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Control of infection of tomato fruits by *Alternaria* and mycotoxin production using plant extracts

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Abstract Tomato fruits are susceptible to infection by *Alternaria* species. In addition, *Alternaria* species may contaminate the fruits with mycotoxins. There is thus interest in control systems to minimise pathogenicity and control toxin production. The objectives of this study were to examine the effect of plant extracts of *Eucalyptus globulus* and *Calendula officinalis* on the growth of strains of *Alternaria alternata* and *A. arborescens*, on pathogenicity of tomato fruits and mycotoxin production. The growth bioassays showed that the ethanolic and chloroformic fractions of *E. globulus* were the most effective in reducing growth of *A. alternata* (66–74 %) and *A. arborescens* (86–88 %), respectively at 2500 μg/g. The effects of plant extracts on mycotoxin biosynthesis were variable and strain dependent. The most effective fractions in decreasing mycotoxin accumulation were the ethanolic and chloroformic extracts of *E. globulus*, which reduced tenuazonic acid by 89 %, alternariol by 75–94 % and almost complete inhibition of alternariol monomethyl ether. All the tested fractions reduced percentage of infected tomato fruits when compared to the controls. The ethanolic and chloroformic fractions of *E. globulus* completely inhibited growth of *A. alternata* and *A. arborescens* on unwounded fruits and reduced the aggressiveness on wounded fruits of strains of both species significantly.

Keywords Tenuazonic acid · Alternariols · Antifungal · *Eucalyptus globulus* · *Calendula officinalis*

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Introduction

Alternaria is a fungal genus that includes both plant-pathogenic and saprophytic species which may affect crops in the field or cause post-harvest decay of plant products. It has been isolated from small grain cereals, citrus fruits, tomato fruit, blueberries, olives, apples (Greco et al. 2012; Logrieco et al. 2009). Tomato fruits (*Lycopersicon esculentum*) are highly susceptible to infection by *Alternaria* species because of the high moisture content, rich nutrient content and thin skin. *Alternaria alternata* causes a disease known as blackmould and *Alternaria arborescens* is the causal agent of stem canker in this plant. These *Alternaria* species are also known to produce many secondary metabolites which play an important role in plant pathogenesis.

Tenuazonic acid (TeA), alternariol (AOH), alternariol monomethyl ether (AME) and altertoxin I (ALTX-I) are considered the main *Alternaria* mycotoxins because of their known toxicity and their frequent presence as natural contaminants in food (Pose et al. 2010). These compounds have been reported as non-host-specific phytotoxins to several crops. In *Arabidopsis thaliana*,

TeA blocked the QB-binding site, impeding the photosynthetic electron transport at the acceptor side of PSII (Chen et al. 2015; Chen et al. 2007). Graf et al. (2012) reported that a mutant strain lacking its ability to produce AOH could only very scarcely colonize tomato tissue, in contrast to the producer wild type strain. This suggests the role of AOH as a key colonization factor during *Alternaria* infection of tomatoes. When these compounds accumulate in edible parts of the plant, such as tomato fruits, they can exert toxic effects on humans and animals. The toxicity of TeA has been reported in chick embryos and several animal species, including guinea pigs, mice, rabbits, dogs, and rhesus monkeys (Solfrizzo et al. 2005). TeA has been related to onyalay, a human haematological disorder occurring in Southern Africa and to oesophageal cancer in Linxian Province, China (Steyn and Rabie 1976). AME and AOH were described as mutagenic, carcinogenic, genotoxic and cytotoxic in microbial and mammalian cell systems (Pavón et al. 2012). Lehmann et al. (2006) have reported the oestrogenic potential of AOH, its inhibitory effects on cell proliferation, and its genotoxic effect in cultured mammalian cells.

While fungicides are the main prevention measure employed to control fungal infections of tomato fruit, some drawbacks have arisen in the last time. The development of microorganisms' resistance and the presence of fungicide residues in food resulted in interest in finding alternative natural compounds (da Cruz Cabral et al. 2013). *Calendula officinalis* has been used topically for the treatment of inflammation and skin wounds. Besides, it has been used in food as a spice and as natural colorant (yellow to orange) due to lycopene and lutein contained in its flowers' extracts (Efstratiou et al. 2012; Re et al. 2009). Its use for food has been approved in USA and appears in the FDA's list of GRAS (Generally Recognized As Safe) substances (Food and Drug Administration, 2013a). Several *Eucalyptus* species were traditionally used in medicine as antiseptics, to treat inflammatory diseases, as analgesics, and against infections of the upper respiratory tract. Numerous biological activities have been associated with eucalyptus extracts, such as antibacterial, antifungal, antioxidant, herbicidal, and acaricidal activity (Hasegawa et al. 2008; Singh et al. 2009; Tyagi and Malik 2011). In particular, *E. globulus* leaves are allowed by FDA for their use as natural flavouring in food (Food and Drug Administration, 2013b). Due to their history of use for a wide variety of applications, both plants have great potential as natural antifungals in foods.

