa saponin extracts with RP-HPLC

RP-HPLC chromatograms for the non-purified and purified quinoa extracts are shown in Fig. 1A and B, respectively. Both RP-HPLC chromatograms contain three main peaks (marked with black arrows) that have been previously reported for quinoa saponins (Muir et al., 2002; San Martín et al., in press).

Table 1 – Mycelial growth (mm) of *B. cinerea* in PDA amended with different saponin doses (mg saponins/ml) of non-purified extract, alkali treated non-purified extract, purified extract, and alkali treated purified extract

Products	Mycelial growth (mm)[a]			
	1 mg/ml[b]	3 mg/ml[b]	5 mg/ml[b]	7 mg/ml[b]
Non-purified extract	48.9 ± 9.8ab	44.7 ± 8.6a	30.9 ± 9.6b	25.9 ± 4.7b
Alkali treated non-purified extract	30.4 ± 8.1b	14.1 ± 1.8b	5.9 ± 5.2c	2.7 ± 4.7c
Purified extract	60.6 ± 7.5a	43.8 ± 4.5a	38.7 ± 6.4ab	35.3 ± 5.7b
Alkali treated purified extract	40.2 ± 9.5ab	14.1 ± 0.9b	9.7 ± 1.7c	2.4 ± 2.8c
PDA	54.9 ± 5.3a	54.9 ± 5.3a	54.9 ± 5.3a	54.9 ± 5.3a

Numbers followed by a different letter within a column are significantly different at $p \leq 0.05$ (Tukey HSD test was used for comparisons).

[a] Values of mycelial growth were determined at 8 days of incubation and are represented by means ± S.D.

[b] Saponin doses in mg/ml.

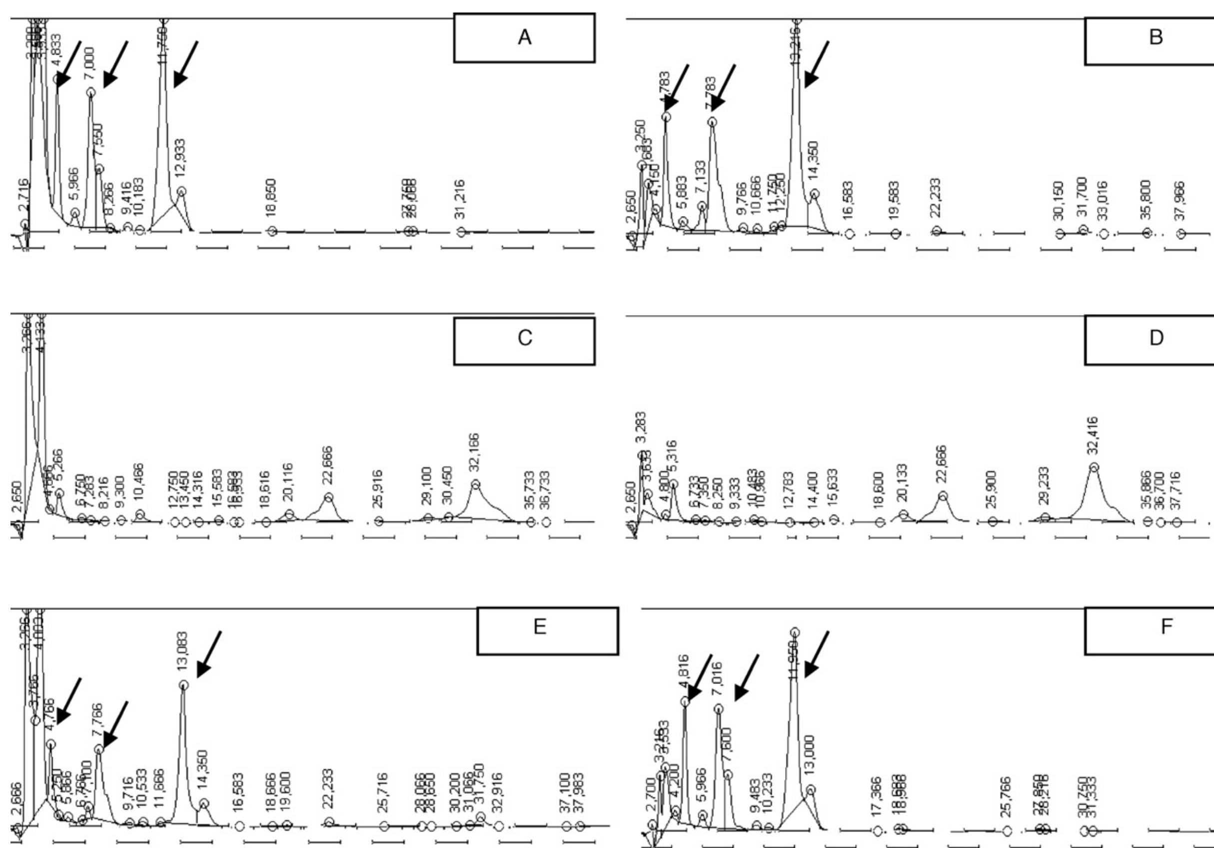

Fig. 1 – RP-HPLC chromatograms. Non-purified extract (A), purified extract (B), alkali treated non-purified extract (C), alkali treated purified extract (D), non-purified extract treated with alkali but without thermal incubation (E), and purified extract treated with alkali and without thermal incubation (F). Peaks marked with black arrows correspond to main quinoa saponins. Absorbance at 210 nm vs. injection time (min).

