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

Ana Aguado, Jean-Michel Savoie, Sylvain Chéreau, Christine Ducos, Maria Aguilar, Nathalie Ferrer, Manuel Aguilar, Laetitia Pinson-Gadais, Florence Richard-Forget

► To cite this version:

Ana Aguado, Jean-Michel Savoie, Sylvain Chéreau, Christine Ducos, Maria Aguilar, et al.. Priming to protect maize from *Fusarium verticillioides* and its fumonisin accumulation. *Journal of the Science of Food and Agriculture*, 2019, 99 (1), pp.64-72. 10.1002/jsfa.9142 . hal-02155923

HAL Id: hal-02155923

<https://hal.science/hal-02155923>

Submitted on 14 Jun 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

Journal:	<i>Journal of the Science of Food and Agriculture</i>
Manuscript ID	JSFA-18-0077.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	26-Apr-2018
Complete List of Authors:	<p>Aguado, Ana; Instituto Andaluz de Investigacion y Formacion Agraria Pesquera Alimentaria y de la Produccion Ecologica, Sustainable Plant Protection</p> <p>Savoie, Jean-Michel; MycSA, UR1264, INRA, 71 Avenue Edouard Bourleaux, F-33883 Villenave d'Ornon Cedex, France, MycSA</p> <p>Chereau, Sylvain; UR1264, INRA, 71 Avenue Edouard Bourleaux, F-33883 Villenave d'Ornon Cedex, France, MycSA,</p> <p>Ducos, Christine; UR1264, INRA, 71 Avenue Edouard Bourleaux, F-33883 Villenave d'Ornon Cedex, France, MycSA</p> <p>Aguilar, Maria; Instituto Andaluz de Investigacion y Formacion Agraria Pesquera Alimentaria y de la Produccion Ecologica, Crop Protection</p> <p>Ferrer, Nathalie; UR1264, INRA, 71 Avenue Edouard Bourleaux, F-33883 Villenave d'Ornon Cedex, France, MycSA</p> <p>Aguilar, Manuel; Instituto Andaluz de Investigacion y Formacion Agraria Pesquera Alimentaria y de la Produccion Ecologica, Agriculture and Environment</p> <p>Pinson-Gadais, Laetitia; UR1264, INRA, 71 Avenue Edouard Bourleaux, F-33883 Villenave d'Ornon Cedex, France, MycSA</p> <p>Forget, Florence; UR1264, INRA, 71 Avenue Edouard Bourleaux, F-33883 Villenave d'Ornon Cedex, France, MycSA</p>
Key Words:	priming, germination, mycotoxins, <i>Fusarium</i> disease, maize

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

ABSTRACT

BACKGROUND

Systemic infection through the seed is one of the routes used by mycotoxinogen pathogen *Fusarium verticillioides* for colonizing maize plants. The prohibition of most of the chemical fungicides by the EU promote the research of plant resistance inducers as an effective and sustainable alternative. Induction of a priming state in maize seeds might affect their susceptibility for contamination and accumulation of fumonisins. This state, obtained by application of a natural fertilizer called Chamae on maize seeds, was investigate in two varieties to control the colonization by the fungus and the accumulation of fumonisins B₁, B₂, and B₃.

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 plant stop their development by necrosis. In a field trial, the 0,01% 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 the fungicide use and environmental contamination.

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, Florence Richard-Forget^b.

^a IFAPA Las Torres Tomejil, Área de Protección de Cultivos, Carretera Sevilla-Cazalla Km 12.2, Alcalá del Río, 41200 Seville, Spain.

^b MycSA, UR1264, INRA, 71 Avenue Edouard Bourleaux, F-33883 Villenave d'Ornon Cedex, France.

* corresponding author: ana.aguado@juntadeandalucia.es

INTRODUCTION

Maize is one of the most important agricultural commodities in the world, and it is used as the major ingredient of dietary staple for humans and animals. In North America and Europe, many maize products are consumed, including breakfast cereals, snacks, softdrinks and beer. Maize is also used to make animal feed. Fumonisin are considered to be hazardous to human and animal health¹⁻⁴. *Fusarium verticillioides* (synonym, *Gibberella fujikuroi*) is one of the most important fungal pathogens in maize causing both pre- and post-harvest losses and being also capable of producing mycotoxins such as Fumonisin B₁ (FB₁) B₂ (FB₂) and B₃ (FB₃), being frequently detected in healthy maize grains^{5,6}. *F. 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 kernels⁷. Insects or other biotic or abiotic agents creating kernels wounds also favour kernel infection⁸. Systemic infection through the seed is another pathway. It starts from fungal conidia or mycelia that either are carried 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 kernels⁹. 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 most of the chemical fungicides by the EU (Directive 91/414/CEE), due to adverse effects of their use on the environment and human health, their toxicity or because pathogens have become resistant to their active substances, have promoted research in the field of plant resistance inducers as an effective and sustainable alternative ¹¹.

