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RESEARCH ARTICLE

Extracts and phenolic compounds from *Zuccagnia punctata* as fungicide seed protectants for corn

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Abstract

The ethanolic leaf extract (TZP) of *Zuccagnia punctata*, its ethereal fraction (Eet), 2′,4′-dihydroxychalcone (DC), 2′,4′-dihydroxy-3′-methoxychalcone (DMC) and 7-hydroxy-3′,4′-dimethoxyflavone (HF) were evaluated as fungicide seed protectants on corn. Microdilution assays on a set of *Fusarium* strains showed minimum inhibitory concentrations (MICs) of 400–800 $\mu g\,mL^{-1}$ (TZP), 50–100 $\mu g\,mL^{-1}$ (Eet), 25–50 $\mu g\,mL^{-1}$ (DC), 50–100 $\mu g\,mL^{-1}$ (DMC) and 200–400 $\mu g\,mL^{-1}$ (HF), with minimum fungicidal concentration (MFC)/MIC = 1. Suspensions of TZP, Eet, DC and DMC at MIC × 20 incorporated to the grains at rates of 1920, 240, 120 and 240 mg dry matter kg^{-1} of grain, respectively, increased the elongation of the primary roots (24–44%) and the number of seminal roots (44–50%). TZP also increased the number of secondary roots. HF was phytotoxic. Suspensions of TZP, Eet, DC and DMC suppressed the endogenous grain mycoflora at levels similar to those recorded for a thiram + carbendazim-based fungicide. Grains treated with TZP (1920 mg kg^{-1}), Eet (240 mg kg^{-1}), DC (120 mg kg^{-1}) and DMC (240 mg kg^{-1}) stimulated the growth of the seedling root system both because of fungal suppression and hormetic effects in greenhouse curative and preventive assays against *Fusarium verticillioides* on a sand/soil substrate. Eet and its chalcones also reduced the severity of seedling blight more than the thiram + carbendazim-based fungicide in preventive assays and led to the same disease severity observed for the fungicide treatment in the curative assays. Our results show that Eet and its chalcones not only were effective seed protectants against *F. verticillioides* and other seedborne fungi, but also improved the early performance of maize seedlings.

Introduction

Fusarium verticillioides (Sacc. Nirenberg) is the most prevalent fungal pathogen in corn worldwide responsible for ear and stalk rots and seedling blight (De Matos *et al.,* 2013). It contaminates the grains with mycotoxins called fumonisins (Sartori *et al.,* 2015) which showed carcinogenic effects in humans and animals (Peiretti-Uzal *et al.,* 2007). Grains used as seed can be infected by *F. verticillioides* in rot ears or after sowing when the hyphae from mycelium or germinating conidia surviving in the stubbles or soil can penetrate the grains through emergent roots or pericarp wounds (Murillo *et al.,* 1999). Infected corn grains ensure the systemic or endophytic transmission of *F. verticillioides* to the plant which can further lead to the development of diseases and/or the accumulation of fumonisins (Carmona & Scandiani, 2011). Fungicides are often applied as seed protectants to the corn grains before sowing. However, their residues remain in the environment and are potentially hazardous for humans and other non-target organisms (Da Cruz Cabral *et al.,* 2013). In addition, the seed protectants currently available are often ineffective on *F. verticillioides*, specially when the fungal inoculum is very high (Carmona & Scandiani, 2011). Several of these active principles are fungistatics at concentrations able to completely suppress fungal growth. This situation together

with their intensive use have favoured the development of fungal resistance (Audenaert *et al.,* 2010; Falcão *et al.,* 2011). Hence, there is a need of low-cost biocidal seed protectants able to control *F. verticillioides*, with new mechanisms of action and unable to have adverse environmental impacts. *Zuccagnia punctata* Cav. (*Fabaceae*), a medicinal plant native from northwest Argentina, might be a source of seed protectants able to solve these issues (Cabrera, 1971). *In vitro* assays showed that the ethanolic leaf extract (TZP) has a promissory antifungal activity on *Fusarium* species, including strains of *F. verticillioides* responsible for corn ear rots (Jiménez *et al.,* 2010, 2014). Their active principles were identified as phenolic compounds recovered in the ethereal fraction (Eet) which was constituted by 48% (w/w) of 2′,4′-dihydroxychalcone (DC), 32% of 2′,4′-dihydroxy-3′-methoxychalcone (DMC) and 5% of 7-hydroxy-3′,4′-dimethoxyflavone (HF) (Jiménez *et al.,* 2016). The above-mentioned chalcones are the major phenolic compounds accumulated in *Z. punctata* leaves (Agüero *et al.*, 2010; Roco *et al.,* 2017). Taking into account these findings, the aim of this work was to evaluate the effectiveness of the ethanolic extract of *Z. punctata*, its Eet and its phenolic compounds DC, DMC and HF as seed protectants in corn, specially against *F. verticillioides*.

