Antifungal activity of secondary plant metabolites from potatoes (*Solanum tuberosum* L.): Glycoalkaloids and phenolic acids show synergistic effects

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ABSTRACT

Aims: To study the antifungal effects of the potato secondary metabolites α-solanine, α-chaconine, solanidine and caffeic acid, alone or combined.

Methods and results: Resistance to glycoalkaloids varied among the fungal species tested, as derived from minimum inhibitory concentration (MICs) assays. Synergistic antifungal activity between glycoalkaloids and phenolic compounds was found. Changes in the fluidity of fungal membranes caused by potato secondary plant metabolites were determined by calculation of the generalized polarization values. The results partially explained the synergistic effect between caffeic acid and α-chaconine and supported findings on membrane disruption mechanisms from previous studies on artificial membranes. Liquid chromatography/mass spectrometry (LC/MS) analysis was used to determine variability and relative amounts of sterols in the different fungal species. The sterol pattern of fungi was related to their taxonomy and to their resistance to potato glycoalkaloids.

Conclusion: Fungal resistance to α-chaconine and possibly other glycoalkaloids is species dependent. The taxonomic classification and the sterol pattern in the fungal membrane play a role in their resistance to glycoalkaloids.

Significance and impact of study: Results contribute to a better understanding of the antifungal mode of action of potato secondary metabolites, which is essential for their potential utilization as antifungal agents in non-food systems.

Keywords: potato secondary metabolites, antifungal synergism, glycoalkaloids, phenolic acids, membrane disruption, LC/MS.

INTRODUCTION

Plants synthesize secondary metabolites as natural defence mechanisms against insects, nematodes, bacteria, viruses and fungi. Members of the Solanaceae family, which includes among others potatoes, tomatoes, and eggplants, produce glycoalkaloids and phenolic compounds (Friedman, 2004). These metabolites are present in vegetables consumed by humans, and their bioactive effects might be either beneficial or dangerous (Friedman, 2004). Therefore, a better understanding of their bioactivity and interactions will contribute to protect consumers and to develop potential applications in fields such as medicine.

Glycoalkaloids are derived from sterols and contain nitrogen and a glycosidic moiety attached to the 3-OH position (Friedman 2004). Glycoalkaloids in the Solanaceae family include α-solanine, α-chaconine, α-tomatine, α-solasonine and α-solamargine (Friedman 2006). Potatoes (*Solanum tuberosum* L.) synthesize mainly the glycoalkaloids α-chaconine and α-solanine, which share the aglycone solanidine but differ in their glycosidic moieties, which are solatriose and chacotriose for α-solanine and α-chaconine, respectively. Among the phenolic compounds found in potatoes, chlorogenic acid makes up about 90%; the rest are mainly caffeic acid and other hydroxycinnamates (Schieber and Aranda Saldaña 2009; Sánchez-Maldonado et al. 2014). Caffeic acid is produced by hydrolysis of chlorogenic acid and possesses antibacterial (Sánchez-Maldonado et al. 2011; Sánchez-Maldonado et al. 2015) and antioxidant activities (Reddy et al. 2007; Dai and Mumper 2010). Caffeic acid shows only weak antifungal activity when compared to other phenolic compounds (Merkl et al. 2010).

Glycoalkaloids from *Solanum* species display anti-allergic, antipyretic, anti-inflammatory, hyperglycemic, antibiotic and anticarcinogenic properties (Schieber and Aranda Saldaña 2009;

Sammani et al. 2014). They are toxic to animals and therefore have anti-herbivore function (Phillips et al. 1996; Yencho et al. 2000). In addition, these compounds have antifungal activity. α-Solanine and α-solamargine demonstrated antifungal properties against 10 different strains (Cipollini and Levey 1997). α-Solasonine and α-solamargine showed synergistic pH dependent activity against *Phoma medicaginis* and *Rhizoctonia solani* (Fewell et al. 1994). α-Chaconine and α-solanine have also shown antifungal activity, with α-chaconine being more active (Fewell and Roddick 1993).

The membrane-disruptive effect of glycoalkaloids has been studied in liposome model membranes. α-Solanine, α-chaconine, and α-tomatine showed membrane-disrupting activity and strong interaction with liposomes containing sterols. Membrane sterols with planar ring structures and a 3 β-OH interacted with glycoalkaloids more efficiently (Keukens et al. 1992). α-Chaconine but not α-solanine disrupted phosphatidylcholine/cholesterol liposomes at pH 7.2. A synergistic effect was observed when both compounds were mixed (Roddick and Rijnenberg 1987). Sugar-sugar interactions were suggested to have an important effect in the membrane disruption mode of action of glycoalkaloids. The proposed mechanism of activity involves insertion of the aglycone in the bilayer, followed by sugar-sugar interactions between the sugar moieties of glycoalkaloids. As a result, a rigid sterol-glycoalkaloid matrix is formed, which disturbs membrane function and causes lysis of the cell (Keukens et al. 1995). This hypothesis is supported by the synergy between α-solanine and α-chaconine, the enhanced leakage caused by glycolipids in liposomes containing sterols, and the loss of activity after cleaving monosaccharides from the glycosidic moiety of glycoalkaloids (Keukens et al. 1995).

Studies using frog embryo cells (Blankemeyer et al. 1992), rabbit erythrocytes, beet cells, and protoplasm of *Penicillium notatum* (Roddick et al. 1988) also indicate membrane disruption by glycoalkaloids. In all cases, α-chaconine has been the most active compound. The interaction of glycoalkaloids with membrane sterols suggests that the sterol profile of fungal membranes modulates glycoalkaloid activity. The sterol profile of fungi is a chemotaxonomic tool (Müller et al. 1994; Mejanelle et al. 2000; Weete et al. 2010) but may also relate to the fungal resistance to glycoalkaloids. To our knowledge, studies investigating the interaction of glycoalkaloids with the sterols in the fungal membrane have not yet been conducted.

In view of the biotic stress that potato tubers are constantly exposed to in their natural environment, it is surprising that the profile of secondary plant metabolites present in potatoes is relatively simple, comprising mainly chlorogenic acid, caffeic acid, α-chaconine and α-solanine (Friedman 2004; Schieber and Aranda Saldaña 2009). Phenolic compounds and alkaloids have shown synergistic activity with other antimicrobial compounds (Daglia, 2012; Cushnie, et al. 2014). From these observations it can be hypothesized that phenolic acids and glycoalkaloids show synergistic antimicrobial effects.

Because of the relevance of potato secondary metabolites in the human diet and their potential application, this study aimed to evaluate the antifungal effects of the secondary metabolites α-solanine, α-chaconine, their aglycone and product of hydrolysis, solanidine, and caffeic acid. Their antifungal effects alone and combined was tested against 5 plant pathogenic fungal strains and 3 spoilage fungal strains. To achieve this objective, minimum inhibitory concentrations (MICs) of these compounds and membrane fluidity of fungi were determined. Additionally, the

relationship between membrane sterol composition of several fungal strains belonging to different species and their resistance to α-chaconine was studied.

MATERIALS AND METHODS

Chemicals

Caffeic acid, cholesterol, cycloartenol, ergosterol and squalene were purchased from Sigma (St. Louis, MO, USA). α-Chaconine, α-solanine and solanidine were obtained from Extrasynthèse (Genay, France).