Protection of maize seeds from infection of *F. verticillioides* by inoculation of antagonists has been experimented. It may reduce the 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 B₁ 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 strategy successfully used to detect and identify quantitative trait loci (QTLs) and candidate genes involved in *F. verticillioides* resistance and low mycotoxin contamination synthesized by this fungus in maize. This tool allows to reveal novel sources of resistance to select maize lines in breeding programs quicker and more efficiently than the classic breeding. Recently, other researchers have got identified 8 QTLs and 43 genes associated with *F. verticillioides* seed rot (FSR) resistance through linkage mapping and GWAS, respectively ¹⁴. By combining transcriptomic data with QTL, 24 candidate genes for resistance to *F. verticillioides* were identified, allowing to make possible the selection of genotypes with both low disease

1 severity and low fumonisin contamination¹⁵.
2

3 The physiological health of the plantlets may also affect the development of the symptoms
4
5
6⁹ and induction of the priming state in the plant through the application of natural
7 fertilizers on seeds could be another way to prepare plants against pathogen attacks.
8

9
10 The aim of the present work was to characterize the influence of a commercial natural
11
12 fertilizer based on vegetal extracts obtained from plant decoctions, Chamae on early
13
14 colonisation of germinating seeds and plantlets by *F. verticillioides* and the
15
16 development of symptoms, including the fumonisin accumulation, and the influence of
17
18 treatments on yield.
19
20
21
22
23
24
25

26 EXPERIMENTALS

27 28 29 30 Seed treatments and inoculation

31 The natural fertilizer Chamae (Saionaimer S.L.) used for seed treatments contains
32
33 principal macronutrients (nitrogen, phosphorus, potassium), secondary macronutrients
34
35 (calcium, magnesium, potassium, sodium and sulphur), and micronutrients (boron, cobalt,
36
37 copper, iron, manganese, molybdenum and zinc). This natural product comes from the
38
39 hydrolysis of crop residues through its decoction that favors the release of nutrients easily
40
41 used by plants.
42
43

44 Different dilutions of Chamae were applied on seeds of two maize varieties (MAS 68 K and
45
46 LG-30681). MAS 68K from Maïsadour (Haut-Mauco, France) is a very late variety of dent
47
48 maize for producing grains. LG-30681, from Limagrain (Saint Beaulieu, France) is a very
49
50 late variety suitable for both grains and forage. Both varieties were obtained in France.
51
52

53 According to the breeder, LG-30681 is characterised by its high tolerance to *Fusarium*
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

ear rot. The seeds were surface sterilized. They were covered with 70% ethanol for 3 min, rinsed with sterile milliQ water and submerged for 15 min in 2.5% sodium hypochlorite solution containing 40 µL of Tween-20 100 mL⁻¹. Hypochlorite was removed and the seeds were immersed again in 70% ethanol for 3 min, rinsed with sterile milliQ water 5 times and dried under a flux of sterilized air. Surface-disinfected seeds were soaked in different dilutions of the natural fertilizer (1%, 0,01%, 0,005% v/v in demineralized water) for 20 hours at room temperature. For drying, they were spread on sterilized filter paper and placed in a laminar flow hood for two hours. In controls, the treatments were not applied to the surface disinfected seeds. Subsequently the seeds were plated on sterile filter paper discs 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 replicate and 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 hours after being deposited in Petri plate with moistened paper.

The strain 0001 of *F. verticillioides* had been first isolated from symptomatic maize plants in Spain and conserved as single spore isolate at the IFAPA Las Torres-Tomejil collection (Sevilla, Spain). It was grown up in 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 in each plate. The spore suspension was filtered through 4 layers of sterile cheesecloth. Spore concentration was determined using a haemocytometer, and the suspension was diluted with sterile milliQ water to obtain a final concentration of 2×10^6 conidia mL⁻¹. When all the seeds had germinated, they were inoculated pipetting 5 µL of the spore suspension (2×10^6 conidia mL⁻¹), in the area of the natural wound that produces the exit

of the rootlet during germination. 8 days after inoculation (d.a.i.), 20 seedlings repetition⁻¹ 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

