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

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Key Words:	priming, germination, mycotoxins, Fusarium disease, maize

1 ***Priming to protect maize from *Fusarium verticillioides* and its fumonisin***
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3 ***accumulation***
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7 **ABSTRACT**
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9 **BACKGROUND**
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11 Systemic infection through the seed is one of the routes used by mycotoxinogen pathogen
12 *Fusarium verticillioides* for colonizing maize plants. The prohibition of most of the chemical
13 fungicides by the EU promote the research of plant resistance inducers as an effective and
14 sustainable alternative. Induction of a priming state in maize seeds might affect their
15 susceptibility for contamination and accumulation of fumonisins. This state, obtained by application
16 of a natural fertilizer called Chamae on maize seeds, was investigate in two varieties to
17 control the colonization by the fungus and the accumulation of fumonisins B₁, B₂, and
18 B₃.
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30 **RESULTS**
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34 After inoculation of *F. verticillioides* on germinating seeds, the colonization by the fungus
35 and the accumulation of fumonisins were significantly lower in seedlings coming from
36 treated seeds, but a significant number of plant stop their development by necrosis. In a field
37 trial, the 0,01% application dilution showed a lower plant density although the level of
38 biomass at harvest was not affected.
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45 **CONCLUSION**
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49 The priming state contributed to the control of *F. verticillioides* development from seed
50 infection and fumonisin accumulation in the early stage of plant growth, without affecting the final
51 crop yield and could
52 reduce the fungicide use and environmental contamination.
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Priming to protect maize from *Fusarium verticillioides* and its fumonisin accumulation

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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

1 residues in the soil ¹⁰ and penetrates young seedlings through the lateral roots and the
2 mesocotyl ⁹.

3
4
5 The prohibition of most of the chemical fungicides by the EU (Directive 91/414/CEE), due
6 to adverse effects of their use on the environment and human health, their toxicity or
7 because pathogens have become resistant to their active substances, have promoted
8 research in the field of plant resistance inducers as an effective and sustainable alternative
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15 ¹¹.

16 Protection of maize seeds from infection of *F. verticillioides* by inoculation of antagonists
17 has been experimented. It may reduce the rotting symptoms and fumonisin accumulation.
18 Treatment of seeds with *Trichoderma harzianum* reduced the levels of fumonisins in
19 different maize cultivars by 56-86% ⁶. Fumonisin B₁ reduction reached up to 94% in grains
20 treated with *Bacillus amyloliquefaciens*, and up to 81% in grains treated with
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34 Breeding for resistance to *Fusarium* is the most economic and environmentally safe
35 strategy. Genome-wide association study (GWAS) is a strategy successfully used to detect
36 and identify quantitative trait loci (QTLs) and candidate genes involved in *F. verticillioides*
37 resistance and low mycotoxin contamination synthesized by this fungus in maize. This tool
38 allows to reveal novel sources of resistance to select maize lines in breeding programs
39 quicker and more efficiently than the classic breeding. Recently, other researchers
40 have got identified 8 QTLs and 43 genes associated with *F. verticillioides* seed rot (FSR)
41 resistance through linkage mapping and GWAS, respectively ¹⁴. By combining
42 transcriptomic data with QTL, 24 candidate genes for resistance to *F. verticillioides* were
43 identified, allowing to make possible the selection of genotypes with both low disease

1 severity and low fumonisin contamination ¹⁵.
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3 The physiological health of the plantlets may also affect the development of the symptoms
4
5 ⁹ and induction of the priming state in the plant through the application of natural
6 fertilizers on seeds could be another way to prepare plants against pathogen attacks.
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10 The aim of the present work was to characterize the influence of a commercial natural
11 fertilizer based on vegetal extracts obtained from plant decoctions, Chamae on early
12 colonisation of germinating seeds and plantlets by *F. verticillioides* and the
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14 development of symptoms, including the fumonisin accumulation, and the influence of
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treatments on yield.