Fungal strains and culture conditions

Eight species of fungi were analyzed for this study: *Aspergillus niger* FUA5001*, Mucor plumbeus* FUA5003*, Penicillium roqueforti* FUA5005 (culture collection of the Food Microbiology Laboratory at the University of Alberta)*, Fusarium graminearum* FG001, *Mycosphaerella pinodes* Is.39, *Alternaria alternata* AA001, *Pyrenophora teres* f. *teres* SK51 and *Pyrenophora tritici-repentis* 332-1 (kindly provided by Dr. S. Strelkov, University of Alberta) (**Table 1)**. The species-specific conditions for sporulation were as follows: *A. niger* FUA5001*, P. roqueforti* FUA5005 and *M. plumbeus* FUA5003 were cultured in PDA plates and incubated in darkness at 25°C under aerobic conditions for 7 days. *F. graminearum* FG001 was cultured in PDA and incubated at 25°C, in a parafilm-sealed petri dish, exposed to 12 h light/ 12 h darkness, alternately, for 5 d. *My. pinodes* Is.39 was streaked in a PDA and incubated in a parafilm--sealed petri dish at room temperature, exposed to 16 h light/ 8 h darkness alternately. *Al. alternata* AA001was first cultured in PDA and incubated in a parafilm-sealed petri dish in the dark at 28 ºC for 3 d. Then, plugs of 70 mm in diameter were subcultured under sterile conditions in S-medium agar, covered with distilled water and incubated at 18 ºC for 1 d. *Py. teres* f. *teres*

isolate SK51 was streaked in V-8 juice-potato dextrose agar, then sealed with parafilm and incubated at 25 ºC exposed to 12 h UV light/12 h darkness, alternately, for 7 d. *Py. tritici-repentis* 331-2 was streaked into V-8 juice agar and incubated under aerobic conditions in darkness at 20 ºC for 12 d or until growth in about 80 % of the plate was observed. Then, mycelia was covered with sterile MiliQ and softly streaked with a sterile loop. Subsequently, the petri dish was incubated for 16 h under aerobic conditions exposed to light at 25 ºC. Then, the petri dish was removed from light and incubated in darkness at 16 ºC for 24 h. When spore development occurred, all fungi could be stored for a maximum of 4 days at 4 °C. After this time, spores germination might occur. Stock cultures containing spores from each strain were prepared and stored at -80 °C into 40% PDB, 60% glycerol.

When sporulation occurred for each fungus, spores from all fungi were removed from the mycelial surface with a sterile spatula and transferred into a saline solution containing 0.9% sodium chloride and 0.1% Tween 80 in water. The saline solution was filtered (filter pore size 20-25 µm) to separate larger particles and homogenized to disperse the spores. The spores were counted using a hemocytometer and resuspended in potato dextrose broth (PDB) to a concentration of 10^4 spores ml^{-1} under sterile conditions.

Determination of the minimum inhibitory concentration (MIC)

The MIC of the compounds was obtained based on the method used by Parente et al. (1995) and Gänzle et al. (1996), modified for fungi. In order to screen the antifungal activity of the secondary metabolites tested, their MICs for *A. niger* FUA5001*, P. roqueforti* FUA5005 and *M. plumbeus* FUA5003 were determined**.** The results demonstrated that α-chaconine was the only

compound having antifungal activity against these 3 strains. Therefore, this compound was selected and its MIC was determined for the remaining 5 fungal strains.

For this purpose, stock solutions of 20 g l^{-1} of caffeic acid and 2.4 g l^{-1} of α-solanine, α-chaconine and solanidine were prepared. α-Solanine was dissolved in methanol/acidified water (0.1 mol l^{-1} HCl) (3:1, v/v). The other compounds were dissolved in pure methanol. The stock solutions and (PDB) were mixed (1:1, v/v), yielding pH values between 5.7 and 6.0 for all samples. Two-fold serial dilutions of the compounds were prepared on 96-wells microtiter plates containing PDB. The final concentration of the compounds ranged from 0.0015 to 0.8 g l^{-1}. The solvents were evaporated over two h under a flow of sterile air. The microtiter plates were inoculated with PDB containing 10^4 fungal spores ml^{-1}. Conditions concerning temperature, light and atmospheric incubation for *A. niger* FUA5001*, P. roqueforti* FUA5005*, M. plumbeus* FUA5003**,** *F. graminearum* FG001, *My. pinodes* Is.39. and *Py. teres* f. *teres* SK51 were the same as mentioned in the section "Fungal strains and culture conditions". *Al. alternata* AA001 was incubated in parafilm-sealed microtiter plates in the dark at 28 ºC**.** *Py. tritici-repentis* 331-2 was incubated under aerobic conditions in darkness at 20 ºC. The antifungal effect of α-chaconine in combination with caffeic acid was also determined from the MIC obtained by critical dilution assay (Parente et al. 1995; Gänzle et al. 1996) for *A. niger* FUA5001*, P. roqueforti* FUA5005 *M. plumbeus* FUA5003**,** *F. graminearum* FG001and *Py. teres* f. *teres* SK51 . α-Chaconine was diluted to concentrations from 0.006 to 0.8 g l^{-1} in media containing 0.05 to 6.7 g l^{-1} of caffeic acid, in a full factorial design.

The MIC was defined as the minimum concentration of the compound that was able to inhibit 100 % of mycelial growth. The mycelial growth was visually monitored on a daily basis and the MICs were determined three days after the positive controls showed mycelial growth.

Generalized polarization in fungal membranes

Integration of α-chaconine, α-solanine and solanidine in the fungal membrane as well as their synergism with caffeic acid was assessed by measuring the LAURDAN generalized polarization (GP) using a method adapted from Molina-Höppner et al. (2004). LAURDAN (2-(dimethylamino)-6-dodecanoylnaphthalene) is a fluorescent dye that is used to study the variations of polarity in natural membranes, including hydration of the bilayer (Parasassi et al. 1994; Parasassi et al. 1998). The GP has been defined as an estimate of wavelength displacements, occurring when lipid bilayers change between gel and fluid phases (Sánchez et al. 2007). The fungal strains were grown as described in the section "Fungal strains and culture conditions", except for the following strains: *Al. alternata* AA001 was streaked in PDA plates that were then sealed with parafilm and incubated in the dark at 28 ºC for 5 d. *Py. Tritici-repentis* 331-2 was streaked into V-8 juice agar and incubated under aerobic conditions in darkness at 20 ºC for 12 d. After all strains agar plates had developed mycelia, plugs of the mycelia (diameter 70 mm) were removed with a sterile scalpel and inoculated in flasks containing 100 ml of sterile PDB. The flasks were incubated for seven days at room temperature with shaking at 150 rpm. The content of the flasks was transferred to centrifugation tubes and centrifuged at 4696 *g* for 5 min. The medium was discarded and the mycelia washed twice by adding saline solution (0.85% NaCl), shaking for 20 s, centrifuging at 4696 *g* for 5 min and discarding the saline solution. For each strain, 10 mg of mycelium was weighted in Eppendorf 1.5 mL tubes, suspended in 300 µl