Materials and methods

Plant material and preparation of the ethanolic leaf extract

Aerial parts of *Z. punctata* were collected during February–March 2015 in Amaicha del Valle (Tucumán Province, Argentina) and identified by comparison with voucher specimens deposited at the Herbarium of the Miguel Lillo Foundation (Argentina). They were air-dried for 5 days. Then, leaves were detached and powdered in a Wiley mill. A portion of the powder (10 g) was extracted by shaking with 100 mL of 96% ethanol for 7 days at 37°C. The extracts were filtered and vacuum-dried at 30°C. The dry residue of the TZP of *Z. punctata* was dissolved in methanol and stored in the dark at 4°C.

Isolation of antifungal constituents from the ethanolic leaf extract of *Z. punctata*

The antifungal compounds DC, DMC and HF were isolated as previously depicted (Jiménez *et al.,* 2016). Briefly, the dry ZTP (300 g) was extracted with diethyl ether in a ratio of 1:4 (w/v). The Eet was vacuum-dried at 30°C, dissolved in methanol and identified as Eet. Dry Eet (800 mg dry matter) was loaded onto a silica gel column (230–400 mesh, 3 cm × 20 cm, 36 g) eluted with 80 mL of *n*-hexane, and 80 mL of *n*-hexane/ethyl acetate (6:1.7 v/v). Five pooled fractions (P1–P5) were collected according to their profiles in thin layer chromatography (TLC) on silica gel developed with *n*-hexane/ethyl acetate (6:1.7 v/v) and visualised under ultraviolet (UV) light (365 nm). The DC and DMC were separated from P2 and P3, and the HF from P5 by high-performance liquid chromatography equipped with a semipreparative IB-SIL RP 18 column (5 μm, 250 mm × 10 mm; Phenomenex, Torrance, CA, USA) and coupled to an UV detector at 254 nm as previously depicted (Jiménez *et al.,* 2016). The identity of the antifungal molecules was confirmed by UV–Vis spectrophotometry and mass spectrometry (Jiménez *et al.,* 2014).

Assayed microorganisms

The strains of *F. verticillioides* (LABI6), *Fusarium graminearum sensu stricto* (LABI25), *Fusarium proliferatum* (LABI36), *Fusarium meridionale* (LABI48) and *Fusarium subglutinans* (LABI51) were provided by the LABIFITO culture collection (Tucumán, Argentina). The microbial strains were preserved in SNA medium (Spezieller Nahrstoffarmer agar: 0.1% of K_2HPO_4, 0.1% $NaNO_3$, 0.05% $MgSO_4.7H_2O$, 0.05% KCl, 0.02% glucose, 0.2% sucrose and 2% agar) at 4°C.

Microdilution assays

Fungal growth was evaluated in 96 flat-bottom well microplates by the microdilution method in liquid Roswell Park Memorial Institute (RPMI) 1640 medium. Protocols were developed according to M38-A and M38-P documents from the National Committee for Clinical Laboratory Standards with some modifications (NCCLS, 2002; Medina *et al.,* 2012). Fungal colonies were grown in Petri dishes for 7 days in solid SNA medium. Then, they were washed with 2 mL of physiological solution (0.9% of NaCl in distilled water) to obtain microconidial suspensions. The asexual spores were counted in a Neubauer chamber, and diluted in RPMI 1640 medium to obtain a density of 1×10^4 microconidia mL^{-1}. The TZP, Eet and its phenolic compounds were assayed in serial twofold dilutions with concentrations comprised between 5 and 500 $\mu g\,mL^{-1}$ which were prepared in liquid RPMI 1640 medium. The final volume in each well was 200 μL, which corresponded to 100 μL of spore suspension and 100 μL of a dilution of TZP, Eet or of an isolated phenolic compound. Controls of growth and sterility were also performed. Nordihydroguaiaretic acid (NDGA) purchased from Sigma-Aldrich (St Louis, MO, USA) and Vendaval thi-carb fv were assayed as positive controls. Each treatment (TZP, Eet, a phenolic compound, NDGA or Vendaval thi-carb fv) had three repetitions per microplate. Each