Few studies have examined the potential of plant extracts to control *Alternaria* species growth (Feng and Zheng 2007; Carvalho et al. 2011; Prakash et al. 2015). To our knowledge, the efficacy of plant extracts in reducing *Alternaria* mycotoxin accumulation has not been investigated yet.

The aim of this work was to study the antifungal activity of extracts from two plants, *Eucalyptus globulus* and *Calendula officinalis*, against strains of two fungal pathogens *Alternaria alternata* and *A. arborescens* on tomato fruit. Thus the effect of solvent extracts of these plants were screened for efficacy against (a) in vitro growth, (b) control of production of three mycotoxins (TeA, AOH, AME) and (c) control of pathogenicity on non-wounded and wounded tomato fruits.

Materials and methods

Fungal species

Two *Alternaria* strains were used in this study. *A. alternata* EGS 34016 is a representative strain obtained from the culture collection of Emory G. Simmons (Mycological Services, Crawfordsville, IN, USA). *A. arborescens* was isolated from tomato fruit affected by blackmould and was identified by morphological classification according to Simmons (2007). Both strains were maintained in Potato Dextrose Agar (PDA) (Pitt and Hocking 2009).

Plant extraction

The plants used for this study, *Eucalyptus globulus* (E.gl) and *Calendula officinalis* (C.o), were purchased from a local herbal drugstore in Buenos Aires, Argentina. The aerial parts of both plants were extracted with solvents with increasing polarity: chloroform, ethanol, methanol and methanol:water (70:30). The extraction was carried out with 200 g of each plant and 2.5 L of each solvent by mechanical agitation at 300 rpm for 6 h at 25 °C. The extracts were filtered through paper and evaporated to dryness in a rotary evaporator at 45 °C. The dry mass obtained was weighted and resuspended in a measured volume of ethanol to produce a stock extract solution of known concentration. These

stock solutions were sterilized by filtration through filters of 0.2 μm pore and store at −20 °C until analysis.

In vitro antifungal assay

The capability of plant extracts to inhibit fungal growth was tested in vitro using Malt Extract Agar (MEA) (Pitt and Hocking 2009). Spore suspensions of seven day-old PDA cultures from *A. alternata* and *A. arborescens* were prepared in a 0.05 % Tween 80 solution at a concentration of 10^6 spores/mL. Each extract was added to the molten medium to obtain final concentrations of 500, 1250 and 2500 μg/g agar. 90 mm plates were inoculated centrally with 2 μL of the spore suspension. All treatments were carried out with three replicates per treatment. Two types of controls were used: inoculated plates without extracts or ethanol (C); and solvent controls (SC) with ethanol added at the same concentration as in the treatments. The Petri plates were incubated at 25 °C for 7 days in the dark. The inhibitory effect was evaluated by measuring two perpendicular diameters of each colony. Growth inhibition was calculated based on the percentage difference of the average diameter compared to control treatments: %I = ((C - T)/C) × 100 where %I is percent inhibition, C is the average diameter of the control cultures, and T is the average diameter of treatment cultures.

Determination of mycotoxins production

To test the effect of plant extracts on production of the three mycotoxins (TeA, AOH and AME), all MEA plates were incubated in the dark for 21 days at 25 °C. Mycotoxin extraction was carried out following the method described by Pose et al. (2010) with minor modifications. Briefly, the colony biomass grown on 20 g of agar was mixed with 80 mL of methanol by mechanical agitation at 300 rpm for 30 min. Then, 25 mL of a 10 % ammonium sulphate solution was added. The mixture was filtered, transferred to a separating funnel, and 20 mL hexane was added. The mixture was shaken and the aqueous phase was extracted. The hexane phase was washed with 25 mL of cold water and was discarded after separation. Two extractions with 20 mL chloroform were conducted to recollect the fraction with neutral metabolites (AME and AOH) which was separated for analysis. The remaining aqueous phase was acidified to pH 2 with HCl 6 N and was extracted twice with 20 mL chloroform. These chloroform fractions were collected in a separating funnel, washed with 15 mL water, filtered through anhydrous sodium sulphate and recollected to analyse acid metabolites (TeA). Both neutral and acid chloroform extracts were evaporated in a rotary evaporator at 40 °C. The residues were dissolved in 4 mL HPLC grade methanol and stored at −18 °C until analysis. Mycotoxin quantification was performed by an HPLC system consisting of a Shimadzu LC 6 A liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a Rheodyne sample valve fitted with a 20 μL loop and a Shimadzu UV detector Model SPD-6 A. The analytical column was a Jupiter 4.6 × 250 mm 5 μ C18 (Phenomenex, USA). The mobile phase was methanol:water (80:20) containing 300 mg/L $ZnSO_4.H_2O$ for AOH and AME, and methanol:water (90:10) containing 300 mg/L $ZnSO_4.H_2O$ for TeA. A flow rate of 0.4 mL/min was used. The wavelength for recording chromatograms was 258 nm for AOH and AME, and 280 nm for TeA. A calibration curve was constructed for quantification purposes using toxin standards and correlating peak area versus concentration. Detection limits of the method were established as the minimum concentration of the toxins detected in the samples that allowed confirmation by the diode array detector (10 μg/kg, 16 μg/kg and 5 μg/kg for AOH, AME and TeA, respectively).