On the eighth day of inoculation, 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 in agitation in an orbital shaker at 0.413 xg and centrifugation (3 min, 1013 xg), the supernatant was filtered through Whatman 2V filter paper. The filtrate was adjusted to pH 6.5 by adding NaOH 10N. Fumonisins were purified by using Bond Elut strong anion Exchange (SAX) Cartridges (Agilent), which were conditioned with 5 mL methanol and 5 mL Methanol/Water (3/1). Then 5 mL of sample was added to the cartridges. The samples were washed by passing through the column of 5 mL of methanol/water (3/1) and 3 mL of pure methanol. To elute the sample from the column, 10 mL of methanol with acetic acid (1%) were 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 analysis with fluorescence detection. Just before injection (20µL), samples were derivatized using o-phthaldialdehyde (OPA) and β-mercaptoethanol mixture (40 mg of OPA are dissolved in 1 mL of methanol, 50 µL of β -mercaptoethanol and 5 mL of Borax 0.1M in water). Chromatographic separation was done with a C18 Kinetex column (150*4.6 mm, 3.5µm) (Phenomenex, France) linked to an HPLC 1100 system (Agilent,) operating in isocratic mode. Mobile phase was composed of Methanol/Water, 0.1M NaH₂PO₄ (77/23, v/v) and pH was adjusted to 3.3 with orthophosphoric acid. Flow rate was 0.6 mL min⁻¹. Excitation wavelength was 335 nm, and emission 440 nm. Total run time was 18 min.

Quantification was performed by using external calibration with FB₁, FB₂ and FB₃ 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 in each repetition, treatment and variety were collected eight days after inoculation. These samples were lyophilised and crushed by liquid nitrogen. 0.1 g of these tissues was used to DNA extraction by DNeasy® Plant Mini Kit according to the manufacturer instructions (Qiagen, Courtaboeuf, France).

The other seedlings not used to extract DNA were planted in pot as it is described in the part “Early stage of maize growth (biomass aerial weight and plants height) and died plants”. So, a second DNA extraction was carried on lyophilized stems and leaves of young plants from pots thirty-six days after inoculation to detect the fungus and to know the possibility of colonization of aerial part of plants. They were ground under liquid nitrogen in a mortar and total DNA were extracted according to the N-cethyl- NNN-trimethyl ammonium bromide (CTAB) procedure, as described in Barroso *et al.*¹⁶ The concentration of nucleic acids (DNA) was determined using a ND-1000 NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE) .

For each analyzed sample, DNA was amplified with primers designed to track *F. verticillioides* DNA. The pair primer Fum 1-654 (5'- CGGTTGTTTCATCATCTCTGA-3') and Fum1-1158 (5'- GCTCCCGATGTAGAGCTTGTT-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 of DNA fungus ng⁻¹ of DNA maize, we also performed the quantification of gene maize actine (accession number 01238) MA-F (TCCTGACACTGAAGTACCCGATTG)/MA-R CGTTGTAGAAGGTGTGATGCCAGTT).

The corresponding melting temperature was 60°C, and the amplified fragment length was 95 base pairs (bp). To know the absolute amount of DNA of both, standard curves of cycle thresholds were generated using known quantities of DNA pure of *F. verticillioides* or of maize (from 0.002 to 20 ng).

Quantitative PCR experiments (qPCR) were performed in a LightCycler® 1.5 System (Roche Applied Science, Meylan, France) and the QuantiFast SYBR Green PCR Kit (Qiagen, Courtaboeuf, France). Each reaction mixture contained 20 ng of DNA (1 µL), QuantiFast SYBR Green PCR Master Mix 1X, 5 mM MgCl₂, and 0.5 mM 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 denaturation at 95 °C, 30 s 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 °Cs⁻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 plants height) and died plants

To evaluate the evolution of the fungus from the seed to the aerial part of the plants and consequences on the plant growth, 9 germinated seeds of each treatment were planted in different pots (14x14x20 cm) filled with 2.5 L of a potting substrate based on 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 it was necessary. Damping-off symptoms as died plants were followed all along 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 Alcal del Rio (Seville, Spain). The soil had the following characteristics: texture silty clay, with a percentage of clay 46.4%, silt 46.7% and sand 8.9%. The percentages of N total Kjeldahl, C.O. and M.O. are 0,12%, 2,3% and 0,78% respectively, with a pH of 8,28 and the conductivity was 1,5 mS/cm. Cation exchange capacity was (meq/100 g) 23.5% with Ca at 8.86%, Mg at 11.06%, K at 1.26%, Na at 2.02. The percentage of base saturation is 99%, exchangeable sodium 6%, carbonates 20.6%, active limestone 11.6%, and the wealth in P is 21.7 ppm and in K 419 ppm.

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

The sowing was performed on March 17th, 2017, using pneumatic testing machines at a high density and subsequent removal of plants required to obtain a density of 95,000-100,000 plants ha⁻¹.

The different treatments were: treated seed with dilutions of natural fertilizer Chamae to 0.01% by imbibitions during 20 hours one day before sowing and seed without any treatment of two different varieties (MAS 68 K and LG-30681). The design was a random blocks with four replicates. Each repetition consisted on two rows 0.75 cm apart and 7 m of length.