of saline solution and stained with 12 µl of saturated ethanolic solution of LAURDAN for 30 min in the dark. Mycelia were washed twice by adding saline solution (0.85% NaCl), shaking for 20 s, centrifuging at 4696 *g* for 2 min and discarding the saline solution. Stock solutions of 2.4 g l^{-1} of α-chaconine and α-solanine (dissolved in methanol) or solanidine (dissolved in methanol acidified water (0.1 mol l^{-1} HCL) (3:1 v/v)), were diluted into a saline solution to a final concentration of 8.5 g l^{-1} of NaCl and one g l^{-1} of α-chaconine, α-solanine or solanidine. After centrifugation, the pellets were resuspended in each of these solutions. The same compounds were also mixed with caffeic acid and diluted into a saline solution to a final concentration of 8.5g l^{-1} of NaCl, 6.7 g $l^{-1}$of caffeic acid and one g l^{-1} of either α-chaconine, α-solanine or solanidine, and tested for synergistic effects on the membrane fluidity. Fluorescence spectra were measured at an excitation wavelength of 360 nm and emission wavelengths from 400 to 600 nm using a Varioskan Flash spectrofluorometer (Thermo Electron Corporation, Nepean, ON, Canada). GP values of solutions were calculated using emission values at 440 nm and 490 nm as previously reported by Bagatolli et al. (2003):

$$GP = (I_{440}-I_{490})/(I_{440}+I_{490})$$

Saponification and extraction of non-saponifiable lipids

The strains were grown in agar and then in PDB as described in the section “Generalized polarization in fungal membranes”. The mycelia that developed in the PDB flasks were washed twice with distilled water, freeze-dried, and stored at -20 ºC.

Saponification and extraction were performed based on the method by (Headly et al. 2002) with some modifications. For this purpose, 200 mg of ground dried mycelia was mixed with six ml of

KOH/water/methanol (6:24:70, w/v/v), left under nitrogen for one min, closed tightly, shaken and held in a 90 ºC water bath for two h; the suspension was shaken every 30 min. Unsaponifiable compounds were extracted three times with 600 ml of petroleum ether. The solvents were removed and dried under nitrogen. The residues were dissolved in methanol to a final concentration of one g l^{-1}.

Thin-layer chromatography (TLC)

Unsaponifiable lipids from the fungal membrane were separated by TLC based on the method described by Marriot (1980) with some modifications. Standard solutions of three g l^{-1} of cholesterol, ergosterol and cycloartenol were prepared, as well as solutions of three g l^{-1} of each sample. Flexible plates coated with silica gel, 10 x 10 cm, were used. The solvent system used for separation consisted of heptane/diisopropyl ether/acetic acid (60:40:4, v/v/v). After air drying, the coated plates were developed in a solvent system of 20 % phosphomolybdic acid in ethanol at 70 ºC for five min.

LC-MS analysis of sterols

Analysis and relative quantification of sterols was performed by LC-MS using an Agilent 1200 series HPLC unit consisting of a degasser, binary pump, autosampler, thermostated column compartment, and diode array detector (Agilent Technologies, Palo Alto, CA, USA), coupled to an Applied Biosystems MDS SCIEX 4000 Q TRAP LC-MS/MS System (AB Sciex, Concord, ON, Canada), which was equipped with an atmospheric pressure chemical ionization (APCI) source operating in positive mode. Sterols were eluted using a Kinetex C18 100A (100 x 3.0 mm, 2.6 µm) column. Isocratic elution was used with a mobile phase consisting of methanol/water (95:5). The run time was 15 min. The flow rate was 0.4 ml min^{-1} and the

injection volume was five μl. The analytes were monitored at 208 nm. Multiple reaction monitoring (MRM) was used for relative quantification. The APCI conditions were as follow: Vaporization temperature, 400 °C; nebulizing gas (GS1), 50 psi; curtain gas, 10 psi; nebulizer current, three μA; declustering potential (DP), 20 V; entrance potential (EP), 10 V and collision exit potential (CXP), 10 V. The collision energy (CE) was optimized for each pair of ions for all compounds using a sample of *P. roqueforti FUA500 ,* which contains all lipids identified in the samples. The values ranged from 30 to 50 eV. The two most abundant transitions (Q1→Q3) for each compound were selected for quantification and confirmation, Q1 always being the parent ion. In order to correct variations in the response and the amount of substance injected, cholesterol was added to each sample as an internal standard (IS) at a final concentration of five mg l^{-1} and the relative quantity of the compounds was calculated by dividing their analyte peak area by the IS peak area. The transitions (Q1→Q3) for all compounds are shown in **Table 4**. Due to possible variances in the response of the compounds, the relative amounts of the sterols can be compared only between samples. Data were acquired and analyzed using Analyst 1.5 software. Results are expressed as means ± standard deviations of three independent experiments.

Statistical analysis

MICs are expressed as means ± standard deviations of three independent experiments. SigmaPlot software (Systat Software, Inc., San Jose, CA, USA) was used to perform statistical analysis. The generalized polarization values were statistically analyzed by one-way ANOVA followed by the Holm–Sidak method for multiple pairwise comparisons if necessary. For all analyses, statistical significance was based on $P < 0.05$.

In order to determine the similarity among the different fungal strains regarding their sterol profile and their resistance to α-chaconine, and the relationships between these variables, principal component analysis (PCA) was performed using JMP software (version 8.0.1, SAS Institute, Inc.). For data analysis on variables measured in different scales, it is recommended to perform PCA on correlation matrix rather than covariance matrix since all the original variables are standardized to unit variance (Borgognone et al. 2001). For this study, PCA was performed on the correlation matrix and no normalization on the data was required. The correlations between fungal sterols and MICs were assessed by Pearson correlation analysis using GraphPad Prism version 5.00 (GraphPad Software).

RESULTS

Minimum inhibitory concentration of potato secondary metabolites

For a first screening of the antifungal activity of the secondary metabolites from potato, MICs of α-chaconine, α-solanine, solanidine and caffeic acid were determined against *A. niger* FUA5001*, M. plumbeus* FUA5003 and *P. roqueforti* FUA5005. Only α-chaconine had antifungal activity against these strains at concentrations below 0.8 g l^{-1}. Caffeic acid had no antifungal activity at 6.7 g l^{-1} against the same three strains. The MICs for α-chaconine were determined for all fungal species. For the purpose of this study, the strains were classified as resistant, with intermediate resistance or sensitive according to their MICs against α-chaconine. The MICs for α-chaconine of the eight species investigated and their classification according to their resistance to the same compound is shown in **Table 2.** Results revealed that *A. niger* FUA5001, *F. graminearum* FG001 and *P. roqueforti* FUA5005 were the most resistant strains, with MICs between 0.67 and >0.9 g l^{-1}. *M. plumbeus* FUA5003 and *M. pinodes* Is.39 showed intermediate resistance, with

MICs between 0.33 and 0.4 g l^{-1} and *Alternaria alternata* AA001, *Py. teres f. teres* SK51 and *P. tritici-pentis* 331-2 were the most sensitive, with MICs between 0.17 and 0.2 g l^{-1}.