Table 1 MIC of fungal growth and MFC of the ethanolic extract of *Z. punctata*, its Eet and its phenolic constituents against *Fusarium* species responsible for maize seedling blight. Each MIC value is the mean of three replications[a]

	LABI6 (*F. verticillioides*)		LABI25 (*F. graminearum sensu stricto*)		LABI36 (*F. proliferatum*)		LABI48 (*F. meridionale*)		LABI51 (*F. subglutinans*)	
	MIC (μg mL^{-1}) E1/E2	MFC/MIC E1/E2	MIC (μg mL^{-1}) E1/E2	MFC/MIC E1/E2	MIC (μg mL^{-1}) E1/E2	MFC/MIC E1/E2	MIC (μg mL^{-1}) E1/E2	MFC/MIC E1/E2	MIC (μg mL^{-1}) E1/E2	MFC/MIC E1/E2
Ethanolic leaf extract	800/800	1.0/1.0	400/400	1.0/1.0	800/800	1.0/1.0	400/400	1.0/1.0	800/800	1.0/1.0
Eet	100/100	1.0/1.0	100/100	1.0/1.0	100/100	1.0/1.0	50/50	1.0/1.0	100/100	1.0/1.0
2′, 4′-Dihydroxychalcone	50/50	1.0/1.0	50/50	1.0/1.0	25/25	1.0/1.0	25/25	1.0/1.0	50/50	1.0/1.0
2′, 4′-Dihydroxy-3′-methoxychalcone	100/100	1.0/1.0	100/100	1.0/1.0	50/50	1.0/1.0	50/50	1.0/1.0	100/100	1.0/1.0
7-Hydroxy-3′, 4′-dimethoxyflavone	400/400	1.0/1.0	200/200	1.0/1.0	400/400	1.0/1.0	200/200	1.0/1.0	400/400	1.0/1.0
35% Thiram +15% carbendazim (Vendaval thi-carb fv)	100/100	2.5/2.5	100/100	2.0/2.0	100/100	2.5/2.5	100/100	2.0/2.0	200/200	2.0/2.0
Nordihydroguaiaretic acid	50/50	2.0/2.0	50/50	2.0/2.0	50/50	2.0/2.0	50/50	2.0/2.0	50/50	2.0/2.0

Eet, ethereal fraction; MFC, minimum fungicidal concentration; MIC, minimum inhibitory concentration.
[a]Values are provided in pairs as means of MIC of Experiment 1 (E1)/MIC of Experiment 2 (E2) or MFC/MIC rate of Experiment 1/MFC/MIC rate of Experiment 2. Each pair of values was obtained for a fungal species and treatment.

microplate was twice repeated and is denoted as Experiment 1 or 2 in Table 1. The microplates were incubated during 72 h at 30°C. Then, the minimum inhibitory concentration (MIC) values were determined as the lowest concentration preventing visible fungal growth. Aliquots of 10 μL collected at MIC and higher concentrations were striated on 2% malt extract–0.5% peptone (MEP) medium contained in Petri dishes and incubated for 72 h at 30°C. The lowest concentration of antifungal compound yielding no growth in the MEP medium was considered the minimum fungicidal concentration (MFC) and was used to calculate the MFC/MIC rate.

Corn grains

The dent corn race ('diente de caballo') with a moisture content of 11% was purchased in the local market. The grains were surface-disinfected with 0.02% NaClO (5 min), subsequently washed (10 min) with sterile distilled water and air-dried at room temperature (about 25°C) for 24 h on top of paper towels placed in Petri dishes. Then, they were used for assays of phytotoxicity, of the endogenous grain mycoflora, of mycelial colonisation and of seed protection.