Fungal pathogenicity and aggressiveness on tomato fruit

To evaluate the inhibitory activity of the plant extracts on *Alternaria* spp. pathogenicity and aggressiveness, an in vivo assay was performed on tomato fruits from the "platense" variety. Healthy tomato fruits of similar size and shape were selected for the experiment. The fruits were first washed with tap water and then submerged in a 1 % sodium hypochlorite solution for two min. After removing from the disinfectant solution they were rinsed with sterile distilled water and dried in a laminar flow. Each tomato fruit was labelled and two delimited regions were indicated. One of the regions was wounded (two mm deep and three mm width) with a sterile scalpel and the other was left unwounded. Each fruit weighting 200 g was sprayed with 10 mL of a 50 mg/mL solution of the corresponding treatment extract in ethanol and were left to dry in a laminar flow cabinet for 30 min. In this way, the final concentration achieved was 2500 μg extract/g of fruit. The sprayer was held 30 cm from the fruit yielding a fine mist in order to achieve a

homogeneous distribution. Two μL of a 10^6 spores/mL suspension prepared as in Section 2.3 was used to inoculate each tomato, both in the wounded and healthy areas. Ten replicates were made for each treatment and each *Alternaria* species, and two independent replicates of the complete experiment were performed. Positive controls were made with fruits inoculated with the same strains. The negative controls were uninoculated fruits sprayed with ethanol. To discard an inhibitory effect due to ethanol, a third type of control (solvent control) was made with inoculated fruits sprayed with ethanol alone. The fruits were incubated at 25 °C for five days. After the incubation period, the number of infected fruits was recorded and the size of the lesion was measured. Pathogenicity was expressed as the percentage of infected fruits and aggressiveness was scored by measuring the lesion diameter.

Statistical analysis

Statistical analysis of fungal growth, mycotoxin content and lesion diameter data was performed by analysis of variance (ANOVA). Means between treatments were compared by the method of Tukey ($p < 0.05$). Statistical analysis was performed by Statistix software v 8.0 (Analytical Software, FL, USA). Least square regression analyses were made to evaluate correlation between growth inhibition data and extract concentration using Microsoft Office Excel 2007. Goodness of fit was evaluated by regression coefficient (R^2) values.

Results

Inhibition of fungal growth by *E. globulus* and *C. officinalis* extracts

Figure 1 shows that the growth of both *Alternaria* species was significantly inhibited when compared to the untreated controls. There was no inhibition of the growth of the *Alternaria* species by the ethanol solvent control treatment. Overall the E.gl extracts were more effective at inhibiting growth than C.o. The chloroformic and ethanolic extracts of E.gl were the most effective treatments resulting in 88 and 86 % inhibition against *A. arborescens* at 2500 μg/g.

The ANOVA showed that all effects (plant, extraction solvent, concentration, fungal strain and their interactions) significantly affected the growth inhibition ($p < 0.01$).

The percentage of inhibition in all cases increased with plant extract concentration. To evaluate the type of relationship existing between both variables, the %I vs extract concentration curves were fitted using different functions (linear, logarithmic and exponential), and the regression coefficient (R^2) were used to assess the goodness of fit. Our results showed that the type of correlation between both variables depended on each extract and fungal species. In a few cases, a linear relationship was observed (e.g. *A. alternata*/E.gl, methanol $R^2 = 0.9996$; *A. arborescens*/E.gl, ethanol $R^2 = 0.9945$). In others, an exponential behaviour was detected (e.g. *A. arborescens*/C.o, methanol $R^2 = 0.9518$). However, in most cases the increment of inhibition described a logarithmic relationship with extract concentration. This response was observed, for example, for *A. alternata* with the ethanolic E.gl extract ($R^2 = 0.932$) or *A. arborescens* with methanol:water E.gl extract ($R^2 = 0.9439$) as well as the ethanolic and methanol:water C.o extracts against both strains ($R^2 = 0.9627$ and $R^2 = 0.8825$ for *A. alternata* and $R^2 = 0.9783$ and $R^2 = 0.9896$ for *A*, *arborescens*, respectively).