When the plant reached its male flowering, at which time it has already its definitive size, the number of productive 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 August 28th, 2017, using harvest testing machines and the yield was quantified in kg ha⁻¹.

Statistical analysis

An ANOVA by variety for treatments was performed for all measured variables (Table 1 and 4). In case of interaction between variety and treatment was not significant, mean values of characters were average of two maize varieties. When interaction were significant, the variables were analysed by ANOVA in each variety. Means were compared using the Least Significant Difference test (LSD) at $P \leq 0.05$ and $P \leq 0.01$. Percentage data were transformed ($\arcsin \sqrt{Y/100}$) 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).

RESULTS

The study was conducted to evaluate the influence of a natural fertilizer applied on seed in order to control fumonisin production, *F. verticillioides* development and agronomic parameters as germination, plants height, died plants, aerial biomass weight of plants and yield. The result showed that fumonisin production and *F. verticillioides* development were reduced on 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 died plants, but lower concentrations minimized this negative impact.

Seed germination

Treatments effect on seed germination is reported in Table 1 and Table 2. There was a significant interaction between variety and treatment and the effect due to both, variety and treatment (Table 1).

The data indicated that 20 hours after placing the seeds on watered filter paper in Petri plates, no germination was observed in controls (non treated seed) of both maize varieties whereas the germination rates in treated seeds were between 80 and 90% for the maize variety MAS 68 K and between 50 and 60% for LG-30681 (Table 2). The differences between the two varieties were significant for all the doses of treatment with the natural fertilizer used for priming (Table 1 and Table 2,). After 48 hours, between 95 and 100% of the seeds germinated (Data 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 fumonisins B₁, B₂ and B₃ and *F. verticillioides* DNA concentration are shown in Table 1, Fig. 1. and Fig. 2. The variation factors maize variety, treatments and the interaction between both were significant to FB₁, FB₂ and fungus DNA quantity (Table 1). In the case of FB₃ the only significant factor was treatment (Table 1).

8 days after *F. verticillioides* inoculation, fumonisin concentrations were significantly higher ($P\leq0.05$) on seedling from MAS 68 K variety than in LG 30681 variety (Fig. 1). In MAS 68 K, all dilutions of the natural product reduced significantly ($P\leq0.05$) fumonisin accumulation (FB₁, FB₂ and FB₃) with regard to inoculated control (Fig. 1.). In the case of LG 30681 variety, 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, FB₁ had the highest concentrations, followed

by FB₂ and finally FB₃ (Fig. 1.).

The same 20 seedlings repetition⁻¹ were used to quantify fungus DNA (Fum 1 gene) as an estimation of the *F. verticillioides* biomass. Significant differences ($P \leq 0.05$) for this parameter were explained by effects of seed treatments and maize varieties (Table 1 and Fig. 2). An interaction between variety and treatment was observed (Table 1). Significant higher quantity of *F. verticillioides* DNA was observed in MAS 68 K seedling than in the variety LG 30681 (Fig. 2.). Significant lower amount ($P \leq 0.05$) of fungal DNA was observed in MAS 68 K variety treated with 0.01% of natural fertilizer (Fig. 2.). In LG 30681 seedlings variety, the 3 doses reduced significantly ($P \leq 0.05$) the amount of fungal DNA measured (Fig. 2.). We note that in negative control seedlings, fungal DNA was not detected. It indicates that there had not been cross contamination during inoculation or incubation (Data not shown).

Plant height, dead plants percentage and weight of aerial biomass of inoculated plants 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 the 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 (Table 1 and Table 3). Nevertheless, we observed that the dilutions 1% and 0,05% of seed treatments incremented significantly ($P \leq 0.05$) the percentage of dead plants (Table 1 and Table 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 control (Table 1 and Table 3).

No significant differences were observed among treatments for the weight of aerial biomass (Table 1 and Table 3).

Number of productive plants, plant height and yield

To determine the effect on seeds of natural fertilizer in a field experiment, we chose the 0.01% dilution applied on seed because it showed the best behaviour in the evaluated parameters under controlled conditions (germination, fumonisin accumulation, plant death and biomass weight aerial), 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 (Table 4 Table 5).

Eighty-four days after sowing, the number of productive plants coming from treated seeds was significantly lower ($P\leq0.05$) than in non treated controls (Table 4 and Table 5). Three months after sowing, when the plants were flowering, the plant height was not significantly different among different treatments (Table 4 and Table 5). The yield, estimated as kg ha^{-1} at the end of the season, was not significantly affected by the seed treatment (Table 4 and Table 5).