Phytotoxicity assays

Methanolic aliquots of TZP, Eet, DC, DMC and HF, and of NDGA dissolved in methanol were evaporated to dryness at 30°C under reduced pressure. The dried residues were weighed, suspended in distilled water and the mixtures stirred (40 cycles min^{-1}) at 30°C to generate homogeneous suspensions at concentrations of MIC × 10 (8.0 mg mL^{-1}, TZP; 1.0 mg mL^{-1}, Eet; 0.5 mg mL^{-1}, DC; 1.0 mg mL^{-1}, DMC; 4 mg mL^{-1}, HF; 1 mg mL^{-1}, NDGA) and MIC × 20 (16 mg mL^{-1}, TZP; 2 mg mL^{-1}, Eet; 1 mg mL^{-1}, DC; 2 mg mL^{-1}, DMC; 8 mg mL^{-1}, HF; 10 mg mL^{-1}, NDGA). Each suspension (120 mL) was incorporated to 1 kg of grains resulting in rates of 960 mg kg^{-1} (MIC × 10) and 1920 mg kg^{-1} (MIC × 20) for TZP, 120 mg kg^{-1} (MIC × 10) and 240 mg kg^{-1} (MIC × 20) for Eet, 60 mg kg^{-1} (MIC × 10) and 120 mg kg^{-1} (MIC × 20) for DC, 120 mg kg^{-1} (MIC × 10) and 240 mg kg^{-1} (MIC × 20) for DMC and 480 mg kg^{-1} (MIC × 10) and 960 mg kg^{-1} (MIC × 20) for HF. The control consisted of surface-sterilised grains treated with 120 mL of water instead of the suspensions. A treatment consisting of a 35% thiram–15% carbendazim-based seed protectant (Vendaval thi-carb fv) applied at the label-recommended rates (700 mg thiram + 300 mg carbendazim kg^{-1} of grain) was also included. The grain samples were air-dried at room temperature for 24 h on the top of paper towels placed in Petri dishes. Four replications of 50 grains were germinated per treatment using the 30- by 60-cm rolled towel standard germination test (ISTA, 2013). The towels were rolled and placed upright in pails and covered with a clear plastic bag. Towels with replications were randomised within pails. The mean germination of grains, of primary root length and of the number of seminal and secondary roots was determined after an incubation of 7 days at 25°C. Two experiments were performed and each one

Table 2 Effects of the ethanolic extract of *Z. punctata*, its Eet and its isolated phenolic compounds assayed at MIC × 20 on the elongation of primary roots, and the number of secondary and seminal roots of maize recorded in the rolled towel standard germination test. Each value is a mean of four replicates (50 grains per replicate)

	Experiment 1			Experiment 2		
Treatment	Primary Roots (cm)	Number of Secondary Roots	Number of Seminal Roots	Primary Roots (cm)	Number of Secondary Roots	Number of Seminal Roots
Ethanolic leaf extract	10.1	12.6	2.3	12.5	10.2	2.2
Eet	9.2	0.2	2.3	8.5	0.2	2.0
2′, 4′-Dihydroxychalcone	8.7	0.3	2.4	8.3	0.2	2.2
2′, 4′-Dihydroxy-3′-methoxychalcone	8.8	0.3	2.4	8.4	0.3	2.0
7-Hydroxy-3′, 4′-dimethoxyflavone	5.2	0.2	1.8	3.9	0.3	1.5
35% Thiram + 15% carbendazim (Vendaval thi-carb fv)	7.3	0.2	1.7	7.1	0.2	1.4
Nordihydroguaiaretic acid	4.3	0.2	1.6	4.0	0.3	1.4
Control (water)	7.2	0.2	1.6	7.8	0.2	1.3
SED	0.15	0.08	0.04	0.18	0.05	0.02
LSD (5%)	0.30	0.2	0.3	0.4	0.3	0.3
d.f.	26	26	26	26	26	26

Eet, ethereal fraction; LSD 5%, least significant difference between two means at the 0.05 level; MIC, minimum inhibitory concentration; SED, standard error of the difference between two means.

consisted of the set of grains treated with the suspensions of *Z. punctata*, Vendaval thi-carb fv and water. They are denoted as Experiments 1 and 2 in Table 2.

Assays on the endogenous grain mycoflora

Grains treated with TZP, Eet, DC and DMC at concentrations of MIC × 20 as described in the phytotoxicity assay were used to test the impact of these suspensions on the endogenous grain mycoflora. Surface-sterilised grains and grains treated with Vendaval thi-carb fv were also tested as negative and positive controls, respectively. Grains were placed in eight Petri dishes, each one containing 20 mL of MEP medium with 2% agar and five grains of a treatment. Then, they were incubated 7 days at 25°C in darkness. At the end of the incubation, the maize grains were observed regarding the presence of fungal colonies using a stereo-binocular microscope. Identification of *Penicillium* spp. and *Aspergillus* spp. was based on morphological features according to Hocking *et al.* (2006) and for *Fusarium* spp. in agreement with Leslie & Summerell (2006). The frequency of occurrence of the fungal genera was calculated by the formula:

$$\%\ \text{of a Fungal genus} = (\text{number of grains affected with a fungal genus/total number of grains}) \times 100$$

The experiment depicted was performed twice. Experiments are denoted as 1 and 2 in Table 3.