Effect of *E. globulus* and *C. officinalis* extracts on mycotoxin production by *Alternaria* spp.

Table 1 shows the effects of plant extracts on mycotoxin biosynthesis. The behaviour was toxin and strain dependant. According to ANOVA, all effects (toxin x strain x plant extract), as well as their interactions, were significant ($p < 0.05$).

A. alternata was not able to produce TeA under any treatment or in the controls. The production of TeA by *A. arborescens* was significantly reduced by all E.gl extracts at all concentrations evaluated, except for methanol:water fraction at 500 μg/g. Increasing the concentration of the extract resulted in lower amount of toxin accumulated by this species. The ethanolic and chloroformic extracts were the most effective ones, reducing around 90 % at 2500 μg/g. The C.o extracts that caused the higher reduction in TeA production were the chloroformic and ethanolic fractions when the concentration in the medium was 1250 μg/g or higher, and the methanolic one at 2500 μg/g, with a maximum reduction of 66 % (chloroformic extract, 1250 μg/g). In a lesser

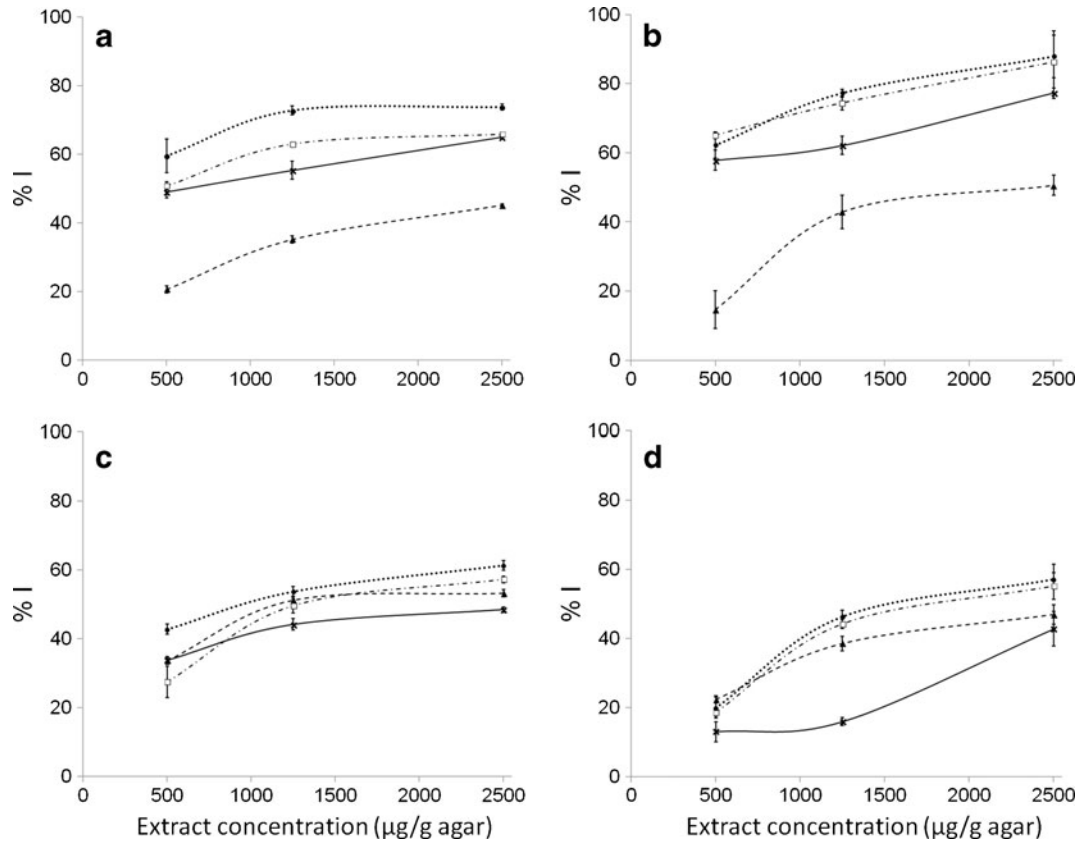

Fig. 1 Growth inhibition (% I) of *Alternaria alternata* and *A. arborescens* by plant extracts obtained with different solvents at three concentrations levels. **a** *A. alternata*/*Eucalyptus globulus* extracts; **b** *A. arborescens*/*E. globulus* extracts; **c** *A. alternata*/*Calendula officinalis* extracts; **d** *A. arborescens*/*C. officinalis* extracts. Bars indicate Standard Errors of the mean. (—x—) MeOH, (- -▲- -) MeOH:H_2O, (– -□- · –) EtOH, (···●···) $CHCl_3$

extent, the methanol:water fraction of this plant at 1250 μg/g was also able to reduce the accumulation of this toxin.