To evaluate synergism between the α-chaconine and caffeic acid, the MIC of α-chaconine was determined at different concentrations of caffeic acid with five fungal strains (**Fig. 1**). Caffeic acid decreased the MIC of α-chaconine in concentrations ranging from 0.05 g l^{-1} to 6.7 g l^{-1}. For most strains, α-chaconine acted synergistically with caffeic acid as an antifungal agent. The MIC for *Py. teres f. teres* SK51*, F. graminearum* FG001, and *A. niger* FUA5001 showed an about 1000-fold decrease at the highest concentration of caffeic acid. The MIC of *P. roqueforti* FUA5005 decreased about 10-fold. For *M. plumbeus FUA5003*, the decrease was two-fold.

Measurement of membrane fluidity by generalized polarization

Generalized polarization was calculated for five fungal strains in order to determine the effect on the fluidity of the membrane caused by α-chaconine, α-solanine and solanidine, either alone or in combination with caffeic acid (**Table 3**). The changes in the GP were species dependent.

The addition of α-chaconine or α-solanine did not change the GP value of the membranes compared with the controls. The GP values decreased when solanidine was present and this change was significant for *Fusarium graminearum*. Caffeic acid in combination with solanidine significantly decreased the GP for *F. graminearum, Mu. Plumbeus* and *Al. alternata,* out of five strains.

The addition of caffeic acid to either α-chaconine, α-solanine or solanidine, generated a decrease in the GP values. This variation was significant for α-chaconine and *A. niger* and *F. graminearum,* the two most resistant strains.

Characterisation and relative quantification of sterols

An initial assessment of the sterol pattern of the fungal strains was performed by TLC separation of the unsaponifiable fraction extracted from fungal membranes (data not shown). Ergosterol and cholesterol were used as standards. The major band appeared at the same retention time for all strains and matched the retention time of the ergosterol standard. However, differences in the small bands were observed. Therefore, further analyses were carried out by LC-APCI-MS (**Fig. S1, Fig. S2**). A total of 16 different sterols was detected in all samples (**Table 4**). The mass spectra of these compounds were characteristic of sterols analysed by APCI, with $[M+H-H_2O]^+$ as the most abundant fragment in all cases (Headly et al. 2002; Sánchez-Machado et al. 2004; Cabañete-Díaz et al. 2007; Romero González et al. 2010).

Since ergosterol was the only standard available, letters from A to P were assigned to the sterols detected in the samples, according to their different *m/z*. The mass spectrum of compound F showed fragments at *m/z* 255, 297, 311, and 381, which matched the mass spectrum of brassicasterol(Mo et al. 2013). Compound A is probably dehydroergosterol or an isomer, with main fragments at *m/z* 395 and 377(McIntosh et al. 2008).

The identity of the remaining compounds could not be elucidated. Their relative quantity was determined by dividing the peak area in the MRM mode by the peak area of the internal standard, cholesterol, in order to compensate for variations in the response and the amount of substance injected. The relative quantities of the sterols found in each fungal strain are shown in **Table 5.** Although an internal standard was used, the MS response might be different between different compounds. Therefore, the relative amount of each compound can only be compared separately between strains. In all strains considered in this study, the most abundant sterols were ergosterol and compound E, the mass spectrum of which showed fragments at *m/z* 445, 427 and

409, which has been observed in 24-epibrassinolide (Huo and Liu 2010). Therefore, compound E might be a brassinosteroid or a derivative. However, brassinosteroids are mainly found in plants and the identity of compound E thus remains to be confirmed.

Relationship between membrane sterols and resistance to glycoalkaloids

Compounds G and H were present the strains resistant to α-chaconine, except in *F. graminearum* FG001, but not in all sensitive strains. Furthermore, two of the three most resistant strains, *A. niger* and *P. roqueforti*, and a strain with intermediate resistance, *M. plumbeus,* show higher amounts of most sterols, compared to the rest of the strains. Moreover, the two most sensitive strains, *Py. teres* and *Py. tritici-repentis,* have the lowest amounts and variety of sterols (**Table 5**). To determine whether there is a correlation between the sterols and resistance to glycoalkaloids, Pearson correlation analysis was performed. P values of 0.09, approaching significance were obtained for sterols G and H. Therefore, a higher amount of the sterols G and H in the fungal membrane is associated with a high MIC value of α-chaconine against any of the tested fungal strains. The relationship between membrane sterols and resistance to glycoalkaloids was also explored with principal component analysis. PCA is a multivariate statistical instrument that aims to explain the variability of a data set with the least possible number of variables (Borgognone et al. 2001, Kilimann et al. 2006). PC1 and PC2 explained 46.5% and 24.6% of the variance, respectively, and together they explain more than 70% of the data variability (Figure S1). Fungal species clustered depending on their taxonomy (**Fig. S3a and Table 1**). The biplot (**Fig. S3b**) suggested an association of sterols H, G, E and ergosterol. *A. niger* FUA5001 and *P. roqueforti* FUA5005, classified as resistant to α-chaconine, appear as positively correlated with higher amounts of the same sterols (G, E and Ergosterol) and to a high MIC.

DISCUSSION

α-Chaconine inhibited growth of 5 out of 8 fungal strains but *A. niger* FUA5001*, P. roqueforti* 5005 and *F. graminearum* FG001 exhibited resistance to α-chaconine. MICs of α-chaconine differed up to nine fold, indicating species- or strain-dependent antifungal activity. The antifungal activity of α-chaconine and α-solanine has previously been found to substantially differ between species, and α-chaconine demonstrated higher antifungal activity compared to α-solanine, (Fewell and Roddick 1993; Fewell and Roddick 1997). In addition, differences in the antifungal activity of α-solanine against 10 strains from several species have been reported (Cipollini and Levey 1997). Furthermore, the lack of antifungal activity of the aglycone solanidine confirms the importance of the sugar moiety in the membrane disruption mechanism of glycoalkaloids proposed by Keukens et al. (1995). The crucial role of the sugar moiety can also be derived from the observation that filamentous fungi detoxify α-chaconine through the removal of sugars (Weltring et al. 1997; Oda et al. 2002).

Synergism between numerous secondary metabolites in plants occurs as a defence mechanism to environmental stress (Ryabushkina 2005). The antifungal synergism between α-chaconine and caffeic acid observed in this study may relate to the defence mechanism against fungal phytopathogens. To our knowledge, synergistic antifungal activity between glycoalkaloids and phenolic compounds from potatoes has not been reported previously.

This is the first *in vitro* study on the disruption of fungal membranes by glycoalkaloids, and the results support previous findings from experiments carried out using liposome model systems (Roddick and Rijnenberg 1987; Keukens et al. 1992; Keukens et al. 1995). The GP values in the liquid phase usually range between 0.3 and -0.3, whereas the values in the gel phase are between

0.5 and 0.6 (Sanchez et al. 2007; Hofstetter et al. 2012). GP values between 0.05 and 0.6 have been reported for bacteria (Ulmer et al. 2002). The GP values of the fungal cells, when not exposed to any of the potato secondary metabolites (blanks), ranged between 0.6 and 0.76, indicating a more organized gel phase as a consequence of the presence of sterols in eukaryotic cells.