EXPERIMENTALS

Seed treatments and inoculation

31 The natural fertilizer Chamae (Saionaimer S.L.) used for seed treatments contains
32 principal macronutrients (nitrogen, phosphorus, potassium), secondary macronutrients
33 (calcium, magnesium, potassium, sodium and sulphur), and micronutrients (boron, cobalt,
34 copper, iron, manganese, molybdenum and zinc). This natural product comes from the
35 hydrolysis of crop residues through its decoction that favors the release of nutrients easily
36 used by plants.
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44 Different dilutions of Chamae were applied on seeds of two maize varieties (MAS 68 K and
45 LG-30681). MAS 68K from Maïsadour (Haut-Mauco, France) is a very late variety of dent
46 maize for producing grains. LG-30681, from Limagrain (Saint Beuzire, France) is a very
47 late variety suitable for both grains and forage. Both varieties were obtained in France.
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53 According to the breeder, LG-30681 is characterised by its high tolerance to *Fusarium*
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6 ear rot. The seeds were surface sterilized. They were covered with 70% ethanol for 3 min, rinsed
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8 with sterile milliQ water and submerged for 15 min in 2.5% sodium hypochlorite solution containing
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10 40 μL of Tween-20 100 mL^{-1} . Hypochlorite was removed and the seeds were immersed again in
11
12 70% ethanol for 3 min, rinsed with sterile milliQ water 5 times and dried under a flux of sterilized air.
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14 Surface-disinfected seeds were soaked in different dilutions of the natural fertilizer (1%, 0,01%,
15
16 0,005% v/v in demineralized water) for 20 hours at room temperature. For drying, they were spread
17
18 on sterilized filter paper and placed in a laminar flow hood for two hours. In controls, the treatments
19
20 were not applied to the surface disinfected seeds. Subsequently the seeds were plated on sterile
21
22 filter paper discs moistened with 1 mL of sterile demineralized water in 90 mm diameter Petri plates
23
24 at 25°C, without light, until germination.
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28 Germination of 40 seeds in each replicate and three replicates was evaluated for each dilution of
29
30 treatment with the natural product and control treatment in the two maize varieties. The percentage
31
32 of germinated seeds was estimated 20 and 48 hours after being deposited in Petri plate with
33
34 moistened paper.
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37 The strain 0001 of *F. verticillioides* had been first isolated from symptomatic maize plants in Spain
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39 and conserved as single spore isolate at the IFAPA Las Torres-Tomejil collection (Sevilla, Spain). It
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41 was grown up in PDA plates at 25°C in the dark for 10 days. A suspension was prepared by
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43 blending infested PDA with 5 mL of sterile distilled water in each plate. The spore suspension was
44
45 filtered through 4 layers of sterile cheesecloth. Spore concentration was determined using a
46
47 haemocytometer, and the suspension was diluted with sterile milliQ water to obtain a final
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49 concentration of 2×10^6 conidia mL^{-1} . When all the seeds had germinated, they were inoculated
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51 pipetting 5 μL of the spore suspension (2×10^6 conidia mL^{-1}), in the area of the natural wound that
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53 produces the exit
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1 of the rootlet during germination. 8 days after inoculation (d.a.i.), 20 seedlings repetition⁻¹
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3 of each treatment and variety were frozen, lyophilized and stored before quantification of
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5 fumonisins and DNA isolation from *F. verticillioides*.
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12 **Extraction and quantification of fumonisins from germinated and inoculated seeds**

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14 On the eighth day of inoculation, 4 g of freeze-dried ground germinated and inoculated
15 seeds were extracted with 20 mL of methanol-water (3:1 v/v). After 15 min in agitation in
16 an orbital shaker at 0.413 xg and centrifugation (3 min, 1013 xg), the supernatant was
17 filtered through Whatman 2V filter paper. The filtrate was adjusted to pH 6.5 by adding
18 NaOH 10N. Fumonisins were purified by using Bond Elut strong anion Exchange (SAX)
19 Cartridges (Agilent), which were conditioned with 5 mL methanol and 5 mL
20 Methanol/Water (3/1). Then 5 mL of sample was added to the cartridges. The samples
21 were washed by passing through the column of 5 mL of methanol/water (3/1) and 3 mL of
22 pure methanol. To elute the sample from the column, 10 mL of methanol with acetic acid
23 (1%) were added. The samples were evaporated to dryness under a nitrogen stream and
24 the pellets were dissolved in 200 µL of methanol before high performance liquid
25 chromatography analysis with fluorescence detection. Just before injection (20µL),
26 samples were derivatized using o-phthaldialdehyde (OPA) and β-mercaptoethanol mixture
27 (40 mg of OPA are dissolved in 1 mL of methanol, 50 µL of β -mercaptoethanol and 5 mL
28 of Borax 0.1M in water). Chromatographic separation was done with a C18 Kinetex
29 column (150*4.6 mm, 3.5µm) (Phenomenex, France) linked to an HPLC 1100 system
30 (Agilent,) operating in isocratic mode. Mobile phase was composed of Methanol/Water,
31 0.1M NaH₂PO₄ (77/23, v/v) and pH was adjusted to 3.3 with orthophosphoric acid. Flow
32 rate was 0.6 mL min⁻¹. Excitation wavelength was 335 nm, and emission 440 nm. Total run
33 time was 18 min.
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1 Quantification was performed by using external calibration with FB₁, FB₂ and FB₃ standard
2 solutions prepared from commercial pure powders (Romer Labs, Tulln, Austria).
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8 **Detection and quantification of total DNA from *F. verticillioides* in maize samples**