Seed protection assays

The suspensions of TZP, Eet, DC, DMC and HF were assayed on corn grains against *F. verticillioides* (LABI6) in curative and preventive assays. In the curative assays, surface-sterilised grains were submersed in a suspension of 10^4 spores mL^{-1} of *F. verticillioides* and then incubated in sterile conditions on filter paper for 48 h at 30°C. Then, 100 infected grains were seeded at a depth of 0.5 cm in 48 cm × 50 cm × 30 cm trays, each one covered with a layer of a substrate of sand/soil (4:1, w/w, 1.5 kg per tray). After sowing, the trays were placed in a greenhouse for 15 days which was maintained at temperatures of 26–29°C under a photoperiod of about 12 h. The trays were watered every 3 days. In the preventive assays, the sand/soil substrate was inoculated with 1 g of rice infected with *F. verticillioides* after exposure to a 5-day-old fungal colony grown in solid MEP medium. Then, grains treated with a suspension were sowed, and the trays were irrigated and incubated as depicted in the curative assays. Vendaval thi-carb fv was applied to the grains instead of the suspensions as positive control in all assays. The negative controls both in preventive and curative assays consisted of surface-sterilised grains treated with sterile distilled water instead of the suspensions. The percentage of seedlings emerged was counted at the end of each assay. Then, the seedlings were carefully recovered from the trays of each treatment, washed in running tap water and observed for symptoms of water-soaked or -discoloured areas. Disease rating was recorded as a root disease index (RDI) based on a scale of 0–5, where 0 = no symptom on roots, 1 = <25% of roots symptomatic for lesions, 2 = 25–49%, 3 = 50–74%, 4 = 75% or greater and 5 = wilted or dead seedlings. Disease severity was calculated as a product of disease incidence × RDI. The mean elongation of the root system, and seedling dry weight were also determined in the maize seedlings. All assays

Table 3 Effects of the ethanolic extract of *Z. punctata*, its Eet and its phenolic components on the endogenous mycoflora of the maize grains. Each value is a mean of eight replications (5 grains per replicate)

	Treatments								
	TZP	Eet	DC	DMC	V	C	SED	LSD 5%	d.f.
Experiment 1									
Fusarium spp.	–	–	–	–	2	59	0.2	5	42
Aspergillus spp.	3	2	2	2	2	13	0.1	2	42
Penicillium spp.	3	–	–	2	–	6	0.2	4	42
Other fungi	1	3	–	–	2	5	0.1	3	42
Experiment 2									
Fusarium spp.	–	–	–	–	2	70	0.3	4	42
Aspergillus spp.	2	3	2	1	1	10	0.2	2	42
Penicillium spp.	2	3	–	1	–	3	0.3	3	42
Other fungi	2	3	–	–	2	5	0.1	3	42

C, control; DC, 2′, 4′-dihydroxychalcone; DMC, 2′, 4′-dihydroxy-3′-methoxychalcone; Eet, ethereal fraction; LSD 5%, least significant difference between two means at the 0.05 level; SED, standard error of the difference between two means; TZP, ethanolic leaf extract; V, 35% thiram + 15% carbendazim (Vendaval thi-carb fv).

Table 4 Mean values of disease severity of the *Fusarium* seedling blight recorded in preventive and curative assays for the ethanolic extract of *Z. punctata*, its Eet and its isolated phenolic constituents. Each value is a mean of three replicates (100 grains per replicate)

	Treatments								
	TZP	Eet	DC	DMC	V	C	SED	LSD 5%	d.f.
Preventive assay									
Experiment 1	1.2	0.4	0.3	0.3	0.8	3.6	0.09	0.3	12
Experiment 2	1.5	0.3	0.2	0.2	0.7	4.4	0.08	0.4	12
Curative assay									
Experiment 1	0.8	0.8	0.7	0.7	0.9	3.9	0.05	0.3	12
Experiment 2	0.9	0.7	0.6	0.5	0.6	4.5	0.04	0.5	12

C, control; DC, 2′, 4′-dihydroxychalcone; DMC, 2′, 4′-dihydroxy-3′-methoxychalcone; Eet, ethereal fraction; LSD 5%, least significant difference between two means at the 0.05 level; SED, standard error of the difference between two means; TZP, ethanolic leaf extract; V, 35% thiram + 15% carbendazim (Vendaval thi-carb fv).

were performed in a completely randomised design with three trays per treatment. Each assay was performed twice and was indicated in Table 4 as Experiment 1 or 2.

