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Chlorogenic acid is a fungicide active against phytopathogenic fungi

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ABSTRACT

Plants synthesize diverse types of secondary metabolites and some of them participate in plant protection against pathogen attack. These compounds are biodegradable and renewable alternatives, which may be envisaged for the control of plant pests and diseases. Chlorogenic acid (CGA) is a phenolic secondary metabolite which accumulates in diverse plant tissues and can be found in several agro-industrial by-products and waste. The aim of this work was to determine whether CGA could control the growth of various plant pathogenic fungi, gaining insight into its mechanism of action. Microscopic analysis showed the complete inhibition of spore germination or reduction of mycelial growth for *Sclerotinia sclerotiorum*, *Fusarium solani*, *Verticillium dahliae*, *Botrytis cinerea* and *Cercospora sojina*. CGA concentrations that did not completely abolish spore germination were able to produce a partial inhibition of mycelial growth. Viability tests and vital dye staining demonstrate that CGA induces fungal cell lysis. Its fungicidal activity involves an early membrane permeabilization of the spores. These results show the antifungal activity of CGA against phytopathogenic fungi relevant in horticulture and agriculture highlighting the potential of CGA-enriched wastes and by-products to be used as biofungicides.

1. Introduction

Synthetic fungicides have been extensively used to protect crops but are now under debate due to the development of resistance as well as environmental concerns. In this context, biofungicides emerge as a promising tool for plant disease control and growing interest is being observed on the search for novel natural compounds with this properties. Plants produce a vast array of secondary metabolites that are supposed to protect them from the attack of microbial pathogens [1]. It has been shown that some of these natural products can act as fungicides and could be useful for disease control in agriculture [2,3]. Consequently, natural products are attracting the attention because they are cheaper, safer and eco-friendly. Among the main groups of natural compounds synthesized by plants are terpenoids, tannins, alkaloids, flavonoids and phenolic acids [4]. Phenolic acids contain a phenolic ring and an organic carboxylic acid function. Two important naturally occurring types of phenolic acids are hydroxybenzoic acids and hydroxycinnamic acids, which are derived from benzoic and cinnamic acid, respectively. Chlorogenic acid (CGA), the ester of caffeic acid and quinic acid, is a hydroxycinnamic acid synthesized by plants via the phenylpropanoid pathway [5,6]. CGA is one of the most widespread soluble phenolic compounds in the plant kingdom and has been reported to be enriched in diverse plant sources such as *Eucommia* and artichoke leaves as well as honeysuckle flowers, potato tubers (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), apple (*Malus domestica*), and coffee beans [7,8].

Until now CGA has mainly been studied as an antioxidant relevant in food sciences [9] since it displays several beneficial properties to human health such as the regulation of glucose and lipid metabolism [10], anticarcinogenic effects [11], reduction of the blood pressure [12], cognitive and neuroprotective effects [13], etc. Nevertheless, CGA can also exert antimicrobial activity. It has been reported that it is able to inhibit the growth of the human pathogenic yeast *Candida albicans* [14] and its application reduces powdery mildew severity in leaves of rose [15]. Besides, transgenic tomato plants with elevated levels of CGA display enhanced resistance towards the bacterial pathogen *Pseudomonas syringae* [16]. This evidence has prompted us to investigate whether CGA could control the growth of different phytopathogenic fungi which produce important loses in crops and horticulture, gaining insight into its mechanism of action. This aim becomes particularly interesting since CGA-enriched extracts may be obtained from several agroindustrial by-products which could be envisaged as bio-fungicides.

2. Materials and methods

2.1. Chemicals

Chlorogenic acid (1, 3, 4, 5-tetrahydroxycyclohexanecarboxylic acid 3-(3,4-dihydroxycinnamate), 3-(3,4-dihydroxycinnamoyl) quinic acid) was purchased from Sigma-Aldrich (C3878); stock solutions 200 mg/ml in ethanol were freshly diluted in water for further use. PDA (Potato

Abbreviations: CGA, chlorogenic acid; ELISA, Enzyme-Linked Immuno Sorbent Assay; OD, optical density; PDA, potato dextrose agar; PDB, potato dextrose broth.

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Dextrose Agar) was from Difco™ (Ref 213400) and PDB (Potato Dextrose Broth) from Sigma Aldrich (P6685). Propidium iodide (Sigma Aldrich, 81845) stock solution 1 mg/ml was freshly prepared diluted in water.

2.2. Fungi

The fungi *Fusarium solani eumartii*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Verticillium dahliae* and *Cercospora sojina* were from the collection of the Instituto de Investigaciones Biológicas, Universidad Nacional de Mar del Plata, Mar del Plata, Argentina. Fungi were grown for 15 days at 25 °C in Petri dishes containing 39 g/l PDA supplemented with 100 μg/ml ampicillin and 100 μg/ml chloramphenicol, except *C. sojina* that was grown without antibiotics.

To collect fungal spores from *C. sojina*, *F. solani*, *B. cinerea* and *V. dahliae* 1 ml of sterile water was poured on the plates and different methods were used depending on the fungus (brushing, released with a spatula or washing with 0.05% Tween 20, respectively). Each suspension obtained was duly filtered through gauze to prevent the passage of mycelial debris that might be in solution together with the spores. *S. sclerotiorum* ascospores were kindly provided by Advanta Semillas S.A.I.C. (Balcarce, Argentina) and were collected from Petri dishes containing imprints of apothecia. All spore suspensions were quantified in a Neubauer chamber under optical microscopy. Collected spores were immediately used or stored at 4 °C for 1–5 days.