The production of AOH by *Alternaria* strains was significantly reduced by almost all E.gl extracts. High levels of toxin inhibition were reached (ranging between 75 and 96 % reduction). The only extracts not effective in reducing this toxin production were methanol and methanol:water at 500 μg/g for *A. alternata*, but both were inhibitory over this concentration. For the rest of E.gl extracts, incrementing the concentration did not result in a higher inhibition of this toxin biosynthesis both for *A. alternata* and *A. arborescens*.

Even though more E.gl treatments were highly effective (≥80 % inhibition) in reducing AOH production for both species, significant reductions were also observed with several C.o extracts. The most effective for *A. alternata* were the ethanolic and chloroformic fractions at 2500 μg/g (94 and 89 %, respectively). With these two fractions the inhibition increased with concentration; while for the other two, 1250 μg/g was the most effective treatment. For *A. arborescens*, the lowest AOH concentrations were detected with 500 μg/g, with the exception of the methanolic extract.

AME biosynthesis by *A. alternata* was not effectively reduced by any extracts at the lowest concentration tested (500 μg/g), but all the other concentrations yielded AME accumulation in amounts significantly lower than the control. On the other hand, for *A. arborescens*, E.gl extracts were able to reduce AME production at all concentration tested, being the optimum treatment the methanolic extract at 500 μg/g (99 % inhibition). The ethanolic and chloroformic fractions were also quite effective in reducing the production of this toxin, reaching inhibition levels of 91 % (ethanolic, 1250 μg/g) and 92 % (chloroformic, 500 μg/g).

Regarding C.o extracts, several treatments increased the toxin biosynthesis by *A. alternata* (indicated by "a"

Table 1 Mycotoxin concentration produced by *Alternaria alternata* and *A. arborescens* in presence of *Eucalyptus globulus* and *Calendula officinalis* extracts

Toxin	Extract	Conc. (μg/g)	Mycotoxin concentration (μg/g) ± standard deviation			
			A. alternata		*A. arborescens*	
			E. globulus	*C. officinalis*	*E. globulus*	*C. officinalis*
TeA	Control	0	n.d.	n.d.	66.4 ± 6.6	66.4 ± 6.6
	Methanol: water	500	n.d.	n.d.	58.7 ± 1.2 [b]	85.2 ± 10.5 [a]
		1250	n.d.	n.d.	54.7 ± 2.8	59.2 ± 0.4
		2500	n.d.	n.d.	33.1 ± 1.8	68.0 ± 27.7 [b]
	Methanol	500	n.d.	n.d.	46.6 ± 1.2	64.6 ± 13.7 [b]
		1250	n.d.	n.d.	42.8 ± 15.9	80.6 ± 5.9 [a]
		2500	n.d.	n.d.	18.7 ± 6.8	39.6 ± 9.6
	Chloroform	500	n.d.	n.d.	18.5 ± 3.7	72.9 ± 6.0 [b]
		1250	n.d.	n.d.	16.0 ± 5.4	22.4 ± 13.8
		2500	n.d.	n.d.	7.2 ± 3.3	28.7 ± 4.8
	Ethanol	500	n.d.	n.d.	32.0 ± 14.7	67.7 ± 0.7 [b]
		1250	n.d.	n.d.	33.9 ± 2.2	44.8 ± 13.7
		2500	n.d.	n.d.	6.8 ± 4.6	43.7 ± 3.9
AOH	Control	0	7.7 ± 1.5	7.7 ± 1.5	49.8 ± 10.9	49.8 ± 10.9
	Methanol: water	500	7.3 ± 2.6 [b]	8.2 ± 3.1[b]	8.4 ± 0.3	9.2 ± 1.9
		1250	0.8 ± 0.3	1.1 ± 0.2	11.3 ± 1.8	10.9 ± 1.4
		2500	2.0 ± 0.3	4.5 ± 0.6	29.1 ± 8.7	32.4 ± 6.4
	Methanol	500	7.1 ± 1.8 [b]	5.3 ± 0.7	2.2 ± 1.9	10.6 ± 0.6
		1250	1.5 ± 0.5	2.8 ± 1.3	6.7 ± 2.0	5.9 ± 0.1
		2500	1.1 ± 0.5	5.0 ± 0.2	11.4 ± 6.5	23.2 ± 7.1
	Chloroform	500	1.07 ± 0.03	1.97 ± 0.02	3.1 ± 0.9	8.7 ± 1.3
		1250	1.9 ± 0.4	1.6 ± 0.2	11.9 ± 4.1	11.2 ± 6.1
		2500	1.4 ± 0.4	0.9 ± 0.4	10.2 ± 2.2	34.6 ± 4.1
	Ethanol	500	0.49 ± 0.02	7.21 ± 0.02 [b]	5.1 ± 4.1	6.8 ± 1.8
		1250	1.4 ± 0.5	4.8 ± 0.2	9.7 ± 2.7	18.5 ± 3.0
		2500	1.3 ± 0.5	0.4 ± 0.1	7.0 ± 2.8	50.8 ± 8.8 [b]
AME	Control	0	6.2 ± 2.8	6.2 ± 2.8	38.7 ± 3.9	38.7 ± 3.9
	Methanol: water	500	9.5 ± 4.0 [b]	16.6 ± 2.0 [a]	6.17 ± 0.03	3.8 ± 0.7
		1250	0.9 ± 0.4	0.53 ± 0.08	4.1 ± 1.4	5.8 ± 0.4
		2500	2.5 ± 0.8	5.4 ± 2.6 [b]	24.9 ± 8.7	34.8 ± 11.7 [b]
	Methanol	500	13.4 ± 3.4 [a]	6.0 ± 2.6 [b]	0.47 ± 0.21	7.3 ± 2.1
		1250	1.7 ± 0.8	2.2 ± 1.7	0.9 ± 0.2	7.8 ± 1.8
		2500	0.4 ± 0.3	8.2 ± 1.8 [b]	14.9 ± 8.7	23.5 ± 7.2
	Chloroform	500	10.1 ± 1.3 [b]	50.5 ± 0.5 [a]	3.1 ± 1.2	14.7 ± 1.6
		1250	0.27 ± 0.06	2.3 ± 0.8	8.6 ± 3.8	14.3 ± 6.9
		2500	0.5 ± 0.4	0.7 ± 0.3	17.7 ± 3.9	46.2 ± 3.8 [b]
	Ethanol	500	12.0 ± 1.0 [a]	32.2 ± 0.7 [a]	3.9 ± 2.6	5.7 ± 0.8
		1250	0.38 ± 0.13	11.5 ± 1.6 [a]	3.3 ± 1.1	31.0 ± 4.2 [b]
		2500	0.07 ± 0.05	0.23 ± 0.07	9.5 ± 5.6	52.8 ± 4.4 [a]

