Research Article

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Priming to protect maize from *Fusarium verticillioides* and its fumonisin accumulation

Ana Aguado,[a*] Jean-Michel Savoie,[b*] Sylvain Chéreau,[b] Christine Ducos,[b] María Aguilar,[a] Nathalie Ferrer,[b] Manuel Aguilar,[a] Laetitia Pinson-Gadais[b] and Florence Richard-Forget[b]

Abstract

BACKGROUND: Systemic infection through the seed is one of the routes used by the mycotoxinogenic pathogen *Fusarium verticillioides* for colonizing maize plants. The prohibition of the use of most chemical fungicides by the EU has promoted research on plant resistance inducers as an effective and sustainable alternative. Induction of a priming state in maize seeds might affect their susceptibility to contamination and accumulation of fumonisins. This state by application of a natural fertilizer called Chamae on maize seeds, was investigated in two varieties to control the colonization by the fungus and the accumulation of fumonisins B_1, B_2 and B_3, germinating seeds, dead plants and yield.

RESULTS: After inoculation of *F. verticillioides* on germinating seeds, the colonization by the fungus and the accumulation of fumonisins were significantly lower in seedlings coming from treated seeds, but a significant number of plants stopped their development by necrosis. In a field trial, the 0.01% (v/v) application dilution showed a lower plant density, although the level of biomass at harvest was not affected.

CONCLUSION: The priming state contributed to the control of *F. verticillioides* development from seed infection and fumonisin accumulation in the early stage of plant growth, without affecting the final crop yield, and could reduce fungicide use and environmental contamination.

Keywords: priming; germination; mycotoxins; *Fusarium* disease; maize

INTRODUCTION

Maize is one of the most important agricultural commodities in the world and is a major ingredient of dietary staple for humans and animals. In North America and Europe, many maize products are consumed, including breakfast cereals, snacks, soft drinks and beer. Maize is also used to make animal feed. Fumonisins are considered to be hazardous to human and animal health.[1–4] *Fusarium verticillioides* (synonym *Gibberella fujikuroi*) is one of the most important fungal pathogens in maize, causing both pre- and postharvest losses and also being capable of producing mycotoxins such as fumonisins B_1 (FB_1), B_2 (FB_2) and B_3 (FB_3), which are frequently detected in healthy maize grains.[5,6] *Fusarium verticillioides* can infect maize via several routes. The exposed silks are the main pathway for the fungus to naturally get into the ear and reach the kernels.[7] Insects or other biotic or abiotic agents creating kernels wounds also favor kernel infection.[8] Systemic infection through the seed is another pathway. It starts from fungal conidia or mycelia that are carried either inside the seeds or on the seed surface, and the fungus develops inside the young plant, moving from the roots to the stalk and finally to the cob and kernels.[9] Systemic infection may also result from inoculum that survives in crop residues in the soil[10] and penetrates young seedlings through the lateral roots and the mesocotyl.[9]

The prohibition of the use of most chemical fungicides by the EU (Directive 91/414/CEE), owing to their adverse effects on the environment and human health, their toxicity or because pathogens have become resistant to their active substances, has promoted research in the field of plant resistance inducers as an effective and sustainable alternative.[11]

Protection of maize seeds from infection by *F. verticillioides* by inoculation of antagonists has been experimented. It may reduce rotting symptoms and fumonisin accumulation. Treatment of seeds with *Trichoderma harzianum* reduced the levels of fumonisins in different maize cultivars by 56–86%.[6] Fumonisin B_1 reduction reached up to 94% in grains treated with *Bacillus amyloliquefaciens* and up to 81% in grains treated with *Microbacterium oleovorans*.[12] *Pediococcus pentosaceus* (strain L006) is able to produce some extracellular metabolites capable of reducing fumonisin production *in vitro* by 75–80% after 20 days of incubation.[13]

Breeding for resistance to *Fusarium* is the most economic and environmentally safe strategy. Genome-wide association study (GWAS) is a method successfully used to detect and identify quantitative trait loci (QTLs) and candidate genes involved in

* *Correspondence to: A Aguado, IFAPA Las Torres Tomejil, Protección Vegetal Sostenible, Carretera Sevilla-Cazalla Km 12.2, Alcalá del Río, 41200, Seville, Spain. E-mail: ana.aguado@juntadeandalucia.es; or J-M Savoie, MycSA, UR1264, INRA, Villenave d'Ornon Cedex, Bordeaux-Aquitaine, France. E-mail: jean-michel.savoie@inra.fr*

a *IFAPA Las Torres Tomejil, Protección Vegetal Sostenible, Carretera Sevilla-Cazalla Km 12.2, Alcalá del Río, Seville, Spain*

b *MycSA, UR1264, INRA, Villenave d'Ornon Cedex, Bordeaux-Aquitaine, France*

F. verticillioides resistance and low mycotoxin contamination synthetized by this fungus in maize. This tool allows to reveal novel sources of resistance to select maize lines in breeding programs more efficiently than in classic breeding. Recently, other researchers have identified eight QTLs and 43 genes associated with *F. verticillioides* seed rot resistance through linkage mapping and GWAS respectively.[14] By combining transcriptomic data with QTLs proposed 24 candidate genes for resistance to *F. verticillioides* were identified, allowing to make possible the selection of genotypes with both low disease severity and low fumonisin contamination.[15]

The physiological health of the plantlets may also affect the development of symptoms,[9] and induction of the priming state in the plant through the application of Chamae on seeds could be another way to prepare plants against pathogen attacks. The aim of the present work was to characterize the influence of this commercial natural fertilizer based on extracts obtaines from plant decoctions, Chamae, on early colonization of germinating seeds and plantlets by *F. verticillioides* and the development of symptoms, including fumonisin accumulation, and the influence of treatments on yield.