DISCUSSION

Maize fumonisins contamination poses a serious risk to human and animal health. The study of new techniques for its control, overall if they are respectful with the environment and they are low cost, is a priority for the sustainable control of this serious problem. The aim of this work has been the study of 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.

Among the various priming techniques, biopriming involves a soaking treatment with beneficial microorganisms or bioactive molecules such as phytohormones ¹⁷. In most cases, previous researches showed a greater rate of germination in treated seed ¹⁸. However, the results obtained by 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 ¹⁹.

Molecular techniques which are used for the improvement of *F. verticillioides* resistance and fumonisins 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 ²⁰.

In this sense, the seeds 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 hours as priming treatment. Due to the very high percentages of germination rates of controls, no improvement of this trait was observed. However, all the treatments decreased the germination delay by at least one day, in comparison with the control plants. In addition to the beneficial effect on germination, priming is also proposed as a way to produce tolerant plants against various stresses ²¹ 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 ²². Thus, an advantage of this technique is that it does not entail the costs normally associated with the activation of an inducible

defence 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 ²³. Systemic infection of maize by *F. verticillioides* could be through the seeds via fungal conidia or mycelia that are carried inside the seeds or coming from inoculums that survives in crop residues in the soil. This is an infection route of ears that have been proposed by several authors ^{9,10}. *F. verticillioides* is also considered as a “root pathogen” responsible of damping-off symptoms ²⁴. 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 behaviour of the two maize varieties used. The amount of fungus DNA was three-fold lower in the variety LG-30681; it is known to be more tolerant than variety MAS 68 K. This lower colonization of *F. verticillioides* in a tolerant variety than in a susceptible variety agree with the observation of Wu *et al.* ²⁴. The three-fold lower development of *F. verticillioides* leaded to a more than ten-fold lower fumonisin accumulation. These micotoxins are known to contribute to virulence in seedlings grown from inoculated maize seeds ^{25,26}. The partial resistance to colonization at seedling 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 ²⁷.

Previous studies showed high level of variability between susceptible and resistant maize lines for the respond to *F. verticillioides* infection. Although both presented similar functional categories of respond genes to infection. Resistant lines had basic defence to the fungus because genes were highly transcribed before infection. However, in susceptible maize lines, the genes were induced after infection. These results suggest that

1 plant basal genes in seed tissue could decrease colonization and fumonisin synthesis by the
2 fungus^{27, 28}.
3

4
5 The dilution of Chamae to 0.01% applied on seeds of the susceptible variety (MAS 68 K) was
6 able to reduce significantly the level of fumonisin accumulation and the biomass of *F.*
7

8 *verticillium* in seedlings during the first days following the inoculation. The level of
9 contamination of seedlings was reduced to the same level as that of the tolerant untreated
10 variety. The tolerant variety did not show any significant response to the treatment for the
11 fumonisin accumulation, but the quantity of fungal biomass was significantly lowered in
12 seedlings treated with the different dilutions of Chamae. These results suggest that the
13 treatments might favour defence responses in seedlings when they are inoculated with the
14 fungus *F. verticillioides*. Interestingly, plant response did not induce a reaction of the used
15 strain of *F. verticillioides* through higher production of fumonisins, but a lower rate of fumonisin
16 accumulation per unit of fungal biomass. Production of oxidative stress protectors is known to
17 be activated (accumulation of stress-related proteins, constitutive expression of
18 pathogenesis-related proteins and antioxydant enzyme activities) as maize responses to *F.*
19 *verticillioides* infection in embryos and adult plants, triggering maize resistance towards this
20 fungus^{29,30}. Ferrigo *et al.*³¹ observed divergent behaviours in *F. verticillioides* populations,
21 face to oxidative stress induced by H₂O₂. Other strains could react differently than the
22 Spanish- one tested here, and a large panel of strains should be tested in further works.
23

24 Mycotoxin contamination of feed often begins in the field but this could continue at harvest,
25 transportation and storage, depending on environmental conditions. Thus, the most effective
26 way to prevent mycotoxin occurrence is to limit the biosynthesis by the fungus when the crop
27 is cultivated¹³.
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