The polarity in the membrane is determined by access of water to the polar-lipid interphase (Parasassi et al. 1998) and strongly dependent on the membrane fluidity, modulated by sterols. A decrease in the LAURDAN GP value occurs when there is a change from the gel phase to a liquid-crystalline phase (Parasassi et al. 1998), which implies an increase in the polarity. The polarity of the fungal membranes was not affected by the addition of α-chaconine or α-solanine. However, a decrease in the GP value, indicating an increase in the polarity of the bilayer, was observed when solanidine was present. These changes were significant for three out of five strains. Since the mechanism of membrane disruption by glycoalkaloids proposed by (Keukens et al. 1995) implies first the insertion of the aglycone in the bilayer and then the complex formation between the sugar moieties, our results confirm that solanidine is able to insert in the phospholipidic bilayer. In the absence of a sugar moiety, hydration of the bilayer occurs. In contrast, if the hydrophilic sugar moieties are present, such as in α-chaconine and α-solanine, in spite of other changes occurring in the membrane, the water concentration of the membranes would remain unchanged, likely due to hydrophilic interactions between the sugar moieties with water molecules present in the surroundings.

The effect on the membrane caused by the three alkaloids was strongly enhanced by caffeic acid. This partially explains the synergistic antifungal effect of α-chaconine and caffeic acid observed. Caffeic acid and solanidine in combination caused the highest polarity change in the membrane.

Saponification with KOH, followed by extraction using organic solvents such as petroleum ether, hexane, pentane or diethyl ether, is the most common method for the recovery of sterols from different matrices such as oil, seaweeds, wetland and fungi (Fujino and Ohnishi 1978; Headly et al. 2002; Sánchez-Machado et al. 2004; Cabañete-Díaz et al. 2007). APCI-MS is a useful tool to identify sterols with standards; and HPLC is used for sterol quantification (Headly et al. 2002; Sánchez-Machado et al. 2004; Cabañete-Díaz et al. 2007). Since the identification of the sterols was not the aim of this study, APCI-MS was used for differentiation between the sterols present in the fungal membrane and the MRM mode was used for relative quantification since it provided higher sensitivity to detect minor components compared to HPLC.

Ergosterol was the second most abundant sterol in all fungal strains analysed, which belong to the phylum Ascomycota. This observation is in agreement with previous findings that high concentrations of ergosterol are found in fungi from the phylum Ascomycota and Basidiomycota (Newell et al. 1988; Pasanen et al. 1999). Compound F, tentatively identified as brassicasterol Mo et al. (2013), has also been found in fungi from the same phylum (Weete et al. 2010).

The sterol pattern of the fungal species relates to their taxonomic classification (Weete et al., 2010). This study demonstrated that fungal resistance to glycoalkaloids as well as the influence of glycoalkaloids are also species specific and relate to their taxonomic position. Moreover, PCA and Pearson correlation analysis suggested that resistance to α-chaconine is related to the sterol pattern, particularly to the presence of compounds G and H. Previous findings that the type of

sterols present in the membrane affects the antifungal activity of glycoalkaloids were based on model studies in liposomes (Keukens et al. 1992).

In conclusion, different fungal strains vary in their resistance to α-chaconine and possibly to other glycoalkaloids. This resistance is likely associated with their taxonomic position and the sterol pattern in their membrane. Synergistic antifungal activity between the potato secondary metabolites α-chaconine and caffeic acid was observed, likely as a plant defense mechanism against fungi. In addition, this study confirms the interaction of glycoalkaloids with fungal membranes, which was previously observed in model studies with liposomes but not in fungal cells. Overall, the results of this investigation improve the knowledge on the antifungal mode of action of potato secondary metabolites, which contributes to a better understanding of their effects in eukaryotic cells, and to the development of possible novel applications of such compounds.

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CONFLICT OF INTEREST

The authors have no conflict of interest to be declared.

REFERENCES

Bagatolli, L.A., Sánchez, S.A., Hazlett, T. and Gratton, E. (2003) Giant vesicles, Laurdan, and two photon fluorescence microscopy: evidence of lipid lateral separation in bilayers. *Methods Enzymol* 360, 481-500.

Blankemeyer, J.T., Stringer, B.K., Rayburn, J.R., Bantle, J.A. and Friedman, J. (1992) Effect of potato glycoalkaloids, α-chaconine and α-solanine, on membrane potential of frog embryos. *J Agric Food Chem* **40**, 2022-2025.

Borgognone, M.G., Bussi, J, Hough, G. (2001). Principal component in sensory analysis: covariance or correlation matrix. *Food Qual Prefer* **12,** 323-326.

Cabañete-Díaz, B., Segura Carretero, A., Fernández-Gutiérrez, A., Belmonte Vega, A., Garrido Frenich, A., Martínez Vidal, J.L. and Durán Martos, J. (2007) Separation and determination of sterols in olive oil by HPLC-MS. *Food Chem* **102**, 593-598.

Cipollini, M.L. and Levey, D.J. (1997) Antifungal activity of *Solanum* fruit glycoalkaloids: implications for frugivory and seed dispersal. *Ecology* **78**, 799-809.

Cushnie, T. P., Cushnie, B., Lamb, A. J.(2014) Alkaloids: An overview of their antibacterial, antibiotic-enhancing and antivirulence activities. *Int J Antimicrob Ag* **44**, 377–386

Daglia, M. (2012). Polyphenols as antimicrobial agents. *Curr Opin Biotechnol* **23,** 174–181

Dai, J. and Mumper, R.S. (2010) Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules* **15**, 7313-7352.

Fewell, A.M. and Roddick, J.G. (1997) Potato glycoalkaloid impairment of fungal development. *Mycol Res* **101**, 597-603.

Fewell, A.M. and Roddick, J.G. (1993) Interactive antifungal activity of the glycoalkaloids α-solanine and α-chaconine. *Phytochemistry* **33**, 323-328.

Fewell, A.M., Roddick, J.G. and Weissenberg, M. (1994) Interactions between the glycoalkaloids solasonine and solamargin in relation to inhibition of fungal growth. *Phytochemistry* **37**, 1007-1011.

Friedman, M. (2006) Potato glycoalkaloids and metabolites: Role in the plant and in the diet. *J Agric Food Chem* **54**, 8655-8681.

Friedman, M. (2004) Analysis of biologically active compounds in potatoes (*Solanum tuberosum*), tomatoes (*Lycopersicon esculentum*), and jimson weed (*Datura stramonium*) seeds. *J Chromatogr A* **1054**, 143-155.

Fujino, Y. and Ohnishi, M. (1978) Characterization and composition of sterols in the free and esterified sterol fractions of *Aspergillus oryzae*. *Lipids* **14**, 663-668.

Gänzle, M.G., Hertel, C. and Hammes, W.P. (1996) Antimicrobial activity of bacteriocin-producing cultures in meat products: modelling of the effect of pH, NaCl, and nitrite concentrations on the antimicrobial activity of sakacin P against *Listeria ivanovii* DSM20750. *Fleischwirtschaft* **76**, 409-412.

Headly, J.V., Peru, K.M., Verma, B. and Robarts, R.D. (2002) Mass spectrometric determination of ergosterol in a prairie natural wetland. *J Chromatogr A*, 149-156.