EXPERIMENTAL

Seed treatments and inoculation

The natural fertilizer Chamae (Saionaimer SL, Barakaldo, Vizcaya, Spain) used for seed treatments contains principal macronutrients (nitrogen, phosphorus and potassium), secondary macronutrients (calcium, magnesium, potassium, sodium and sulfur) and micronutrients (boron, cobalt, copper, iron, manganese, molybdenum and zinc). This natural product comes from the hydrolysis of crop residues to a decoction that favors the release of nutrients easily used by plants.

Different dilutions of Chamae were applied on seeds of two maize varieties (MAS 68 K and LG 30681). MAS 68 K from Maïsadour (Haut-Mauco, France) is a very late variety of dent maize for producing grains. LG 30681 from Limagrain (Saint Beauzire, France) is a very late variety suitable for both grains and forage. Both varieties were obtained in France. According to the breeder, LG 30681 is characterized by its high tolerance to *Fusarium* ear rot. The seeds were surface sterilized. They were covered with 700 mL L^{-1} ethanol for 3 min, rinsed with sterile Milli-Q water and submerged for 15 min in 25 mL L^{-1} sodium hypochlorite solution containing 400 µL L^{-1} Tween 20. Hypochlorite was removed and the seeds were immersed again in 700 mL L^{-1} ethanol for 3 min, rinsed with sterile Milli-Q water five times and dried under a flux of sterilized air.

Surface-disinfected seeds were soaked in different dilutions of the natural fertilizer (1, 0.01 and 0.005% v/v in demineralized water) for 20 h at room temperature. For drying, they were spread on sterilized filter paper and placed in a laminar flow hood for 2 h. In controls, the treatments were not applied to the surface-disinfected seeds. Subsequently the seeds were plated on sterile filter paper disks moistened with 1 mL of sterile demineralized water in 90 mm diameter Petri plates at 25 °C, without light, until germination.

Germination of 40 seeds in each of three replicates was evaluated for each dilution of treatment with the natural product and control treatment in the two maize varieties. The percentage of germinated seeds was estimated 20 and 48 h after deposition on Petri plates with moistened paper.

The strain 0001 of *F. verticillioides* used had been first isolated from symptomatic maize plants in Spain and conserved as a single-spore isolate in the IFAPA Las Torres-Tomejil collection (Seville, Spain). It was grown up on potato dextrose agar (PDA) plates at 25 °C in the dark for 10 days. A suspension was prepared by blending infested PDA with 5 mL of sterile distilled water on each plate. The spore suspension was filtered through four layers of sterile cheesecloth. Spore concentration was determined using a hemocytometer, and the suspension was diluted with sterile Milli-Q water to obtain a final concentration of 2×10^6 conidia mL^{-1}.

When all seeds had germinated, they were inoculated by pipetting 5 µL of spore suspension (2×10^6 conidia mL^{-1}) in the area of the natural wound that produces the exit of the rootlet during germination. Eight days after inoculation (DAI), 20 seedlings per repetition of each treatment and variety were frozen, lyophilized and stored before quantification of fumonisins and DNA isolation from *F. verticillioides*.

Extraction and quantification of fumonisins from germinated and inoculated seeds

At 8 DAI, 4 g of freeze-dried ground germinated and inoculated seeds were extracted with 20 mL of methanol/water (3:1 v/v). After 15 min of agitation in an orbital shaker at $0.413 \times g$ and centrifugation (3 min, $1013 \times g$), the supernatant was filtered through Whatman 2V filter paper. The filtrate was adjusted to pH 6.5 by adding 10 mol L^{-1} NaOH. Fumonisins were purified using Bond Elut strong anion exchange (SAX) cartridges (Agilent), which were conditioned with 5 mL of pure methanol and 5 mL of methanol/water (3:1 v/v). Samples of 5 mL were added to the cartridges. The samples were washed by passage through a column of 5 mL of methanol/water (3:1 v/v) and 3 mL of pure methanol. To elute the samples from the column, 10 mL of methanol with acetic acid (10 mL L^{-1}) was added. The samples were evaporated to dryness under a nitrogen stream and the pellets were dissolved in 200 µL of methanol before high-performance liquid chromatography (HPLC) analysis with fluorescence detection. Just before injection (20 µL), samples were derivatized using a mixture of *o*-phthaldialdehyde (OPA) and β-mercaptoethanol (40 mg of OPA dissolved in 1 mL of methanol, 50 µL of β-mercaptoethanol and 5 mL of 0.1 mol L^{-1} borax in water). Chromatographic separation was done with a C18 Kinetex column (150 mm × 4.6 mm, 3.5 µm; Phenomenex, France) linked to an HPLC 1100 system (Agilent, France) operating in isocratic mode. The mobile phase was composed of methanol/water (77:23 v/v) and 0.1 mol L^{-1} NaH_2PO_4 and the pH was adjusted to 3.3 with orthophosphoric acid. The flow rate was 0.6 mL min^{-1}. The excitation wavelength was 335 nm and the emission wavelength was 440 nm. The total run time was 18 min.