After infection, *F. verticillioides* might develop systemic colonization of young plants. In the present work, after planting of seedling 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, the extraction of DNA from the stem of the plants was carried out and the presence of *F. verticillioides*' DNA was studied by quantitative-PCR. Whereas 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 sterilisation (Data not showed). The aerial biomass was not significantly affected in the plants from treated seeds inoculated with *F. verticillioides*. According to the work of Howard ³² and Duncan and Howard ³³, *F. verticillioides* does not form penetrating structures that break the epidermis, which can hinder a 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 few mycelium 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 control whereas they were shown at the lowest dilutions 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 mycelium as was observed by Oren *et al.* ⁹ in physiologically weakened plants. In their study, this always leaded to stop the development of the 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 defences induced by seed treatments may be associated to a plant energy cost that could result into losses of productive plants, production or even plant size³⁴. Benefits from induced resistance responses via the application of different products might be limited by the inherent costs of defence³⁵. The costs associated with direct activation of defences also could be associated with loss of yield or detrimental effects on other agronomic factors of plants. Such costs were observed with direct activation of the defense system in plants²¹, but not when a priming state was induced through application jasmonic acid on seeds¹⁹. In the present work, the fertilizer applied on seed had a minimal impact on the height and the aerial biomass weight of 1 month old plants and it did not affected final yield (kg ha⁻¹), despite a lowering of the number of plant with standard height and regular ear size (productive plants) in each repetition.

CONCLUSION

In conclusion, according to our results, provoking a priming state by applying the natural fertilizer Chamae on maize seeds could improve germination and limit the colonization of seedlings by *F. verticillioides* and its associated fumonisin accumulation. 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 of planting density. The 0.01% dilution of the seed treatment is the most recommended since it improved the germination, reduced the amount of fungus DNA and fumonisins production by fungus, did not increment significantly

death plants and did not reduce the yield. Therefore, the priming can be recommended as a low cost and effective technique in the control of the fumonisins production and the development of *F. verticillioides* from seed infection, which does not affect the crop production.

ACKNOWLEDGEMENTS

The authors would like to thank the technical staff of the Mycology and Food Safety Unit (MycSA-INRA, Bordeaux, France) for their assistance.

References

[1] Franceschi S, Bidoli E, Barón A and Vecchia CL, Maize and risk of cancers of the oral cavity, pharynx, and esophagus in northeastern Italy. *J Natl Cancer Inst.* **82**: 1407–1411 (1990).

[2] Gelderblom WCA, Kriek N, Marasas WFO and Thiel PG, The effect of fumonisin B₁ on the growth of bacteria. *Carcinogenesis* **12**: 1247–1251 (1991).

[3] Norred WP and Voss KA, Toxicity and role of fumonisin in animal diseases and human esophageal cancer. *J Food Protect* **57**: 522-527 (1994).

[4] Pitt JI and Tomaska L, Are mycotoxins a health hazard in Australia?. *Food Aust J* **53**: 545–559 (2001).

[5] Niderkorn V, Morgavi DP, Aboab B, Lemaire M and Boudra H, Cell wall component and mycotoxin moieties involved in the binding of fumonisin B₁ and B₂ by lactic acid bacteria. *J Appl Microbiol* **108**: 977-985 (2009).

[6] Nayaka SC, Niranjana SR, Shankar ACU, Raj SN, Reddy MS, Prakash HS and Mortensen CN, Seed biopriming with novel strain of *Trichoderma harzianum* for the

- control of toxigenic *Fusarium verticillioides* and fumonisins in maize. *Arch Phytopathology Plant Protect* **43**: 264–282 (2010).
- [7] Munkvold GP and Carlton WM, Influence of inoculation method on systemic *Fusarium moniliforme* infection of maize plants grown from infected seeds. *Plant Dis* **81**: 211-216 (1997).
- [8] Munkvold GP, Epidemiology of *Fusarium* diseases and their mycotoxins in maize ears. *Eur J Plant Pathol* **109**: 705–713 (2003).
- [9] Oren L, Ezrati S, Cohen D and Sharon A, Early events in the *Fusarium verticillioides*–maize interaction characterized by using a green fluorescent protein-expressing transgenic isolate. *Appl Environ Microb* **69**: 1695–1701 (2003).
- [10] Rheeder JP and Marasas WFO, *Fusarium* species from plant debris associated with soils from maize production areas in the Transkei Region of South Africa. *Mycopathologia* **143**: 113-119 (1998).
- [11] van Aubel G, Buonatesta R, and Van Cutsem P, COS-OGA: A novel oligosaccharidic elicitor that protects grapes and cucumbers against powdery mildew. *Crop Prot* **65**: 129-137 (2014).
- [12] Pereira P, Nesci A, Castillo C and Etcheverry M, Impact of bacterial biological control agents on fumonisin B₁ content and *Fusarium verticillioides* infection of field-grown maize. *Biol Control* **53**: 258-266 (2010).
- [13] Dalié D, Pinson-Gadais L, Atanasova-Penichon V, Barreau C, Deschamps A and Richard-Forguet F, Impact of *Pediococcus pentosaceus* strain L006 and its metabolites on fumonisin biosynthesis by *Fusarium verticillioides*. *Food Control* **23**: 405-411(2012).
- [14] Ju M, Zhou Z, Mu C, Zhang X, Gao J, Liang Y, Chen J, Wu Y, Li X, Wang S, Wen J, Yang L, Jianyu Wu J, Dissecting the genetic architecture of *Fusarium verticillioides* seed rot resistance in maize by combining QTL mapping and genome-wide association