Hofstetter, S., Denter, C., Winter, R., McMullen, L.M. and Gänzle, M.G. (2012) Use of the fluorescent probe LAURDAN to label and measure inner membrane fluidity of endospores of *Clostridium* spp. *J Microbiol Methods* **91**, 100.

Huo, F. and Liu, H. (2010) Fragmentation investigation of brassinosteroid compounds by ion trap and quadrupole time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom* **24**, 3325-3334.

Keukens, E.A.J., De Vrije, T., Fabrie, C. H. J. P., Demel, R.A., Jongen, W.M.F. and De Kruijff, B. (1992) Dual specificity of sterol-mediated glycoalkaloid induce membrane disruption. *BBA-Biomembranes* **1110**, 127-136.

Keukens, E.A.J., De Vrije, T., Van den Boom, C., De Waard, P., Plasman, H.H., Thiel, F., Chupin, V., Jongen, W.M.F. and De Kruijff, B. (1995) Molecular basis of glycoalkaloids induced membrane disruption. *Biochim Biophys Acta* **1240**, 216-228.

Kilimann, K.V., Hartmann, C., Delgado, A., Vogel, R.F., Gänzle, M.G. (2006). Combined high pressure and temperature induced lethal and sublethal injury of *Lactococcus lactis*-Application of multivariate statistical analysis. *Int J Food Microbiol* **109,** 25-33.

Liu, J., Cao, T., Feng, J., Chang, K., Hwang, S. and Strelkov, S.E. (2013) Characterization of the fungi associated with ascochyta blight of field pea in Alberta, Canada. *Crop Prot* **54**, 55-64.

Marriot, M.S. (1980) Inhibition of sterol byosynthesis in *Candida albicans* by imidazole-containing antifungals. *J Gen Microbiol* **117**, 253-255.

McIntosh, A.L., Atshaves, B.P., Huang, H., Gallegos, A.M., Kier, A.B. and Schroeder, F. (2008) Fluorescence techniques using dehydroergosterol to study cholesterol trafficking. *Lipids* **43**, 1185-1208.

Mejanelle, L., Lopez, J.F., Gunde-Cimerman, N. and Grimalt, J.O. (2000) Sterols of melanized fungi from hypersaline environments. *Org Geochem* **31**, 1031-1040.

Merkl, R., Hradkova, I., Filip, V. and Smidrkal, J. (2010) Antimicrobial and antioxidant properties of phenolic acids alkyl esters. *Czech J Food Sci* **28**, 275-279.

Mo, S., Dong, L., Hurst, W.J. and Van Breemen, R.B. (2013) Quantitative analysis of phytosterols in edible oils using APCI liquid chromatography–tandem mass spectrometry. *Lipids* **48**, 949-956.

Molina-Höppner, A., Doster, W., Vogel, R.F. and Gänzle, M.G. (2004) Protective effect of sucrose and sodium chloride for *Lactococcus lactis* during sublethal and lethal high-pressure treatments. *Appl Environ Microbiol* **70**, 2013-2020.

Müller, M.M., Kantola, R. and Kitunen, V. (1994) Combining sterol and fatty acid profiles for the characterization of fungi. *Mycol Res* **98**, 593-603.

Newell, S.Y., Arsuffi, T.L. and Fallon, R.D. (1988) Fundamental procedures for determining ergosterol content of decaying plant material by liquid chromatography. *Appl Environ Microbiol* **54**, 1876-1879.

Oda, Y., Saito, K., Ohara-Takada, A. and Mori, M. (2002) Hydrolysis of the potato glycoalkaloid a-chaconine by filamentous fungi. *J Biosci Bioeng* **94**, 321-325.

Parasassi, T., Giusti, E., Gratton, E., Monaco, E., Raimond, M., Ravagnan, G. and Sapora, O. (1994) Evidence for an increase in water concentration in bilayers after oxidative damage of phospholipids induced by ionizing radiation. *Int J Radiat Biol* **65**, 329-334.

Parasassi, T., Krasnowska, E.K., Bagatolli, L. and Gratton, E. (1998) Laurdan and Prodan as polarity-sensitive fluorescent membrane probes. *J Fluoresc* **8**, 365-373.

Parente, E., Brienza, C., Moles, M. and Ricciardi, A. (1995) A comparison of methods for the measurement of bacteriocin activity. *J Microbiol Methods* **22**, 95-108.

Pasanen, A.L., Yli-Pietila, K., Pasanen, P., Kalliokoski, P. and Tarhanen, J. (1999) Ergosterol content in various fungal species and biocontaminated building materials. *Appl Environ Microbiol* **65**, 138-142.

Phillips, B.J., Hughes, J.A., Phillips, J.C., Walters, D.G., Anderson, D. and Tahourdin, C.S. (1996) A study of the toxic hazard that might be associated with the consumption of green potato tops. *Food Chem Toxicol* **34**, 439-449.

Reddy, M.K., Gupta, S.K., Jacob, M.R., Khan, S.I. and Ferreira, D. (2007) Antioxidant, antimalarial and antimicrobial activities of tannin-rich fractions, ellagitannins and phenolic acids from *Punica granatum* L. *Planta Med* **73**, 461-467.

Roddick, J.G. and Rijnenberg, A. L. (1987) Synergistic interaction between the potato glycoalkaloids α-solanine and α-chaconine in relation to lysis of phospholipid/sterol liposomes. *Phytochemistry* **26**, 1325-1328.

Roddick, J.G., Rijnenberg, A.L. and Osman, S.F. (1988) Synergistic interaction between potato glycoalkaloids α-solanine and α-chaconine in relation to destabilization of cell membranes: ecological implications. *J Chem Ecol* **14**, 889-902.

Romero-Gonzalez, R., Garrido Frenich, A. and Martinez Vidal, J.L. (2010) Chapter 64: Liquid chromatography-mass spectrometry determination of sterols in olive oil. In *Olives and olive oil and health and disease prevention* ed. Victor, P. and Ronald, W. pp. 591-601. San Diego CA, USA: Elsevier.

Ryabushkina, N.A. (2005) Synergism of metabolite action in plant responses to stresses. *Russian J Plant Physiol* **52**, 547-552.

Sammani, A., Shammaa, E., Chehna, F. and Rahmo, A. (2014). The *in-vitro* toxic effect of the glycoalkaloids for some *Solanum* species against the *LIM-1863* cell line. *Phcog J* **6(4)**, 23-31.

Sánchez, S.A., Tricerri, M.A., Gunther, G. and Gratton, E. (2007) Laurdan generalized polarization: from cuvette to microscope. In *Modern Research and Educational Topics in Microscopy.* ed. Méndez-Vilas, A. and Díaz, J. pp. 1007-1014: FORMATEX.

Sánchez-Machado, D.I., Lopez-Hernandez, J., Paseiro-Losada, P. and Lopez-Cervantes, J. (2004) An HPLC method for the quantification of sterols in edible seaweeds. *Biomed Chromatogr* **18**, 183-190.