Quantification was performed using external calibration with FB_1, FB_2 and FB_3 standard solutions prepared from commercial pure powders (Romer Labs, Tulln, Austria).

Detection and quantification of total DNA from *F. verticillioides* in maize samples

Twenty seedlings of each repetition, treatment and variety were collected at 8 DAI. These samples were lyophilized and crushed in liquid nitrogen. Then 0.1 g portions of these tissues were used for DNA extraction with a DNeasy® Plant Mini Kit (Qiagen, Courtaboeuf, France).

The other seedlings not used to extract DNA were planted in pots as described in the next section. A second DNA extraction was carried on lyophilized stems and leaves of young plants from

pots at 36 DAI to detect the fungus and determine the possible colonization of the aerial part of plants. They were ground under liquid nitrogen in a mortar and their total DNA was extracted according to the *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (CTAB) procedure as described by Barroso *et al.*[16]

The concentration of nucleic acids (DNA) was determined using an NanoDrop ND-1000 spectrophotometer (NanoDrop Products, Wilmington, DE, USA).

For each analyzed sample, DNA was amplified with primers designed to track *F. verticilloides* DNA. The pair primer Fum 1-654 (5′- CGGTTGTTCATCATCTCTGA-3′) and Fum1-1158 (5′-GCTCCCG ATGTAGAGCTTGTT C-3′) was designed to amplify a 504-bp fragment (Tm, 60 °C) from the polyketide synthase gene FUM1 (GenBank accession number AF155773) of *F. verticillioides*. To normalize the quantification as ng fungus DNA ng^{-1} maize DNA, we also performed the quantification of *maize actin* gene (accession number J01238) using primers MA-F(TCCTGACACTGAAGTACCCGATTG) and MA-R(CGTTGTAGAAGGTGTGATGCCAGTT).[17] Quantification were determined using standard curves of cycle thresholds were generated using known quantities (0.002 – 20 ng) of pure DNA of *F. verticillioides* or maize.

Quantitative PCR (qPCR) experiments were performed in a LightCycler® 1.5 System (Roche Applied Science, Meylan, France) with a QuantiFast SYBR Green PCR Kit (Qiagen). Each reaction mixture (1 µL) contained 20 ng of DNA, 1× QuantiFast SYBR Green PCR Master Mix, 5 mmol L^{-1} $MgCl_2$ and 0.5 mmol L^{-1} of each primer. The amplification conditions consisted of a first denaturation step for 5 min at 95 °C, followed by 40 cycles of 10 s of denaturation at 95 °C and 30 s of combined annealing/extension at 60 °C. The melting curve was generated using the following profile: 15 s at 95 °C, 15 s at 65 °C and 15 s at 95 °C with a 0.1 °C s^{-1} transition. Assays for each sample were performed in triplicate.

Lack of non-specific qPCR amplification or primer-dimer formation was checked by melting curve analysis in each run.

Early stage of maize growth (biomass aerial weight and plant height) and dead plants

To evaluate the evolution of the fungus from the seed to the aerial part of the plants and the consequences on plant growth, nine germinated seeds of each treatment were planted in individual pots (14 cm × 14 cm × 20 cm) filled with 2.5 L of a potting substrate based on a blend of weakly decomposed white sphagnum peat and clay granules (Substrate 5, Klasmann-Deilman GmbH, Germany). The plants were cultivated for 28 days in a greenhouse. Temperature and air humidity were recorded. Mean temperature during the cultivation period was 22 °C, with maximum at 30.2 °C and minimum at 15.0 °C. Mean air humidity was 59.1%, with 84.5% as maximum and 37.0% as minimum. The substrate was regularly humidified when necessary. Damping-off symptoms as dead plants were followed throughout the culture. Height (cm) and aerial biomass weight (g) of plants were measured at the end of this growing period.

Number of productive plants, plant height and yield

An assay under field conditions was performed in Alcalá del Rio (Seville, Spain). The soil is of silty clay texture with (w/w) 46.4% clay, 46.7% silt and 8.9% sand. The proportions (w/w) of total Kjeldahl nitrogen, organic carbon (O.C.) and organic matter (O.M.) are 0.12, 2.3 and 0.78% respectively, with a pH of 8.28 and a conductivity of 1.5 mS cm^{-1}. Cation exchange capacity is (meq per 100 g) 23.5%, with calcium at 8.86%, magnesium at 11.06%, potassium at 1.26% and sodium at 2.02%. Other characteristics are (w/w) 99% base saturation, 6% exchangeable sodium, 20.6% carbonate, 11.6% active limestone, 21.7 ppm phosphorus and 419 ppm potassium.