- analysis. *Sci Rep* **7**: 1-11 DOI: 10.1038/srep46446 (2017)
- [15] Maschietto V, Colombi C, Pirona R, Pea G, Strozzi F, Marocco A, Rossini L, Lanubile A, QTL mapping and candidate genes for resistance to Fusarium ear rot and fumonisin contamination in maize. *BMC Plant Biology*: **17:20**: 1-21. DOI 10.1186/s12870-017-0970-1 (2017).
- [16] Barroso G, Perennes D and Labarère JA, 'miniprep' method for RFLP analysis and dsRNAs detection perfected in the cultivated fungus *A. aegerita*. In: Science and Cultivation of Edible Fungi, ED. by Elliot TJ. Rotterdam: Balkema, AA, pp. 87-94. (1995).
- [17] Paparella S, Araújo SS, Rossi G, Wijayasinghe M, Carbonera D and Balestrazzi A, Seed priming: state of the art and new perspectives. *Plant Cell Report* **34**: 1281-1293 (2015).
- [18] Di Girolamo G and Barbanti L, Treatment conditions and biochemical processes influencing seed priming effectiveness. *It J Agron* **7**: 178-188 (2012).
- [19] Worrall D, Geoff HH, Jason PM, Marcin G, Croft P, Taylor Jane ET, Nigel DP and Roberts RM, Treating seeds with activators of plant defence generates long-lasting priming of resistance to pests and pathogens. *New Phytol* **193**: 770-778 (2012).
- [20] Robertson-Hoyt LA, Jines MP, Balint-Kurti P, Kleinschmidt CE, White DG, Payne GA, Maragos CM, Molnar TL, Holland JB, QTL mapping for Fusarium ear rot and fumonisin contamination resistance in two maize populations. *Crop Sci* **46**: 1734-43 (2006)
- [21] van Hulten M, Pelser M, van Loon LC, Pieterse CMJ and Ton J, Costs and benefits of priming for defense in Arabidopsis. *P Nat Acad Sci* **103**: 5602-5607 (2006).
- [22] Conrath U, Beckers GJM, Flors V, García-Agustín P, Jakab G, Mauch F and MauchMani B, Priming: getting ready for battle. *Mol Plant Microbe In* **19**: 1062-1071

(2006).

- [23] Frost CJ, Mescher MC, Dervinis C, Davis JM, Carlson JE and de Morales CM, Priming defense genes and metabolites in hybrid poplar by the green leaf volatile cis-3hexenyl acetate. *New Phytol* **180**: 722-734 (2008).
- [24] Wu L, Wang X, Xu R and Li H, Difference between resistant and susceptible maize to systematic colonization as revealed by DsRed-labeled *Fusarium verticillioides*. *The Crop J* **1**: 61–69 (2013).
- [25] Desjardins AE, Plattner RD, Nelsen TC and Leslie JF, Genetic analysis of fumonisin production and virulence of *Gibberella fujikuroi* mating population A (*Fusarium moniliforme*) on maize (*Zea mays*) seedlings. *Appl Environ Microb* **61**: 79-86 (1995).
- [26] Glenn AE, Zitomer NC, Zimeri AM, Williams LD, Riley RT and Proctor RH, Transformation-mediated complementation of a FUM gene cluster deletion in *Fusarium verticillioides* restores both fumonisin production and pathogenicity on maize seedlings. *Mol Plant Microbe In J* **21**: 87-97 (2008).
- [27] Lanubile A, Logrieco A, Battilani P, Proctord RH, Marocco A, Transcriptional changes in developing maize kernels in response to fumonisin-producing and nonproducing strains of *Fusarium verticillioides*. *Plant Sci* **210**: 183– 192 (2013).
- [28] Lanubile A, Bernardi J, Marocco A, Logrieco A, Paciolla C, Differential activation of defense genes and enzymes in maize genotypes with contrasting levels of resistance to *Fusarium verticillioides*, *Environ Exp Bot* **78**: 39–46 (2012).
- [29] Maschietto V, Lanubile A, De Leonardis S, Marocco A, Paciolla C, Constitutive expression of pathogenesis-related proteins and antioxidant enzyme activities triggers maize resistance towards *Fusarium verticillioides*. *J Plant Physiol* **200**: 53-61 (2016).
- [30] Campo S, Carrascar M, Coca M, Abian J and San Segundo B, The defense response of germinating maize embryos against fungal infection: a proteomics approach.

Proteomics **4**: 383 – 396 (2004).