9
10 Twenty seedlings in each repetition, treatment and variety were collected eight days after
11 inoculation. These samples were lyophilised and crushed by liquid nitrogen. 0.1 g of these
12 tissues was used to DNA extraction by DNeasy® Plant Mini Kit according to the
13 manufacturer instructions (Qiagen, Courtaboeuf, France).
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18 The other seedlings not used to extract DNA were planted in pot as it is described in the part
19 “Early stage of maize growth (biomass aerial weight and plants height) and died plants”. So,
20 a second DNA extraction was carried on lyophilized stems and leaves of young plants from
21 pots thirty-six days after inoculation to detect the fungus and to know the possibility of
22 colonization of aerial part of plants. They were ground under liquid nitrogen in a mortar and
23 total DNA were extracted according to the N-cethyl- NNN-trimethyl ammonium bromide
24 (CTAB) procedure, as described in Barroso *et al.*¹⁶ The concentration of nucleic acids (DNA)
25 was determined using a ND-1000 NanoDrop spectrophotometer (NanoDrop Products,
26 Wilmington, DE) .
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38 For each analyzed sample, DNA was amplified with primers designed to track *F. verticillioides*
39 DNA. The pair primer Fum 1-654 (5'- CGGTTGTTTCATCATCTCTGA-3') and Fum1-1158 (5'-
40 GCTCCCGATGTAGAGCTTGTT-3') was designed to amplify a 504-bp fragment (T_m, 60°C)
41 from the polyketide synthase gene FUM1 (GenBank accession number AF155773) of
42 *F. verticillioides*.
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49 To normalize the quantification as ng of DNA fungus ng⁻¹ of DNA maize, we also
50 performed the quantification of gene maize actine (accession number 01238) MA-F
51 (TCCTGACACTGAAGTACCCGATTG)/MA-R CGTTGTAGAAGGTGTGATGCCAGTT).
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1 The corresponding melting temperature was 60°C, and the amplified fragment length was
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3 95 base pairs (bp). To know the absolute amount of DNA of both, standard curves of cycle
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5 thresholds were generated using known quantities of DNA pure of *F. verticillioides* or of
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7 maize (from 0.002 to 20 ng).
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9
10 Quantitative PCR experiments (qPCR) were performed in a LightCycler® 1.5 System
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12 (Roche Applied Science, Meylan, France) and the QuantiFast SYBR Green PCR Kit
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14 (Qiagen, Courtaboeuf, France). Each reaction mixture contained 20 ng of DNA (1 µL),
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16 QuantiFast SYBR Green PCR Master Mix 1X, 5 mM MgCl₂, and 0.5 mM of each primer.
17

18 The amplification conditions consisted of a first denaturation step for 5 min at 95 °C,
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20 followed by 40 cycles of 10 s denaturation at 95 °C, 30 s combined annealing/extension at
21
22 60 °C. The melting curve was generated using the following profile: 15 s at 95°C, 15 s at
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24 65°C, and 15 s at 95°C with a 0.1 °Cs⁻¹ transition. Assays for each sample were performed
25
26 in triplicate.
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29 Lack of non-specific qPCR amplification or primer-dimer formation was checked by melting
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31 curve analysis in each run.
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33 34 **Early stage of maize growth (biomass aerial weight and plants height) and died** 35 36 **plants**

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38 To evaluate the evolution of the fungus from the seed to the aerial part of the plants and
39
40 consequences on the plant growth, 9 germinated seeds of each treatment were planted in
41
42 different pots (14x14x20 cm) filled with 2.5 L of a potting substrate based on blend of
43
44 weakly decomposed white sphagnum peat and clay granules (Substrate 5, Klasmann-
45
46 Deilman GmbH, Germany). The plants were cultivated for 28 days in a greenhouse.
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48 Temperature and air humidity were recorded. Mean temperature during the cultivation
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50 period was 22°C with maximum at 30.2°C and minimum at 15.0°C. Mean air humidity was
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52 59.1 % with 84.5 % as maximum and 37.0 % as minimum. The substrate was regularly
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1 humidified when it was necessary. Damping-off symptoms as died plants were followed all
2 along the culture. Height (cm) and aerial biomass weight (g) of plants were measured at
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4 the end of this growing period.
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10 **Number of productive plants, plant height and yield**