Normal agronomic techniques were adopted. Briefly, the previous crop was maize and the field was ploughed each year.

Sowing was performed at high density on 17 March 2017 using pneumatic testing machines, and subsequent removal of plants was required to obtain a density of 95 000 – 100 000 plants ha^{-1}.

Seeds of two different varieties (MAS 68 K and LG 30681) were subjected to two different treatments: (1) seed treated with 0.01% dilution of natural fertilizer Chamae by imbibition for 20 h, 1 day before sowing, and (2) seed without any treatment. The design was a random blocks with four replicates. Each repetition consisted of two rows 0.75 cm apart and of 7 m length.

When the plant reached male flowering, at which time it already has its definitive size, the number of productive plants, plants was measured by counting plants with standard height and regular ear size in each replicate in one row. The height of ten plants was also measured.

The harvest was performed on 28 August 2017 using harvest testing machines, and the yield was quantified in kg ha^{-1}.

Statistical analysis

Analysis of variance (ANOVA) by variety for treatments was performed for all measured variables. When the interaction between variety and treatment was not significant, mean values of characteristics were averaged over both maize varieties. When interaction was significant, the variables were analysed to ANOVA in each variety. Means were compared using the least significant difference (LSD) test at $P \leq 0.05$ and $P \leq 0.01$.

Percentage data were transformed ($\sin^{-1}(\sqrt{Y/100})$) and ANOVA was used to test for statistically significant effects of independent variables. All statistical analyses were performed with Statistix 9.0 (Analytical Software, Tallahassee, FL, USA).

RESULTS

The study was conducted to evaluate the influence of a natural fertilizer Chamae applied on seed in order to control fumonisin production, *F. verticillioides* development and agronomic parameters such as germination, plant height, dead plants, aerial biomass weight of plants and yield. The results showed that fumonisin production and *F. verticillioides* development were reduced in inoculated maize plants by the application of natural fertilizer on seeds and the impact of this fertilizer on agronomic parameters; however, the highest concentration of this natural fertilizer had a negative impact on the number of dead plants, but lower concentrations minimized this negative impact.

Seed germination

Treatment effects on seed germination are reported in Tables 1 and 2. There was a significant interaction between variety and treatment and significant effects due to both variety and treatment (Table 1).

The data indicated that 20 h after placing the seeds on watered filter paper in Petri plates, no germination was observed in controls (non-treated seeds) of both maize varieties, whereas the germination rates in treated seeds were between 80 and 90% for MAS 68 K and between 50 and 60% for LG 30681 (Table 2). The differences between the two varieties were significant for all doses of treatment with the natural fertilizer used for priming (Tables 1 and 2). After 48 h, between 95 and 100% of the seeds germinated (data

Table 1. ANOVA for studied parameters under controlled conditions: seed germination, fumonisin (FB1, FB2 and FB3) production, quantification of *Fusarium verticillioides* DNA, height and weight of aerial parts of 1-month-old plants

Source of variation	DF	Parameter															
		Germination rate		Fumonisin concentration						DNA quantification		% of dead plants 8 days after inoculation		Plant height(cm)		Biomass weight(g)	
				FB1		FB2		FB3									
		MS	P	MS	P	MS	P	MS	P	MS	P	MS	P	MS	P	MS	P
Treatment (T)	3	8454.51	0.0000***	12 305.1	0.0121*	1583.96	0.0041**	40.55	0.0274*	0.00607	0.0041**	0.0038	0.0222*	68.18	0.3978	3.17	0.6908
Variety (V)	1	1926.04	0.0000***	73 444.4	0.0001***	8866	0.0000***	226.97	0.4335	0.09401	0.0000***	0.0006	0.0000***	81.34	0.2724	3.05	0.4714
Repetition (R)	2	0.26	0.9917	200.1	0.9184	211.66	0.4144	5.42	0.1055	0.00140	0.4144	0.0025	0.4236	91.62	0.2623	11.98	0.1506
T × V	3	232.29	0.0032**	9968.6	0.0245*	1452.97	0.0058**	37.20	0.6091	0.00122	0.0058**	0.0006	0.5181	48.03	0.5656	6.98	0.3321
Error	14	31.21		2334.7		225.47		5.77		0.00153		0.0009		63.21		5.61	

DF, degrees of freedom; MS, mean square;
*$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$.

not shown). The treatment did not affect the germination power of seeds and no difference between the varieties was significant, but it was observed that seed treatments anticipated the germination.

F. verticillioides growth and fumonisin production in inoculated seedlings

The fumonisin and *F. verticillioides* DNA concentrations are shown in Table 1 and Figs 1 and 2. The variation factors maize variety and treatment and the interaction between them were significant for FB_1, FB_2 and fungus DNA (Table 1). In the case of FB_3, the only significant factor was treatment (Table 1).