[31] Ferrigo D, Raiola A, Bogialli S, Bortolini C, Tapparo A and Causin R, In Vitro Production of Fumonisins by *Fusarium verticillioides* under oxidative stress induced by H₂O₂. *J Agr Food Chem* **63**: 4879-4885 (2015).

[32] Howard RJ, Breaching the outer barriers—cuticle and cell wall penetration, In: Carroll G and Tudzynski P editors. *Plant Relationships* (Vol. 5A): 43–60. Springer-Verlag, New York (1997).

[33] Duncan KE and Howard RJ, Biology of maize kernel infection by *Fusarium verticillioides*. *Mol Plant Microbe In J* **23**: 6–16 (2010).

[34] Cipollini D, Purrington CB and Bergelson J, Costs of induced responses in plants. *Basic Appl Ecol* **4**: 79-89 (2003).

[35] Heil M, Hilpert A, Kaiser W and Linsenmair KE, Reduced growth and seed set following chemical induction of pathogen defence: does systemic acquired resistance (SAR) incur allocation costs? *J Ecol* **88**: 645-654 (2000).

1
2
3
4
5

Table 1. Analysis of Variance (ANOVA) for the studied parameters under controlled conditions: seed germination, fumonisins production (FB ₁ , FB ₂ and FB ₃), quantification of <i>F. verticillioides</i> DNA, height and weight of aerial parts of 1 month old plants																	
Parameters																	
Germination Rate				Fumonisin concentration				DNA quantifications			Percentage of died plants days after inoculation		Plant Height (cm)		Biomass weigh (gr)		
				FB1		FB2		FB3									
Source variation	df ^a	MS ^b	P	MS ^b	P	MS ^b	P	MS ^b	P	MS ^b	P	MS ^b	P	MS ^b	P	MS ^b	P
Treatments (T)	3	8454.51	0.0000***	12305.1	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***	73444.4	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
Repetitions (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 X 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	
*P< 0.05, ** P< 0.001, ***P < 0.0001; ^a degree freedom; ^b Means square																	

16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47

- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9
- 10
- 11
- 12
- 13
- 14
- 15
- 16
- 17
- 18
- 19
- 20
- 21
- 22
- 23
- 24
- 25
- 26
- 27
- 28
- 29
- 30
- 31
- 32
- 33
- 34
- 35
- 36
- 37
- 38
- 39
- 40
- 41
- 42
- 43
- 44
- 45
- 46
- 47

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

Treatments	Control	Control inoculated	Natural fertilizer application dilutions (%) on seed and inoculated on seedling		
			1	0,01	0,005
	<i>Heigh plant (cm)</i>				
	65.00 a	61.00 a	59.86 a	59.83 a	55.67 a
	<i>Weigh of aerial biomass (g)</i>				
	9.13 a	8.69 a	7.65 a	7.69 a	7.44 a
	<i>Died plants</i>				
	0 a	0 a	49.60 c	11.10 ab	27.75 bc
Same letters indicate non-significant differences among treatments and different letters indicate significant differences according to LSD test ($P \leq 0.05$)					

Parameters

Source variation	df ^a	Number of productive plants per block 84 days after sown		Height plant (84 days after sown) in flowering		Yield (Kg/Ha)	
		MS ^b	P	MS	P	MS	P
Treatments (T)	1	60.06	0.0355*	0.00045	0.7846	50457	0.8943
Variety (V)	1	10.56	0.3272	0.00019	0.8594	7.2E+07	0.0002***
Repetitions (R)	3	1.72	0.9102	0.00566	0.4387	7E+06	0.09
T X V	1	3.06	0.5905	0.02038	0.091	3575722	0.2755
Error	9	9.84		0.051		2738860	

* $P < 0.05$, *** $P < 0.0001$; ^a degree freedom; ^b Means square

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		
Treatments	Treated seed with doses 0.01%	Non treated seed
	<i>Height plant (cm)</i>	
	2.71 a	2.71 a
	<i>Number of productive plants</i>	
	48.75 a	52.62 b
	<i>Yield (kg ha⁻¹)</i>	
	10605 a	10505 a
Same letters indicate non-significant differences among treatments and different letters indicate significant differences according to LSD test ($P \leq 0.05$)

Figure 1. Accumulation of fumonisins: **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 2 varieties (MAS 68 K and LG 30681). 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 ($P \leq 0.05$).

Figure 2. Quantification of *Fusarium verticillioides* DNA as the ratio *Fum1* gene amplification to *maize actin* gene amplification, in seedlings of two maize varieties (MAS 68 K and LG 30681), 8 days after inoculation.

Cl: 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 different letters indicate significant differences among treatments and varieties according to LSD test ($P \leq 0.05$).