Sánchez-Maldonado, A.F., Schieber, A. and Gänzle, M.G. (2011) Structure-function relationship of the antibacterial activity of phenolic acids and their metabolism by lactic acid bacteria. *J Appl Microbiol* **111**, 1176-1184.

Sánchez-Maldonado, A.F., Mudge, E., Gänzle, M.G. and Schieber, A. (2014). Extraction and fractionation of phenolic acids and glycoalkaloids from potato peels using acidified water/ethanol-based solvents. *Food Res Int* **65 (Part A)**, 27-34.

Sánchez-Maldonado, A.F., Schieber, A. and Gänzle, M.G. (2015). Plant defence mechanisms and enzymatic transformation products and their potential applications in food preservation: Advantages and limitations. *Trends Food Sci Technol* **46**, 49-59.

Schieber, A. and Aranda Saldaña, M.D. (2009) Potato peels: A source of nutritionally and pharmacologically interesting compounds-A review. *Food* **3**, 23-29.

Ulmer, H.M., Herberhold, H., Fahsel, S., Gänzle, M.G., Winter, R. and Vogel, R.F. (2002) Effects of pressure-induced membrane phase transitions on inactivation of HorA, an

ATP-dependent multidrug resistance transporter, in *Lactobacillus plantarum*. *Appl Environ Microbiol* **68**, 1088-1095.

Weete, J.W., Abril, M. and Blackwell, M. (2010) Phylogenic distribution of fungal sterols. *Plos One* **5**, e10899.

Weltring, K.M., Wessels, J. and Geyert, R. (1997) Metabolism of the potato saponins α-chaconine and α-solanine by *Gibberella pilicaris*. *Phytochemistry* **46**, 1005-1009.

Yencho, G.C., Kowalski, S.P., Kennedy, G.G. and Sanford, L.L. (2000) Segregation of leptine glycoalkaloids and resistance to colorado potato beetle (*Leptinotarsa decemlineata* (Say)) in F2 *Solanum tuberosum* (4x) x S. chacoense (4x) potato progenies. *Am J Potato Res* **77**, 167-178.

Zhang, C., Brandt, M.J., Schwab, C. and Gänzle, M.G. (2010) Propionic acid production by cofermentation of *Lactobacillus buchneri* and *Lactobacillus diolivorans* in sourdough. *Food Microbiol* **27**, 390-395.

FIGURE LEGENDS

Figure 1. MIC of α-chaconine at different concentrations of caffeic acid for 4 fungal strains. *A. niger* FUA5001 (●), *F. graminearum* FG001 (■), *P. roqueforti* FUA5005 (▲), *M. plumbeus* FUA5003 (◊), *Py. teres f. teres* SK51 (□). Values are means ± standard deviation (n=3)

SUPPORTING INFORMATION LEGENDS

Figure S1. Chromatogram of the sterols found in *P. roqueforti FUA5005*, which contains all sterols present in the strains investigated. The compounds were detected at a wavelength of 208 nm.

Figure S2. Mass spectra of the sterols detected in the fungal membranes; compounds are: A, B, C, Ergosterol, E, F, G, H, I, J, K, L, M, N, O and P. The *m/z* in bold were used for the relative quantification in the MRM for the transitions (Q1→Q3), Q1 always being the parent ion.

Figure S3. Biplot of principal component analysis of the fungal strains related to their amount of normalized sterols from the membrane and their MIC. Strains: *A. niger* FUA5001 (●), *F. graminearum* FG001 (■), *P. roqueforti* FUA5005 (▲) *M. plumbeus* FUA5003 (□), *M. pinodes* (⚹), *Alternaria alternata* AA001 (○), *Py. teres f. teres* SK51 (□), *Py. tritici-repentis* 332-1 (△). Ergosterol (Erg).

Interpretation: The sum of the principal components shows how much of the variability of the data is explained by these two principal components. Strains that appear close to each other are similar and different to those that appear far in the biplot. The length of the line of each variable (sterols and MIC) is a representation of how well the variable is represented by the principal components plotted in the biplot. Variables in the same area are highly positively correlated, **always that the line is long enough to show that is well represented by those components**. The biplot shows that principal components 1 and 2, explain more than 70% of the variability of the data for this analysis. *Alternaria alternata* AA001*, Py. teres f. teres S*K51, and *Py. tritici-repentis* 332-1, from the family Pleospora, are clustered together in the left low quadrant, as well as *M. pinodes,* which belongs to the same order, *Pleosporales*. *A. niger* FUA5001 and *P. roqueforti* FUA5005*,* which belong to the same subphylum *Pezizomycotina* but to a different class, *Eurotiomycetes* clustered together, in the upper left side of the chart. *F. graminearum* FG001, from the same subphylum, is also located in the left side. *M. plumbeus* FUA5003 is taxonomically different from all the other tested strains; it belongs to the phylum Zygomycota

and is located in the right side of the score plot, far from all others. There is a high correlation between the presence of sterols H, G, E and ergosterol. There is also a correlation between these sterols and a high MIC, however, the length of the MIC in the loading plot shows that the representation of the MIC by PC1 and PC2 is less than it is for the sterols. *A. niger* FUA5001 and *P. roqueforti* FUA5005, classified as resistant to α-chaconine, seems to be positively correlated with higher amounts of the same sterols (H, G, E and Ergosterol) and to a high MIC. *M. plumbeus* FUA5003, with intermediate resistance, was correlated with sterols A, C, I, J, K, L, M, N, O and P. *F graminearum, My. pinodes Is.39, Alternaria alternata* AA001, *Py. teres f. teres* SK51, and *P. triticirepentis* 332-1 were not correlated to any of the sterols.

TABLES

Table 1. Fungal strains investigated in this study

Species (isolate)	**Class**	**Family**	**Occurrence/ pathogenicity**
Aspergillus niger FUA5001*	*Eurotiomycetes*	*Aspergillaceae*	Spoilage mold
Penicillium roqueforti FUA5005, from blue cheese (1)*	*Eurotiomycetes*	*Aspergillaceae*	Spoilage mold
Alternaria alternata AA001†	*Dothideomycetes*	*Pleosporaceae*	Brown spot or brown spot in several plants including citrus
Pyrenophora teres* f. *teres SK51, from barley†	*Dothideomycetes*	*Pleosporaceae*	Net blotch of barley
Pyrenophora tririci-repentis 331-2, from infected wheat†	*Dothideomycetes*	*Pleosporaceae*	Tan spot in wheat
Mycosphaerella pinodes Is.39, from infected peas (2)†	*Dothideomycetes*	*Didymellaceae*	Ascochyta blight in peas
Fusarium graminearum (FG001†)	*Pyrenomycetes*	*Nectriaceae*	Fusarium head blight on wheat and barley
Mucor plumbeus FUA5003*	*Mucormycotina*	*Mucoraceae*	Spoilage mold

*Strain collection of the Food Microbiology Laboratory at the University of Alberta; †Dr. Strelkov collection (University of Alberta)

References: **(1)** (Zhang et al. 2010); **(2)** (Liu et al. 2013)

Table 2. MICs of α-chaconine to the fungal strains and classification according their resistance