Eight days after *F. verticillioides* inoculation, fumonisin concentrations were significantly higher ($P \leq 0.05$) in seedlings of MAS 68 K variety than in those of LG 30681 variety (Fig. 1). In MAS 68 K, all dilutions of the natural product reduced fumonisin (FB_1, FB_2 and FB_3) accumulation significantly ($P \leq 0.05$) with regard to inoculated control (Fig. 1.). In the case of LG 30681, the accumulation of these mycotoxins showed lower values than in the other variety, and it was not affected by treatments on seeds (Fig. 1.). Regarding the proportion of different fumonisins detected, FB_1 had the highest concentrations, followed by FB_2 and finally FB_3 (Fig. 1.).

The same 20 seedlings per repetition were used to quantify fungus DNA (*Fum1* gene) as an estimation of the *F. verticillioides* biomass. Significant differences ($P \leq 0.05$) in this parameter were explained by effects of both seed treatments and maize variety (Table 1 and Fig. 2). An interaction between variety and treatment was observed (Table 1). A significantly higher quantity of *F. verticillioides* DNA was observed in MAS 68 K seedlings than in the variety LG 30681 seedlings (Fig. 2.). A significantly lower amount ($P \leq 0.05$) of fungus DNA was observed in MAS 68 K seedlings treated with 0.01% of natural fertilizer (Fig. 2.). In LG 30681 seedlings, all three doses reduced significantly ($P \leq 0.05$) the amount of fungus DNA measured (Fig. 2.). We note that fungus DNA was not detected in negative control seedlings. This indicates that there had been no cross-contamination during inoculation or incubation (data not shown).

Plant height, dead plant percentage and weight of aerial biomass of plants inoculated with *F. verticillioides*

No interaction between variety and treatment was detected (Table 1), so the results for these parameters are represented as the means of the two varieties (Table 3).

One month after transplantation of the seedlings, the seed treatments and inoculation with *F. verticillioides* had not a significant effect on the plant height and weight of aerial biomass of plants under control condition (Tables 1 and 3). Nevertheless, we observed that the dilutions 1 and 0.05% of seed treatments increased significantly ($P \leq 0.05$) the percentage of dead plants (Tables 1 and 3). In both inoculated and non-inoculated controls, dead plants were not observed (Table 3). The dilution 0.01% of seed treatment showed dead plants, but this parameter was not significantly different from the inoculated and non-inoculated controls (Tables 1 and 3). No significant differences were observed among treatments for the weight of aerial biomass (Tables 1 and 3).

Number of productive plants, plant height and yield

To determine the effect of natural fertilizer on seeds in a field experiment, we chose the 0.01% dilution applied on seed, because it showed the best behavior of the parameters evaluated under controlled conditions (germination, fumonisin accumulation,

Table 2. Effect of seed treatments on germination

	Germination (%)							
	Variety LG-30681				Variety MAS 68 K			
	Natural fertilizer dilution (%)				Dilution (%)			
	Control	1	0.01	0.005	Control	1	0.01	0.005
Natural fertilizer application	0c	55.83a	67.50ab	65a	0c	83.33ab	86.66a	90a

Different letters indicate significant differences according to LSD test ($P \leq 0.05$).