Statistical analysis

Data from each experiment performed in the phytotoxicity, endogenous mycoflora and seed protection assays were analysed using one-way analysis of variance. Means were subjected to Fisher's least significant difference at 5% level of significance. The statistical analyses were performed in STATGRAPHICS Centurion XVI 16.1.03 (Herndon, Virginia,USA).

Results and discussion

Microdilution assays

The TZP, Eet and the isolated phenolic constituents showed different inhibitory activities on the growth of the five strains of *Fusarium* assayed. Table 1 shows the MIC values obtained in Experiment 1 (E1) and 2 (E2) on each *Fusarium* strain. The TZP exerted the lowest inhibitory effect on the *Fusarium* species (MIC E1/E2 = 800/800 μg dry matter mL^{-1} on *F. verticillioides, F. proliferatum* and *F. subglutinans;* MIC E1/E2 = 400/400 μg dry matter mL^{-1} on *F. graminearum sensu stricto* and *F. meridionale*). The highest inhibitory activity was observed for both DC (MIC E1/E2 = 50/50 μg dry matter mL^{-1}, *F. verticillioides, F. graminearum sensu stricto* and *F. subglutinans;* MIC E1/E2 = 25/25 μg dry matter mL^{-1}, *F. proliferatum* and *F. meridionale*) and Vendaval thi-carb fv (MIC E1/E2 = 100/100 μg dry matter mL^{-1} on all the *Fusarium* species, except for *F. subglutinans*). The lignan NDGA was also assayed as reference of antifungal effect because it is the major antifungal principle found in the alcoholic extract of the creosote bush (*Larrea tridentata*) which is currently used as a botanical fungicide on vegetable crops (Vogt *et al.,* 2013; Merino-Ramos *et al.,* 2017). NDGA showed the same antifungal activity or 2-fold less inhibitory activity than DC and was 8–16- and 4–8-fold more active than TZP and HF, respectively. It showed MIC values equal or twofold lower than those recorded for Eet

and DMC. However, the ethanolic extract, its Eet and its constituents killed the fungal strains at their MIC values (MFC/MIC = 1) while NDGA and Vendaval thi-carb fv showed a biostatic rather than a biocidal effect in a range of concentrations of 2.5 × MIC and 2 × MIC, respectively. Drugs with fungistatic activity in a wide range of concentrations are undesirable as antifungal agents because they expose a large part of the fungal population to a strong directional selection towards resistant strains (Anderson, 2005). Our data indicate that the leaf metabolites of *Z. punctata* are fungicides not only on *F. verticillioides* but also on several *Fusarium* species responsible for seedling blight. Several reports support the broad antifungal effect observed in the current work for the TZP of *Z. punctata* and its metabolites DC and DMC. The ethanolic extract and its *n*-hexane fraction completely suppressed the growth of soybean pathogens such as *Phomopsis longicolla*, *Alternaria alternata*, *Fusarium equiseti*, *Colletotrichum truncatum* and *Sclerotium bataticola* at MIC values of 100–500 and 125–1000 $\mu g\,mL^{-1}$, respectively (Svetaz *et al.,* 2004). The ethanolic extract also suppressed the growth of the yeasts *Saccharomyces carlsbergensis* (MIC = 200 $\mu g\,mL^{-1}$) and *Rhodotorula* spp. (MIC = 400 $\mu g\,mL^{-1}$), and the mycelial expansion of *Rhizoctonia solani*, *Macrophomina phaseolina*, *Penicillium notatum*, *Aspergillus niger* and *Basidiomycetes* species responsible for wood rots at concentrations of 400–1600 μg dry matter mL^{-1} (Quiroga *et al.,* 2001; Jiménez *et al.,* 2010). The DC and DMC also inhibited the growth of yeast and dermatophytes responsible for human diseases (Svetaz *et al.,* 2007). Several modes of action were depicted for chalcones but their mechanisms of action on fungal cells are yet unclear. Chalcones inhibit the activity of glutathione-*S*-transferases often involved in drug resistance. This effect is likely because of the reaction of the ketovinyl moiety in the chalcone molecules with thiol groups of enzymes (Sivakumar *et al.,* 2009). It was shown that DC and DMC are not able to disrupt fungal membranes or inhibit the growth of cell walls (Svetaz *et al.,* 2007). They synergize the action of antifungal drugs of clinical use such as amphotericin B, ketoconazole and echinocandins as well as compounds of agricultural use such as epoxiconazole and prothioconazole, and of food use such as potassium sorbate and calcium propionate (Jiménez *et al.,* 2014). This situation is more promising as their use as seed protectants either alone or as part of plant extracts seems to exert mechanisms of action different from those of the current antifungal compounds used in human clinics and agriculture.