STRAIN	MIC	SDEV	RESISTANCE
A. niger 5001	>0.9	0	Resistant
F. graminearum FG001	>0.9	0	Resistant
P. roqueforti FUA5005	0.67	0.23	Resistant
M. plumbeus FUA5003	0.40	0.00	Intermediate
My. pinodes Is.39	0.33	0.12	Intermediate
Al. alternata AA001	0.20	0.00	Sensitive
Py. teres f. teres SK51	0.17	0.06	Sensitive
Py. tritici-repentis 331-2	0.17	0.06	Sensitive

Table 3. Generalized polarization of fungal membranes after addition of glycoalkaloids α-chaconine (α-C), α-solanine (α-S), solanidine (S), caffeic acid (CA)

Compound	**Generalized polarization values**									
	A. niger FUA5001		***F. graminearum FG001***		***M. plumbeus FUA5003***		***Alternaria alternata AA001***		***Py. teres f. teres SK51***	
Blank *	0.60 ± 0.04	**a, b**	0.72 ± 0.09	**a**	0.60 ± 0.10	**a**	0.72 ± 0.11	**a**	0.76 ± 0.12	**a**
α-C	0.67 ± 0.09	**a**	0.69 ± 0.02	**a,b**	0.47 ± 0.05	**a,b**	0.69 ± 0.005	**a**	0.70 ± 0.05	**a**
α-C+CA	0.55 ± 0.01	**b**	0.49 ± 0.11	**c**	0.52 ± 0.19	**a**	0.50 ± 0.19	**a, b**	0.57 ± 0.18	**a**
α-S	0.53 ± 0.02	**b**	0.73 ± 0.04	**a**	0.71 ± 0.10	**a**	0.59 ± 0.04	**a**	0.71 ± 0.09	**a**
α-S+CA	0.51 ± 0.04	**b**	0.59 ± 0.06	**b,c**	0.50 ± 0.19	**a**	0.53 ± 0.20	**a, b**	0.62 ± 0.02	**a**
S	0.58 ± 0.05	**a, b**	0.49 ± 0.01	**c**	0.49 ± 0.10	**a**	0.61 ± 0.02	**a**	0.69 ± 0.11	**a**
S+CA	0.56 ± 0.03	**b**	0.53 ± 0.10	**c**	0.21 ± 0.13	**b**	0.39 ± 0.16	**b**	0.63 ± 0.23	**a**

n=3; GP values in the same column with different letters differ significantly ($P < 0.05$)

* MeOH/H_2O/formic acid (50:50:1)

Table 4. Retention times and fragments determined for the extracted sterols.

Compound	**Retention time** (min)	*m/z* $[M+H]^+$	$[M+H-H_2O]^+$	Other
A	4.55	395	377	209
B	5.13	397	379	271
C	5.42	425	407	267
Ergosterol	5.96	397	379	295
E	5.96	445	427	409
F	5.95	399	381	297
G	6.48	411	393	295
H	6.54	459	441	423
I	6.86	399	381	213
J	7.05	413	395	325
K	7.06	383	365	145
L	7.57	423	405	149
M	7.58	409	391	149
N	8.39	427	409	241
O	8.41	441	423	149
P	8.44	437	419	149

Table 5. Relative amounts of unsaponifiable lipids from the fungal membrane of different fungal strains

	A. niger FUA5001	*F. gramin.* FG001	*P. roqueforti* FUA505	*M. plumbeus* FUA5003	*My. pinodes* Is.39	*Al. alternata* AA001	*Py. teres* SK51	*Py. tritici-rep.* 331-2
Resistance to α-chaconine*	**R**	**R**	**R**	**I**	**I**	**S**	**S**	**S**
Unsaponifiable lipids†,‡	**Peak area of unsaponifiable lipid/Peak area of internal standard**							
A	0 ± 0	68 ± 20	113 ± 10	246 ± 29	141 ± 16	0 ± 0	0 ± 0	13 ± 7
B	0 ± 0	0 ± 0	257.5 ± 57.1	71 ± 12	87 ± 5	82 ± 16	0 ± 0	26 ± 8
C	53 ± 14	15 ± 7	53.7 ± 58.2	153 ± 17	0 ± 0	26 ± 6	31.9 ± 6.1	18 ± 8
D, Erg	1520 ± 79	808 ± 248	1338 ± 342	1122± 152	943 ± 71	1016 ± 85	1197 ± 153	1057± 169
E	4468 ± 372	2781 ± 915	3202 ± 687	3156 ± 629	2439 ± 521	2947 ± 244	3059 ± 311	3368 ± 555
F	230 ± 10	119 ± 36	417 ± 96	346 ± 37	189 ± 24	458 ± 120	297 ± 67	232 ± 73
G	86 ± 7	0 ± 0	82 ± 23	12 ± 2	0 ± 0	0 ± 0	0 ± 0	0 ± 0
H	72 ± 12	0 ± 0	61 ± 11	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
I	62 ± 2	62± 13	42 ± 4	188 ± 23	8 ±3	81 ± 8	147 ± 24	48 ± 14
J	36 ± 3	52 ±17	71 ± 20	100 ± 19	20 ± 3	35 ± 9	28 ± 6	14 ± 4
K	52 ± 4	67 ± 15	41 ± 8	102 ± 12	49 ± 11	50 ± 2	71 ± 11	32 ± 4
L	24 ± 2	11 ± 4	25 ± 7	149 ± 20	77 ± 23	39 ± 3	84 ± 70	0 ± 0
M	211 ± 38	63± 18	162 ± 26	938 ± 105	548 ± 197	328 ± 133	37 ± 12	0 ± 31
N	40 ± 3	81 ± 3	46 ± 17	267 ± 36	31 ± 6	64 ± 10	51 ± 18	21 ± 50

O	**9 ± 1**	**51 ± 16**	**32 ± 11**	**140 ± 9**	**26 ± 13**	**15 ± 4**	**11 ± 4**	**4 ± 2**
P	**20 ± 4**	**117± 32**	**81 ± 34**	**313 ± 64**	**82 ± 8**	**34 ± 8**	**29 ± 11**	**7 ± 11**

Values are means ± standard deviation, n=3

*Resistant (R), intermediate resistance (I), sensitive (S).

†Shades in cells correspond to comparisons between the amounts of each sterol among the 8 fugal strains. Darker shades indicate high relative amounts and lighter shades indicate low relative amounts.

‡The main *m/z* of the compounds are in **Table 3.**

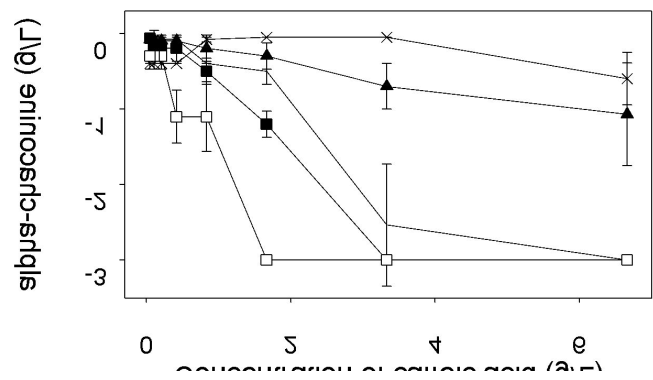

Figure 1 Sánchez-Maldonado et al.