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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.

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