Phytotoxicity assays

Several phenolic compounds and phenolic compounds-based extracts completely suppress fungal growth *in vivo* at concentrations higher than those required *in vitro* (Sampietro *et al.,* 2013) and the antifungal *in vivo* concentrations are often phytotoxic for the crop plants (Christian & Goggi, 2008). For this reason, we checked the phytotoxicity of suspensions of TZP, Eet, DC, DMC and HF when assayed at concentrations of MIC × 10 and MIC × 20 on corn grains by the rolled towel standard germination test. Grains treated with the suspensions at the MIC × 10 concentration did not differ significantly in germination compared to those treated with water in the control treatment (data not shown). Seedlings generated from these grains also showed similar mean values of primary root elongation, number of secondary roots and number of seminal roots compared to those generated from grains in the control treatment. Experiments performed with the suspensions at the concentration of MIC × 20 are shown in Table 2. The elongation of primary roots was promoted in the range of 40–60% by TZP and 20–33% by Eet, DC and DMC. The four suspensions mentioned increased in 43–69% the number of seminal roots. TZP also increased in 63–51% the number of lateral roots compared to the control. The suspensions MIC × 20 of HF and NDGA inhibited the elongation of the primary roots by 28–50% and 40–49%, respectively. The thiram + carbendazim-based fungicide applied at the doses recommended by the manufacturer for seed protection did not show promotive effects on root growth. Except for the number of secondary roots, our results suggest that the chalcones contributed to the growth-promoting effects induced by TZP and Eet on the root system of maize. The reasons for this stimulatory activity are difficult to understand because they rely on a whole host of factors. Roots exposed to increasing concentrations of a phenolic compound often show a sequential hormetic pattern on root elongation of no effect, a promotive effect (as observed at MIC × 20 for the chalcones, the ethanolic extract and the Eet), and an inhibitory effect (Belz, 2007). The promotive effects on root expansion as those recorded for chalcones in this study are often explained as an overcompensation of plant growth when the last one is subjected to a mild stress generated by a phytotoxin applied at a sublethal concentration (Calabrese & Baldwin, 2001). Phenolic compounds can act by several mechanisms on root growth including interference of indole acetic acid metabolism, mitochondrial metabolism and respiration, photosynthesis, protein synthesis and ion uptake and transport (Sampietro & Vattuone, 2006). In our experiments, chalcones might act as many polyphenols inhibiting auxin decarboxylation which in turn can increase the relative content of auxins in the seedlings. In grasses, high auxin levels have been reported as responsible for the proliferation of the number of seminal roots which are adventitious roots

Table 5 Mean values of emerged plants, radical length and dry weight of seedlings recorded in preventive and curative assays for the ethanolic extract of *Z. punctata*, its Eet and its isolated phenolic constituents. Each value is a mean of three replicates (100 grains per replicate)

	Treatments								
	TZP	Eet	DC	DMC	V	C	SED	LSD 5%	d.f.
Percentage of emerged plants									
Preventive assay									
Experiment 1	79	84	80	80	80	35	1.2	14	12
Experiment 2	82	80	78	82	82	25	1.4	15	12
Curative assay									
Experiment 1	80	82	83	83	85	42	1.0	16	12
Experiment 2	78	79	85	85	89	38	1.2	17	12
Radical length (cm)									
Preventive assay									
Experiment 1	16.2	15.4	15.5	15.4	11.2	9.0	0.13	0.5	12
Experiment 2	17.5	16.2	16.5	16.0	12.5	10.0	0.10	0.6	12
Curative assay									
Experiment 1	15.8	16.5	15.9	16.2	12.3	9.0	0.09	1.0	12
Experiment 2	16.5	16.8	16.0	16.8	11.9	9.0	0.15	0.9	12
Radical weight (mg)									
Preventive assay									
Experiment 1	75.4	82.0	85.5	86.2	53.0	35.1	0.61	14	12
Experiment 2	85.0	90.0	88.0	87.2	45.0	29.0	0.55	16	12
Curative assay									
Experiment 1	71.2	77.0	78.9	73.0	55.0	30.4	0.58	12	12
Experiment 2	68.0	69.0	70.5	72.1	50.0	28.0	0.45	14	12

C, control; DC, 2′, 4′-dihydroxychalcone; DMC, 2′, 4′-dihydroxy-3′-methoxychalcone; Eet, ethereal fraction; LSD 5%, least significant difference between two means at the 0.05 level; SED, standard error of the difference between two means; TZP, ethanolic leaf extract; V, 35% thiram + 15% carbendazim (Vendaval thi-carb fv).

of embryonic origin (McSteen, 2010). Some phenolic compounds such as ferulic and *p*-coumaric acid applied at low concentrations also increased the number of secondary roots in plant seedlings (Sampietro *et al.,* 2006). Hence, the root-branching activity of TZP might also be because of the action of phenolic compounds other than the chalcones. The phytotoxicity of HF and NDGA led us to exclude them from further assays.