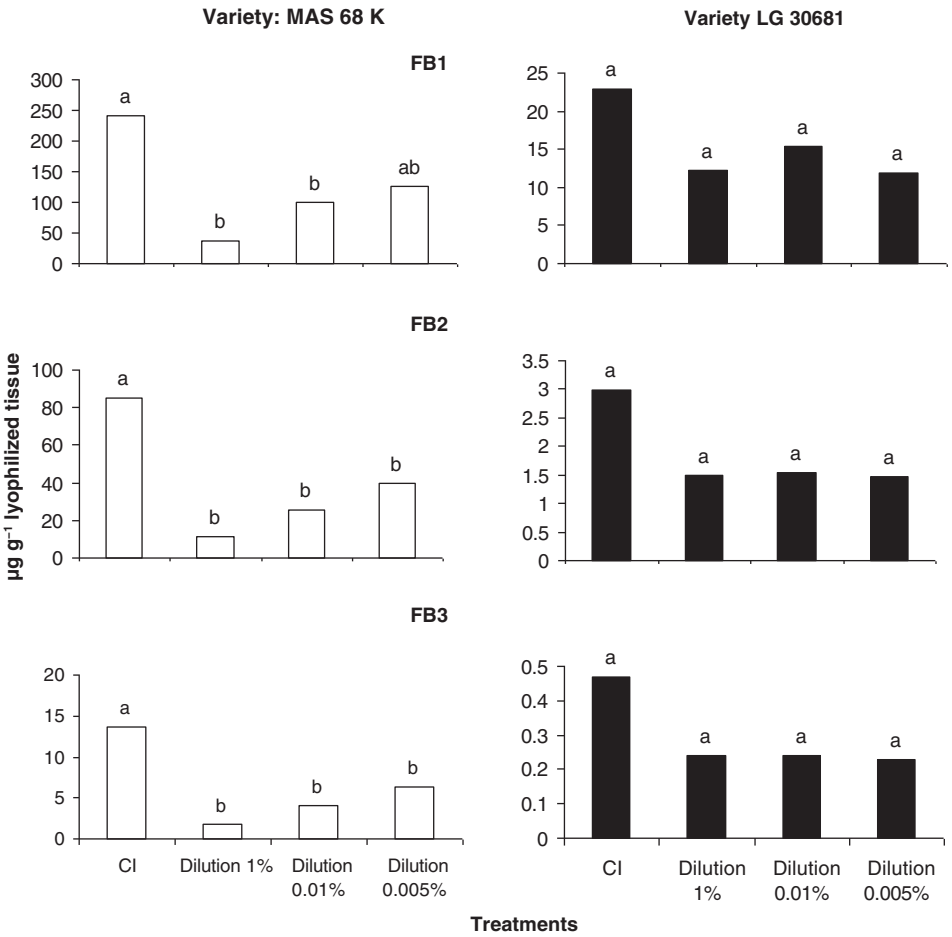

Figure 1. Accumulation of FB1, FB2 and FB3 8 days after inoculation by *Fusarium verticillioides* in seedlings of maize previously treated with different doses of a natural fertilizer on seeds of two varieties (MAS 68 K and LG 30681): CI, control inoculated plants and non-treated seed; doses 1%, inoculated plants and seed treated with natural fertilizer diluted at 1% (v/v); doses 0.01%, inoculated plants and seed treated with natural fertilizer diluted at 0.01% (v/v); doses 0.005%, inoculated plants and seed treated with natural fertilizer diluted at 0.005% (v/v). Bars with the same letters indicate non-significant differences among treatments and bars with different letters indicate significant differences among treatments according to LSD test ($P \leq 0.05$).

plant death and aerial biomass weight), compared with the effect of control seed, on number of productive plants, plant height and yield in the two maize varieties (MAS 68 K and LG 30681) under field conditions (Tables 4 and 5).

Eighty-four days after sowing, the number of productive plants coming from treated seeds was significantly lower ($P \leq 0.05$) than that from non-treated controls (Tables 4 and 5).

Three months after sowing, when the plants were flowering, the plant height was not significantly different among different treatments (Tables 4 and 5).

The yield, estimated as kg ha^{-1} at the end of the season, was not significantly affected by the seed treatment (Tables 4 and 5).

DISCUSSION

Maize fumonisin contamination poses a serious risk to human and animal health. The study of new techniques for its control, overall if they are respectful to the environment and are of low cost, is a priority for the sustainable control of this serious problem. The aim of this work was to study the influence of the priming state on maize plants caused by the application of Chamae in the seed state.

Seed priming is a pre-sowing technique for regulating the germination process by triggering the pre-germinative metabolism that is normally activated during the early phase of germination. It is proposed as a way to achieve faster and better seed germination.

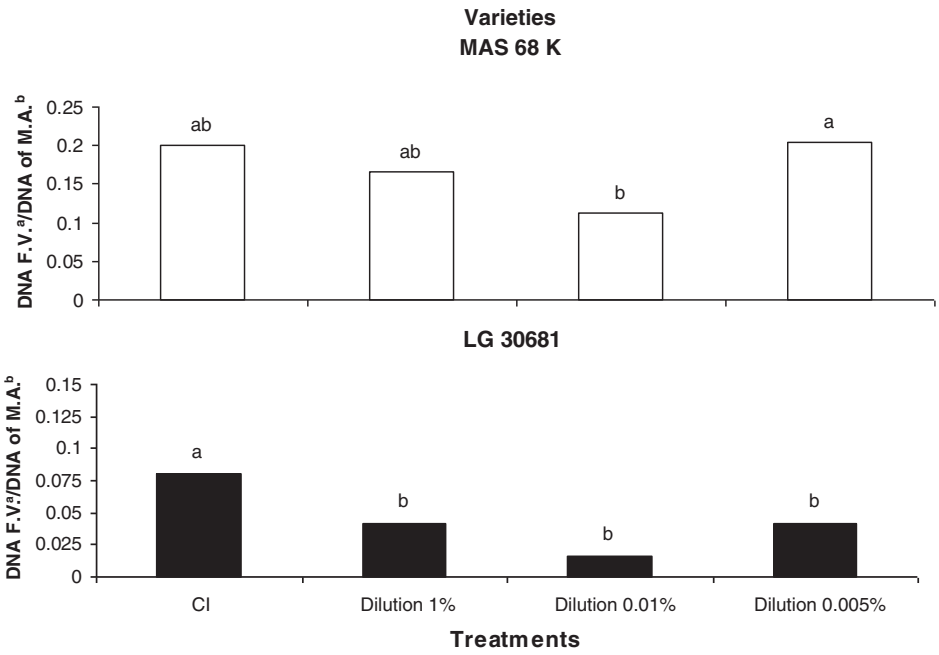

Figure 2. Quantification of *Fusarium verticillioides* DNA as ratio of *Fum1* gene amplification to *maize actin* gene amplification in seedlings of two maize varieties (MAS 68 K and LG 30681) 8 days after inoculation: CI, control inoculated plants and non-treated seeds; doses 1%, inoculated plants and seed treated with natural fertilizer diluted at 1% (v/v); doses 0.01%, inoculated plants and seed treated with natural fertilizer diluted at 0.01% (v/v); doses 0.005%, inoculated plants and seed treated with natural fertilizer diluted at 0.005% (v/v). Bars with the same letters indicate non-significant differences among treatments and bars with different letters indicate significant differences among treatments according to LSD test ($P \leq 0.05$).