LC/MS chromatogram of quinoa husk showed that the main saponins are based on phytolaccagenic acid, hederagenin and oleanolic acid (Madl et al., 2006; San Martín et al., in press). Hence, the three main peaks that show up in the RP-HPLC chromatograms are related to these main saponin structures. In the purified extract (Fig. 1B), the UV absorbing impurities that elute in the early part of the chromatogram (e.g., elution time < 4 min) have been reduced due to dialysis-ultrafiltration.

The chromatograms of the samples treated with alkali, but without thermal incubation (Fig. 1E and F), are similar to those shown in Fig. 1A and B, indicating that without temperature, saponins are not affected by the alkaline treatment.

Fig. 1C shows the RP-HPLC chromatogram of alkali treated non-purified extract, while Fig. 1D shows the RP-HPLC chromatogram of alkali treated purified extract. Both chromatograms show that the three major peaks present in the untreated products (Fig. 1A and B) disappear completely and new peaks appear at longer elution times, indicating the presence of more hydrophobic compounds.

This is in agreement with previous reports, where the LC/ESI and MS/MS analysis indicate that alkali treated quinoa husks contain large molecular weight saponin derivatives with more hydrophobic profile than untreated quinoa saponins (San Martín et al., in press).

3.2. *Antifungal activity on B. cinerea*

Table 1 shows the mycelial growth of *B. cinerea* on PDA amended with alkali treated and untreated products at different saponin doses. There are significantly differences of mycelial growth between alkali treated and untreated products at 3, 5 and 7 mg saponins/ml (Table 1). Both alkali treated non-purified extract and alkali treated purified extract are more antifungal than non-purified extract and purified extract at the same doses of saponins. These results show that alkali treated non-purified extract can be 10 times more active than non-purified extract at 7 mg saponins/ml, while the alkali treated purified extract can be 15 times more active than purified extract at 7 mg saponins/ml.

Conidial germination assays of *B. cinerea* on PDA amended with different products were evaluated during 96 h. Fig. 2 shows that there is no conidial germination, even at 96 h, at 5 mg saponins/ml of alkali treated non-purified extract. A delay in conidial germination is shown with 5 mg saponins/ml of alkali treated purified extract. Both extracts treated with alkali but without thermal incubation, at 5 mg saponins/ml, shows the same behavior of conidial germination as that of the control treatment (Fig. 2). Clearly, only the products that contain alkaline treated saponins had an inhibitory effect on

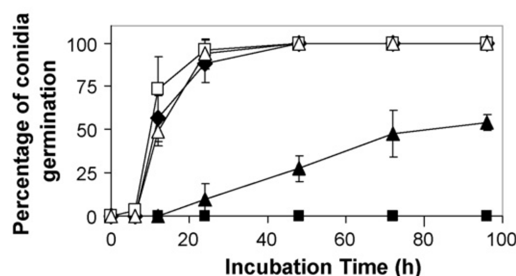

Fig. 2 – Effect of alkali treated products on germination of *B. cinerea* conidia at doses of 5 mg saponins/ml. Control condition (♦), alkali treated non-purified extract (■), non-purified extract treated with alkali but without thermal incubation (□), alkali treated purified extract (▲), and purified extract treated with alkali but without thermal incubation (△).

conidial germination. This suggests that the antifungal activity is not due to the presence of salts in conjunction with saponins, but to the alkaline treated saponins.

Table 2 shows that the untreated quinoa extracts (non-purified extract and purified extract) had no effect on the germination of *B. cinerea* conidia, even at 7 mg saponin/ml. However, after alkali treatment, the ID_{50} and ID_{95} values for alkali treated non-purified extract were 3.0 and 4.4 mg saponins/ml, respectively. Similarly, alkali treated purified extract with ID_{50} and ID_{95} values of 4.8 and 7.2 mg saponins/ml, respectively. Products following the alkali treatment protocol, but without thermal incubation had no effect on the germination of *B. cinerea* conidia at the doses tested (Table 2).

In summary, the results of Tables 1 and 2 and Fig. 2 shows that untreated quinoa saponins have little effect on mycelial growth of *B. cinerea* and no effect on conidial germination. These results are in agreement with findings of Woldemichael and Wink (2001), in which the total saponin fraction of *C. quinoa* showed little antifungal activity against *Candida albicans*. However, when quinoa saponins were treated with alkali, their antifungal activity against *B. cinerea* increased significantly. This is probably due to the formation of more hydrophobic saponins that have a greater affinity with the sterols present in the fungal cell membranes.