Assays on the endogenous grain mycoflora

Field application of fungicides often suppresses some fungal species resulting in a higher incidence of others (Martinez, 2012). This situation should not occur if the fungicide has a wide control spectrum. To check this possibility, the endogenous fungal population of the maize grains was characterised for the presence of *Aspergillus*, *Penicillium* and *Fusarium* species as well as for its response to the TZP, Eet, DC and DMC assayed at rates of 1920, 240, 120 and 240 mg kg^{-1} of grain, respectively (Table 3). As observed in the control treatment of both experiments, *Fusarium* showed the highest incidence in the maize grains (59–70%) followed by *Aspergillus* (13–10%) and *Penicillium* (3–6%). Other fungi that could not be identified were found at a low percentage (5% in both experiments). Fungal populations were found in values of 1–4% in the grains treated with the suspensions of TZP, Eet, DC or DMC. These data indicated that the suspensions applied to the grains had an efficient control of seedborne fungi of maize and a spectrum of fungal control as wide as that of Vendaval thi-carb fv.

Seed protectant assays

Fusarium verticillioides contaminates the corn grains during both pre- and post-sowing (Sampietro *et al.,* 2013), so it was desirable to know if TZP (1920 mg kg^{-1} of grain), Eet (240 mg kg^{-1} of grain), DC (120 mg kg^{-1} of grain) and DMC (240 mg kg^{-1} of grain) have both a preventive and a curative effect. Table 4 indicates that seedlings grown from grains treated with Eet, DC and DMC expressed less disease severity (9–22-fold lower compared to control) than the thiram + carbendazim-based fungicide (5–6-fold lower than the control) in preventive assays and the same disease severity as that observed for the fungicide in the curative assays. In the case of TZP, it showed a lower suppressor power of the *Fusarium* seedling blight than Vendaval thi-carb fv in the preventive assays (threefold lower than in the controls of Experiments 1 and 2) and the same effect under curative assays. The emergence of maize seedlings from grains treated with TZP, Eet, the chalcones and Vendaval thi-carb fv was on

average twofold higher than from grains of the control treatments in both the curative and preventive assays (Table 5). The suspensions of the *Z. punctata* metabolites increased both root elongation and seedling weight by factors of 1.6–1.8 and 1.7–1.8 (preventive assay) and 2.4–2.8 and 2.3–2.7 (curative assay) compared to the controls, respectively. Vendaval thi-carb fv showed a more restricted increase in root length (1.2–1.3-fold, preventive assay; 1.3–1.4-fold, curative assay) and seedling weight (1.4–1.6-fold, preventive assay; 1.7–1.9-fold, curative assay). These data suggest that TZP and Eet increased the elongation of the root system and the weight of maize seedlings in the greenhouse assays not only because of a lower incidence of *F. verticillioides*, but also because of hormetic effects likely mediated by chalcones. They also show for the first time the usefulness of the chalcones and/or the chalcones-enriched Eet as seed protectants and root growth promoting agents in maize. Although several natural products and plant extracts have been proposed as seed protectants, to the best of our knowledge, phenolic-rich essential oils from some plant species and chitosan are the only ones tested on maize grains with a good control on *Fusarium* and without adverse effects on seedling growth (Christian & Goggi, 2008; Lizarraga-Paulín *et al.,* 2011). However, the former (tested in both curative and preventive treatments) controlled fungal soil and seedborne pathogens at levels below those recorded for commercial fungicides. The latter was tested only as a preventive agent and stimulated seedling length, leaf elongation and stem thickness, although it did not stimulate the growth of the root system as observed for TZP, Eet, DC and DMC. Both DC and DMC are natural constituents of foods, beverages and propolis (Vattuone *et al.,* 2013), showed cytoprotective properties on the gastroduodenal tract of rats (De la Rocha *et al.,* 2003) and were non-toxic in lethality assays on *Artemia salina* (Jiménez *et al.,* 2014). Hence, further research should be focused on the usefulness of these compounds as preservatives in stored grains.

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