Among the various priming techniques, biopriming involves a soaking treatment with beneficial microorganisms or bioactive molecules such as phytohormones.[18] In most cases,greater germination of seed was showed a greater rate of germination in treated seed.[19] However, the results obtained by rate of various priming techniques in seeds are heterogeneous according to the different species, the quality of the seed or the conditions of germination, among other parameters.[20]

Molecular techniques which are used for the improvement of *F. verticillioides* resistance and fumonisin production are efficient in breeding programs. They allow quick selection of lines in maize. However, the complex genetic basis of these characters and the great environment effect in their phenotypic expression make the location of QTLs and the efficiency of marker-assisted selection (MAS) more difficult.[21]

In this sense, the seed priming technique could improve or help the effect obtained through breeding of resistant maize varieties, being interesting for studies on an integrated disease management combining both techniques.

In the present study, we used three different dilutions of Chamae soaking of maize seeds for 20 h as priming treatment. Owing to the very high germination rates of controls, no improvement in this trait was observed. However, all treatments decreased the germination time by at least 1 day in comparison with control plants.

In addition to the beneficial effect on germination, priming is also proposed as a way to produce plants tolerant against various stresses[22] by the initiation in the plant of a 'preparation' state that does not confer resistance in itself but allows the acceleration of induction of the defences once the plant faces such stress. The plant is then able to respond better than other plants that have not experienced it.[23] Thus an advantage of this technique is that it does not entail the costs normally associated with the activation of an inducible defense response, since the defensive response is triggered only if the plant recognizes some of these types of stress, and without major ecological or physiological effects.[24] Systemic infection of maize by *F. verticillioides* could be through the seeds via fungal conidia or mycelia that are carried inside the seeds or come from inocula that survive in crop residues in the soil. This is an infection route of ears that has been proposed by several authors.[9,10] *Fusarium verticillioides* is also considered as a 'root pathogen' responsible for damping-off symptoms.[25] To determine whether plants might respond to priming agents at the seed stage, in the present study, we infected seedlings of two maize varieties with spores of a Spanish strain of *F. verticillioides*. We observed that the amount of DNA from the fungus was significantly lowered with the different dilutions of Chamae applied on seeds, with a different behavior of the two maize varieties used. The amount of fungus DNA was threefold lower in variety LG 30681; it is known to be more tolerant than variety MAS 68 K. This lower colonization by *F. verticillioides* in a tolerant variety than in a susceptible variety agree with the observation of Wu *et al.*[25] The threefold lower development of *F. verticillioides* led to more than tenfold lower fumonisin accumulation. These mycotoxins are known to contribute to virulence in seedlings grown from inoculated maize seeds.[26,27] The partial resistance to colonization at seedling stage and limitation of fumonisin production might be a component of the claimed low susceptibility of LG 30681 to *Fusarium* ear rot. The same results were observed also in adult plants and not only at the seedling stage.[28]

Previous studies showed a high level of variability between susceptible and resistant maize lines in their response to *F. verticillioides* infection, although both presented similar functional categories of genes responsive to infection. Resistant lines showed basic defense against the fungus, because the genes were highly transcripted before infection. However, in susceptible maize lines, the genes were induced after infection. These results suggest that

Table 3. Effect of seed treatments on plant height, weight of aerial biomass and died plants of maize inoculated with *Fusarium verticillioides* under control conditions as average of two varieties (MAS 68 K and LG 30681)

Parameter	Control	Control inoculated	Natural fertilizer application dilution (%) on seed and inoculated on seedling		
			1	0.01	0.005
Plant height (cm)	65.00a	61.00a	59.86a	59.83a	55.67a
Weight of aerial biomass (g)	9.13a	8.69a	7.65a	7.69a	7.44a
Dead plants (%)	0a	0a	49.60c	11.10ab	27.75bc

Same letters indicate non-significant differences among treatments and different letters in the same row indicate significant differences according to LSD test ($P \leq 0.05$).

Table 4. ANOVA for studied parameters under field conditions: number of productive plants, plant height and yield

Source of variation	DF	Parameter					
		Number of productive plants per block 84 days after sowing		Plant height (84 days after sowing) at flowering		Yield (kg ha^{-1})	
		MS	*P*	MS	*P*	MS	*P*
Treatment (T)	1	60.06	0.0355*	0.00045	0.7846	50 457.7	0.8943
Variety (V)	1	10.56	0.3272	0.00019	0.8594	7.2×10^7	0.0002***
Repetition (R)	3	1.72	0.9102	0.00566	0.4387	7×10^6	0.09
T × V	1	3.06	0.5905	0.02038	0.091	3 575 722	0.2755
Error	9	9.84		0.051		2 738 860	

DF, degrees of freedom; MS, mean square;
$^{*}P < 0.05$; $^{***}P < 0.0001$.