2.3. Antifungal activity assay

The assay for inhibition of fungal spores germination was performed on microslides. The incubation mixture contained 1000 spores, 2% sucrose and the CGA sample, in a final volume of 20 μl. Controls were performed replacing the CGA solution with the same volume of water. After 16 h of incubation at 25 °C and 100% relative humidity the slides were evaluated for the presence of hyphae under a light microscope (Eclipse E200 microscope, Nikon).

The ability of CGA to inhibit mycelial growth tests was quantified using flat microtitre plates containing PDB medium, as described in Regente & de la Canal 2000 [17], with minor modifications. Briefly, different concentrations of CGA and 2×10^4 *F. solani* conidia were incubated at 25 °C in a final volume of 100 μl. Fungal growth was monitored at 550 nm in an ELISA plate reader. Quantitative tests were performed by triplicate and repeated at least twice. Growth curves were compared using a regression analysis (Infostat/L).

2.4. Evans blue and propidium iodide staining

After evaluation of the antifungal activity in microslides, Evans Blue dye was added to a final concentration of 0.05% and incubated for 10 min at room temperature. Fungal cells were observed by optical microscopy [18].

To test membrane permeabilization assays were performed in microslides as previously described, but incubated for only 1 h. After treatment, propidium iodide was added to a final concentration of 50 μg/ml and observed under fluorescence microscopy using an Eclipse E200 microscope (Nikon) equipped with an epifluorescence unit and a G-2E/C filter set containing an excitation filter at 540/25 nm, a suppressor filter at 630/60 nm and a dichroic mirror at 565 nm [19,20].

2.5. Viable cell counting

The evaluation of the fungicidal activity of CGA was assessed by measuring the radial growth of the fungus in PDA plates. One thousand spores of *F. solani* were incubated in tubes during 24 h at 25 °C in the presence of 2% sucrose and different concentrations of CGA (5 μg/μl and 15 μg/μl) or water as a negative control. Subsequently, 20 μl of each treatment were seeded in the center of a PDA plate. Plates were incubated at 25 °C and the diameter of the colony was measured at 24, 48 and 72 h after inoculation [21]. For all the treatments 3 replicates were made and the results were compared by ANOVA (Infostat/L).

3. Results

3.1. Chlorogenic acid inhibits spore germination and mycelial growth of plant pathogenic fungi

As a first approach to evaluate the effect of CGA on phytopathogenic fungi, spores/conidia from *Sclerotinia sclerotiorum*, *Fusarium solani*, *Verticillium dahliae*, *Botrytis cinerea and Cercospora sojina* were incubated overnight in microslides in the presence of different concentrations of CGA and then analyzed by optic microscopy. Fig. 1 illustrates the antifungal activity of CGA; spores incubated in water (control) germinated producing hyphae while a complete inhibition of the spore germination was observed for all fungi when 15 μg/μl CGA was present. A lower concentration of this phenolic acid abolished germination or reduced mycelial growth, depending of the fungus. *S. sclerotiorum* seemed particularly affected by CGA displaying not only the inhibition of germination but also accumulation of materials around the ascospores, suggesting cell lysis (Fig. 1). On the other hand, *B. cinerea* and *F. solani* appeared to be less sensitive to CGA action, since some conidia were able to germinate and outgrowth hyphae even if mycelial proliferation was clearly reduced. This latter effect was also verified for *F. solani* growing in rich medium in microwells. As shown in Fig. 2 fungal growth monitored as OD 595 nm was reduced in the presence of CGA 10 μg/μl. *F. solani* growth inhibition by different doses of CGA was also assessed in liquid medium by evaluation of optical density and comparison of fungal growth in the absence of added phenolic compound. As seen in Fig. 3, a clear dose-dependent growth inhibition is observed, attaining a 96% after treatment with 10 μg/μl CGA.

3.2. CGA is a fungicide

In order to assess the effect of CGA on fungi a variety of viability assessment methods were used. First we have performed staining with the vital dye Evans Blue [22]. After overnight incubation of *F. solani* with 15 μg/μl CGA conidia did not germinate and appeared blue stained indicative of the penetration of the dye into non-viable cells (Fig. 4). Moreover, a detailed observation evidenced protrusions in conidia that seem to be cell leakage.

To confirm if CGA was killing fungal cells acting as a fungicide, plate viability assays were performed. For this, spores of *F. solani* were incubated in the presence of CGA for 24 h at 25 °C. They were then plated in the center of Petri dishes containing PDA to evaluate spore survival and the ability to develop a radial growth. Fig. 5A illustrates the growth inhibition observed for low doses of CGA and the absence of fungal growth observed when the spores were incubated with15 μg/μl CGA. Quantification of this effect revealed that significant differences occur between the control and the two doses of CGA after 24 h of plating. Measurements at 48 h and 72 h did not show statistical differences for the lowest dose but incubation of the spores with 15 μg/μl CGA completely abolished fungal growth (Fig. 5B). This observation confirms that CGA is acting as a fungicide and not as a fungistatic, since a recovery of fungal growth would be expected upon dilution of the spores in PDA if they were still alive.

3.3. Effect of CGA on fungal cell membrane

As an insight into the mode of action of CGA we have evaluated fungal membrane permeabilization through the use of the fluorescent dye propidium iodide which is excluded from viable cells. Cells with compromised membrane integrity uptake the probe and appeared red-labeled upon binding of the dye to DNA. So, *F. solani* spores were incubated in the presence of CGA, further stained with the dye and