TeA tenuazonic acid, *AOH* alternariol, *AME* alternariol monomethyl ether, *n.d.* not detected

[a] values significantly higher than their correspondent control ($p < 0.05$)

[b] values not significantly different than their correspondent control ($p < 0.05$)

in Table 1). The most effective in reducing toxin concentration was the ethanolic fraction at 2500 μg/g. Interestingly, the same treatment caused a significant rise in AME accumulation by *A. arborescens*. The highest inhibitions for *A. arborescens* were obtained with methanol:water and ethanolic extracts at 500 μg/g (90 and 85 %, respectively).

Impact of *E. globulus* and *C. officinalis* extracts on *Alternaria* spp. pathogenicity and aggressiveness on tomato fruits

The effect of plant extracts on pathogenicity and aggressiveness of *A. alternata* and *A. arborescens* on tomato fruits is shown in Table 2.

As expected, pathogenicity was higher when the skin was wounded. All fractions tested caused a reduction in the percentage of infected fruits with respect to the controls. The solvent control showed that ethanol had no effect on the fungal pathogenicity. The E.gl extracts produced a higher reduction in the pathogenicity of both *Alternaria* species. The ethanolic and chloroformic fractions of E.gl were the most effective, either when applied on wounded or unwounded fruits. Regarding C.o, only the chloroformic extract completely inhibited *Alternaria* spp. growth when inoculated on undamaged fruits.

The type of lesion observed in wounded and unwounded fruits was different. When the tomato fruit was injured, the size of the lesion was always equal to the diameter of the fungal colony. In the case of inoculation without wound, a soft lesion outer the edge of the colony was observed. In both cases the value of aggressiveness recorded corresponded to the entire lesion.