Table 5. Effect of seed treatments on plant height, number of productive plants and yield as average of two varieties (MAS 68 K and LG 30681) under field conditions

Parameter	Seed treated with dilution 0.01%	Non-treated seed
Plant height (cm)	2.71a	2.71a
Number of productive plants	48.75a	52.62b
Yield (kg ha^{-1})	10 605a	10 505a

Same letters indicate non-significant differences among treatments and different letters in the same row indicate significant differences according to LSD test ($P \leq 0.05$).

plant basal genes in seed tissue could decrease colonization and fumonisin synthesis by the fungus.[28,29]

The 0.01% dilution of Chamae applied on seeds of the susceptible variety (MAS 68 K) was able to reduce significantly the level of fumonisin accumulation and the biomass of *F. verticillium* in seedlings during the first few days following inoculation. The level of contamination of seedlings was reduced to the same level as that of the tolerant untreated variety. The tolerant variety did not show any significant response to the treatment for fumonisin accumulation, but the quantity of fungal biomass was significantly lowered in seedlings treated with the different dilutions of Chamae. These results suggest that the treatments might favor defense responses in seedlings when they are inoculated with the fungus *F. verticillioides*. Interestingly, plant response did not induce a reaction of the used strain of *F. verticillioides* through higher production of fumonisins, but a lower rate of fumonisin accumulation per unit of fungal biomass. Production of oxidative stress protectors is known to be activated (accumulation of stress-related proteins, constitutive expression of pathogenesis-related proteins and antioxidant enzyme activities) as maize response to *F. verticillioides* infection of maize in embryos and adult plants, triggering maize resistance towards this fungus.[30,31] Ferrigo *et al.*[32] observed divergent behaviours in *F. verticillioides* populations faced with oxidative stress induced by H_2O_2. Other strains could react differently than the Spanish one tested here, and a large panel of strains should be tested in further works.

Mycotoxin contamination of feed often begins in the field, but this could continue at harvest, transportation and storage, depending on environmental conditions. Thus the most effective way to prevent mycotoxin occurrence is to limit the biosynthesis of the fungus when the crop is cultivated.[13]

After infection, *F. verticillioides* might develop systemic colonization of young plants. In the present work, after planting of seedlings in a horticulture substrate in pots, we obtained either plants that developed normally without symptoms or plants that died before they reached their first month of growth. Because of the possibility of plant colonization by the fungus, we wanted to know if, after inoculation in seedlings, this colonization of the stem had occurred when the plants were already 1 month old. Therefore DNA was extracted from the stem of the plants and the presence of *F. verticilloides'* DNA was studied by quantitative PCR. In this case, no symptoms were observed on plants whatever the treatments, and fungus DNA was not detected by PCR in the stems of the inoculated plants after surface sterilization (data not shown). The aerial biomass was not significantly affected in the plants from treated seeds inoculated with *F. verticillioides*. According to the work of Howard[33] and Duncan and Howard,[34] *F. verticillioides* does not form penetrating structures that break the epidermis, which can hinder systemic infection. Oren *et al.*[9] observed asymptomatic systemic infection characterized by infection of only certain tissues, intercellular growth of a limited number of fungal hyphae

and reproduction of the fungus in a few cells without invasion of other cells. In the stems, they detected only a few mycelia of *F. verticillioides* green fluorescent protein expressing transgenic isolate. We cannot exclude that such asymptomatic colonization occurred in the present experiment with levels of mycelium that were not detectable. In our experiment, necrotrophic symptoms were not observed in non-treated controls, whereas they were shown at the lowest dilution or highest concentration of Chamae. The fungus probably used the nutrients from the fertilizer coating the seeds for its external development and then rotted mesocotyl and main root cells with massive production of fungal mycelia, as observed by Oren *et al.*[9] in physiologically weakened plants. In their study, this always led to stopping the development of plants and their death. This negative effect might be modulated under other circumstances of *F. verticillioides* inoculum potential and soil environment, where the space could be occupied before by competitors.

The defenses induced by seed treatments may be associated to a plant energy cost that could result in losses of productive plants, production or even plant size.[35] Benefits from induced resistance responses via the application of different products might be limited by the inherent costs of defense.[36] The costs associated with direct activation of defenses also could be associated with loss of yield or other agronomic factors of plants. Such costs were observed with detrimental effects on direct activation of the defense system in plants,[22] but not when a priming state was induced through application of jasmonic acid on seeds.[20] In the present work, the fertilizer applied on seed had a minimal impact on the height and aerial biomass weight of 1-month-old plants and did not affect the final yield (kg ha^{-1}), despite a lowering of the number of plants with standard height and regular ear size (productive plants) in each repetition.

CONCLUSION

According to our results, provoking a priming state by applying the natural fertilizer Chamae on maize seeds could improve germination and limit the colonizationof the seedling by *F. verticillioides* and its associated fumonisin accumulation in seedlings. However, depending on the dilution of fertilizer applied, further plant death was observed, and finally the number of productive plants under field conditions was lowered, but without affecting the final yield, suggesting a higher level of biomass production per plant. This situation can be amended with an increase in planting density. The 0.01% dilution of the seed treatment is the most recommended, since it improved germination and reduced the amount of fungus DNA and fumonisin production by fungus, did not increment signicantly death plants and did not reduced the yield. Therefore, the priming can be recommended as a low-cost and effective technique to control *F. verticillioides* infection and fumonisin production without affecting crop production.

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