In the conidial germination assays, alkali treated non-purified extract showed a slightly higher activity than alkali treated purified extract, suggesting that the initial non-purified extract contain some compounds that may contribute to the antifungal properties. There are reported six flavonols glycosides that have been isolated from quinoa seeds (Zhu et al., 2001). Flavonoids, and related polyphenols, are involved in protecting plants against microbial invasion (Harbone and Williams, 2000) and it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms.

Based on the literature, it can be suggested that some of these phenolic compounds of low molecular weight that are removed during dialysis-ultrafiltration could contribute to the slightly higher antifungal activity of alkali treated non-purified extract.

Table 2 – 50% inhibitory doses (ID_{50}) and 95% inhibitory doses (ID_{95}), (in mg saponins/ml) measured by conidia germination of *B. cinerea*, of non-purified extract, alkali treated non-purified extract, non-purified extract treated with alkali but without thermal incubation, purified extract, alkali treated purified extract, and purified extract treated with alkali but without thermal incubation

Products	Antifungal activity[a]	
	ID_{50}[b]	ID_{95}[b]
Non-purified extract	n.e.	n.e.
Alkali treated non-purified extract	3	4.4
Non-purified extract treated with alkali[c]	n.e.	n.e.
Purified extract	n.e.	n.e.
Alkali treated purified extract	4.8	7.2
Purified extract treated with alkali[c]	n.e.	n.e.

n.e.: No inhibition effect at the doses proved on assays.

[a] Values, in mg saponins/ml, were determined at 24 h of incubation.

[b] The regression lines of the logarithm of the compound concentration transformed in probit were highly significant (correlation coefficients between 0.95 and 0.99, $p \leq 0.05$).

[c] Alkali treated products but without thermal incubation.

3.3. *Effect of quinoa saponins on permeabilization of B. cinerea membrane*

To determine the effect of the products on the loss of membrane integrity of *B. cinerea*, we used an assay based on the uptake of the fluorogenic dye SYTOX Green (Thevissen et al., 1996). This substance can only penetrate cells that have compromised plasma membranes, and it fluoresces upon binding to DNA. Fig. 3A shows germinated *B. cinerea* conidia treated with 5 mg saponins/ml of non-purified extract. It can be observed that no uptake of SYTOX Green was detected after incubation with 0.2 μM of the fluorogenic dye. Also, no uptake of SYTOX Green was observed with purified extract at the same doses (data not shown).

In contrast, Fig. 3B shows that the incubation of germinated *B. cinerea* conidia with 0.2 μM SYTOX Green and 5 mg saponins/ml of alkali treated non-purified extract resulted in an uptake of the fluorescence dye and staining of nuclei in the hyphae. The same positive results were obtained with alkali treated purified extract at the same doses (data not shown).

Fig. 3C shows germinated *B. cinerea* conidia treated with 5 mg saponins/ml of non-purified extract treated with alkali but without thermal incubation and no uptake of SYTOX Green was detected. Also no uptake of SYTOX Green was observed in purified extract treated with alkali but without thermal incubation at the same doses (data not shown).

These results indicate that the plasma membrane of *B. cinerea* was only permeabilized in the presence of alkali treated saponins, probably because the interaction between the hydrophobic saponin derivatives and membrane sterols cause the loss of membrane integrity and the formation of transmembrane pores. However, more experimental work is needed to determine the exact mechanism of action of alkali treated saponins on fungal cells.

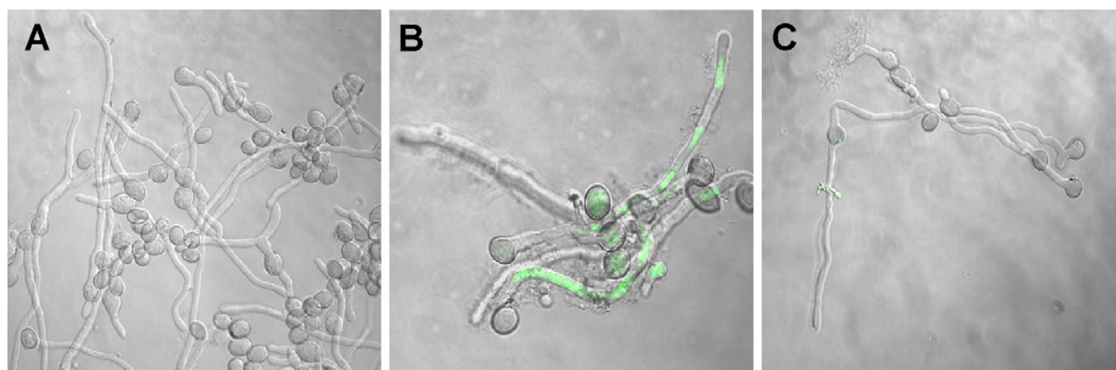

Fig. 3 – Germinated *B. cinerea* conidia were treated with 5 mg saponins/ml of different products and then treated with 0,2 μM SYTOX Green. After 4 h of incubation, SYTOX Green uptake was detected by laser scanning microscopy. Non-purified extract (A), alkali treated non-purified extract (B), and non-purified extract treated with alkali but without thermal incubation (C).

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