The size of the lesion caused by *Alternaria* on tomato fruits, which was recorded as a measure of aggressiveness, was significantly reduced by all extracts except by the methanol:water fraction of both plants. The maximum reduction was reached by *E. globulus*

Table 2 Effect of plant extracts on pathogenicity and aggressiveness of *Alternaria alternata* and *A. arborescens* on tomato fruits

Treatment	*A. alternata*				*A. arborescens*			
	Pathogenicity[1]		Aggressiveness[2]		Pathogenicity[1]		Aggressiveness[2]	
	W	U	W	U	W	U	W	U
Positive control[3]	100	40	19.3 [a]	13.2 [a]	100	35	23.1 [a]	14.4 [a]
Negative control[4]	0	0	-	-	0	0	-	-
Solvent control[5]	100	40	20.9 [a]	12.8 [a]	100	35	22.7 [a]	14.6 [a]
Eucalyptus globulus								
Methanol:water	75	30	20.2 [a]	13.5 [a]	40	25	21.9 [a]	13.7 [a]
Methanol	55	15	15.2[c]	3.5 [c]	25	20	11.8[c]	6.2 [c]
Chloroform	25	0	5.8[f]	0 [d]	10	0	4.4 [d]	0 [e]
Ethanol	15	0	6.7[e]	0 [d]	20	0	5.1 [d]	0 [e]
Calendula officinalis								
Methanol:water	90	35	19.5 [a]	12.7 [a]	65	30	22.3 [a]	11.4 [b]
Methanol	75	30	16.4 [b]	9.1 [b]	60	25	15.1 [b]	5.9 [c]
Chloroform	35	0	8.7[d]	0 [d]	55	0	10.9 [c]	0 [e]
Ethanol	40	10	9.1[d]	3.9 [c]	40	10	11.2 [c]	3.4 [d]

(1) % of infected fruits. Mean of two independent experiments

(2) Mean lesion diameter (mm). Mean of ten replicates from two independent experiments, excluding zero values. Values in a column followed by the same letter are not significantly different ($p < 0.05$)

(3) Inoculated fruits untreated with plant extract or ethanol

(4) Uninoculated fruits sprayed with ethanol

(5) Inoculated fruits sprayed with ethanol

W wounded, *U* unwound

chloroformic and ethanolic extracts with inhibitions ranging between 65 and 81 % on wounded tomatoes.

Discussion

This study has shown that some extracts of E.gl were very effective at inhibiting growth, *Alternaria* toxins production and pathogenicity of strains of the two species examined.

To our knowledge, no data are available in the literature about the effect of these plants on *Alternaria* spp. However, several studies have demonstrated their antimicrobial potential on different bacterial and fungal species (Efstratiou et al. 2012; Soliman and Badeaa 2002; Tyagi and Malik 2011; Vilela et al. 2009).

Alternaria species have been used as target organism to test new natural antifungals. *A. alternata* growth was inhibited by plant derived extracts, such as coriander essential oil (Alves-Silva et al. 2013), methanolic extracts of *Artemisia annua* leaves, *Anadenanthera colubrina* barks and a mixture of flowers and leaves of *Ruta graveolens* (Carvalho et al. 2011), cassia oil (Feng and Zheng 2007), and *Cicuta virosa* L. var. *latisecta* Celak essential oil (Tian et al. 2011). The essential oil from another eucalyptus species, *E. teretecornis*, also proved to be effective in reducing *A. alternata* growth (Guleria et al. 2012). In the present study, all extracts tested showed a fungistatic effect on *A. alternata* and *A. arborescens*, being ethanolic and chloroformic fractions from *E. globulus* the most effective ones. Our results also showed that the percentage of inhibition in all cases increased with the extract concentration. The type of correlation between concentration of the antimicrobial and growth inhibition observed in our work was dependent on each extract and fungal species, with a logarithmic relationship as the most common response. This correlation could be associated with the achievement of a concentration in which active compounds are at their saturation level.

Many studies on the ability of plant compounds to reduce fungal growth do not take into account that the presence of this stress factor may trigger mycotoxin synthesis. In consequence, their biosynthesis could be augmented if the fungus can still grow in presence of the plant extract. Thus, inhibitory effects on growth are not always associated with reductions on mycotoxin accumulation (Bluma et al. 2008a). Even though *Alternaria* is a frequent contaminant in food commodities, its toxins are little studied, perhaps due to the lack of regulation in terms of its presence in food. This issue has a particular importance in tomato products, given that, in developing countries, fruits with high fungal contamination are frequently used for industrialized derived products (tomato pasta, puree, etc), with a consequent higher mycotoxin accumulation.

In our work, the effect of the extract on the amount of mycotoxin synthesised varied with the toxin and the strain studied. The ethanolic and chloroformic E.gl extracts showed high levels of inhibition for these metabolites production by *Alternaria* spp. at most doses assayed. In consequence, these fractions could have a promising application in foods where the three mycotoxins can simultaneously accumulate. It is worth mentioning that certain extracts produced an increment in the synthesis of some of these metabolites with respect to the corresponding control values. Moreover, some of them resulted inhibitory at a certain concentration, but enhanced toxin production at another. The highest increment of toxin biosynthesis was observed for AME production by *A. alternata* in presence of the chloroformic and ethanolic C.o fractions at 500 μg/g. It is interesting to remark that, in general, both *Alternaria* spp. studied showed an opposite trend with respect to AOH and AME production. For *A. alternata*, when the extract concentration was increased, at least the same reduction or, in some cases a higher one, was observed on the mycotoxin accumulation. Meanwhile, *A. arborescens* produced higher toxin amounts or not significantly different from the control when extract concentration was raised. Other authors have reported a similar behaviour regarding aflatoxigenesis. The decrease or raise in the amount of aflatoxin produced by several *Aspergillus* species was dependent on the concentration of the extract or essential oil in combination with environmental conditions (Bluma et al. 2008a; Bluma and Etcheverry 2008b; Garcia et al. 2011). For this reason, the effect of vegetal extracts on mycotoxin biosynthesis must be evaluated to determine the adequate dose for the development of natural antifungals. It is essential to avoid the use of suboptimal doses that could result in a reduction on fungal growth development but an increment in mycotoxin accumulation.

According to Schafer (1994), pathogenicity is the capacity of a fungus to cause disease, a qualitative measure, while aggressiveness was defined by Shaner et al. (1992) as a description of the rate at which a level of disease is reached, with more aggressive pathogens reaching this level faster. Confirmation of in vivo

activity is especially important due to possible interactions between food matrices and the bioactive compounds, which could result in decreasing their efficacy (da Cruz Cabral et al. 2013). In our study, the in vitro inhibition caused by the extracts was higher than in the in vivo assay when carried out on wounded fruits for both strains. A good correlation was observed between the in vitro inhibition of growth and the reduction both in the pathogenicity and aggressiveness of the strains.

Both plant species tested have a wide history of safe use in a great variety of applications, including food purposes. To our knowledge, they have never been tested as antifungals against *Alternaria* and their metabolites.

A relationship between the chemical structure of the main component of a plant extract and its antimicrobial activity has been postulated (Kumar et al. 2010; Prakash et al. 2010; Souza et al. 2005). Data from literature indicates that the major components of eucalyptus essential oil are monoterpenes, from which 1,8-cineole, α-pinene, limonene, p-cymene and α-terpineol are the main compounds (Ben Jemâa et al. 2012; Tyagi and Malik 2011; Wallis et al. 2011). Both chloroform and ethanol can dissolve many of the monoterpenes, which could be associated to their antifungal activity. Benyahia et al. (2005) reported that a chloroformic extract of eucalyptus leaves yielded cladocalol, ursulolactone acetate, ursolic acid, 3β-acetate-12,20(29)-lupadien-28-oic acid, β-sitosterol, and the flavonoid eucalyptine. On the other hand, *C. officinalis* has been reported to contain a high number of carotenoids, such as flavoxanthin, lutein, rubixanthin, β-carotene, γ-carotene and lycopene (Pintea et al. 2003). Carotenoids are known to be bioactive compounds and several of them have shown antimicrobial activities.

However, it is difficult to correlate the fungitoxic activity to single compounds or classes of compounds. Vilela et al. (2009) reported that 1,8-cineole, the major component of *E. globulus* essential oil, was not as effective as the complete essential oil when tested against *Aspergillus flavus* and *A. parasiticus*. This could indicate that the major oil constituent is not the only component responsible for limiting fungal growth. The potential of plant extracts to inhibit microbial growth relies on their wide variety of chemical compounds. There is evidence that even minor components have a critical part in antimicrobial activity, and it seems that the inhibitory effects are the result of their synergistic or at least additive action (Vilela et al. 2009). The advantages of applying crude plant extracts instead of single compounds would include minimizing the chances of resistance development in fungi and reducing the higher costs involved in purifying steps. Therefore, whole plant extracts appear to be more promising in commercial application than isolated compounds (da Cruz Cabral et al. 2013).

Conclusions

The analysis of various extracts of *E. globulus* and *C. officinalis* resulted in finding fractions that showed promising prospects for their utilization to reduce post-harvest *Alternaria* decay in tomatoes and subsequent mycotoxin accumulation. Among them, the chloroformic and ethanolic extracts from *E. globulus* were the most efficient, presenting a great potential for the development of new natural antifungals. In particular, the ethanolic extract of *E. globulus* at a dose of 2500 μg/g, was the most effective at reducing *Alternaria* growth in vitro and in vivo, and showed the maximum reduction in mycotoxin accumulation.

More studies are needed to evaluate the impact on the flavour of tomatoes after application of the extracts and the residual level of volatiles after storage. Their use in a hurdle technology strategy, in combination with other methods of low environmental impact could be a promising alternative. Another point to be further investigated is their effect on the rest of the natural microbiota of tomatoes, or other phytopathogens that might infect these fruits.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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