11 An assay under field conditions was performed in Alcal del Rio (Seville, Spain). The soil
12 had the following characteristics: texture silty clay, with a percentage of clay 46.4%, silt
13 46.7% and sand 8.9%. The percentages of N total Kjeldahl, C.O. and M.O. are 0,12%,
14 2,3% and 0,78% respectively, with a pH of 8,28 and the conductivity was 1,5 mS/cm.
15 Cation exchange capacity was (meq/100 g) 23.5% with Ca at 8.86%, Mg at 11.06%, K at
16 1.26%, Na at 2.02. The percentage of base saturation is 99%, exchangeable sodium 6%,
17 carbonates 20.6%, active limestone 11.6%, and the wealth in P is 21.7 ppm and in K 419
18 ppm.
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30 The normal agronomic techniques were adopted. Briefly, the previous crop was maize,
31 and the field was ploughed each year.
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34 The sowing was performed on March 17th, 2017, using pneumatic testing machines at a
35 high density and subsequent removal of plants required to obtain a density of 95,000-
36 100,000 plants ha⁻¹.
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40 The different treatments were: treated seed with dilutions of natural fertilizer Chamae to
41 0.01% by imbibitions during 20 hours one day before sowing and seed without any
42 treatment of two different varieties (MAS 68 K and LG-30681). The design was a random
43 blocks with four replicates. Each repetition consisted on two rows 0.75 cm apart and 7 m
44 of length.
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51 When the plant reached its male flowering, at which time it has already its definitive size,
52 the number of productive plants was measured by counting plants with standard height
53 and regular ear size in each
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1 replicate in one row. The height of ten plants was also measured.

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3 The harvest was performed on August 28th, 2017, using harvest testing machines and the
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5 yield was quantified in kg ha⁻¹.
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10 **Statistical analysis**

11 An ANOVA by variety for treatments was performed for all measured variables (Table 1
12 and 4). In case of interaction between variety and treatment was not significant, mean
13
14 values of characters were average of two maize varieties. When interaction were
15
16 significant, the variables were analysed by ANOVA in each variety. Means were compared
17
18 using the Least Significant Difference test (LSD) at $P \leq 0.05$ and $P \leq 0.01$. Percentage data
19
20 were transformed ($\arcsin \sqrt{Y/100}$) and ANOVA was used to test for statistically significant
21
22 effects of independent variables. All statistical analyses were performed with Statistix 9.0
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24 (Analytical Software, Tallahassee, FL).
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32 **RESULTS**

33 The study was conducted to evaluate the influence of a natural fertilizer applied on seed in
34 order to control fumonisin production, *F. verticillioides* development and agronomic
35 parameters as germination, plants height, died plants, aerial biomass weight of plants and
36 yield. The result showed that fumonisin production and *F. verticillioides* development were
37 reduced on inoculated maize plants by the application of natural fertilizer on seeds and the
38 impact of this fertilizer on agronomic parameters; however the highest concentration of this
39 natural fertilizer had a negative impact on the number of died plants, but lower
40 concentrations minimized this negative impact.
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53 **Seed germination**

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1 Treatments effect on seed germination is reported in Table 1 and Table 2. There was a
2 significant interaction between variety and treatment and the effect due to both, variety and
3 treatment (Table 1).
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8 The data indicated that 20 hours after placing the seeds on watered filter paper in Petri
9 plates, no germination was observed in controls (non treated seed) of both maize varieties
10 whereas the germination rates in treated seeds were between 80 and 90% for the maize
11 variety MAS 68 K and between 50 and 60% for LG-30681 (Table 2). The differences
12 between the two varieties were significant for all the doses of treatment with the natural
13 fertilizer used for priming (Table 1 and Table 2.). After 48 hours, between 95 and 100% of
14 the seeds germinated (Data not shown). The treatment did not affect the germination
15 power of seeds and no difference between the varieties was significant, but it was
16 observed that seed treatments anticipated the germination.
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30 ***F. verticillioides* growth and fumonisin production in inoculated seedlings**

31 The fumonisins B₁, B₂ and B₃ and *F. verticillioides* DNA concentration are shown in Table
32 1, Fig. 1. and Fig. 2. The variation factors maize variety, treatments and the interaction
33 between both were significant to FB₁, FB₂ and fungus DNA quantity (Table 1). In the case of
34 FB₃ the only significant factor was treatment (Table 1).
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40 8 days after *F. verticillioides* inoculation, fumonisin concentrations were significantly higher
41 ($P \leq 0.05$) on seedling from MAS 68 K variety than in LG 30681 variety (Fig. 1). In MAS 68
42 K, all dilutions of the natural product reduced significantly ($P \leq 0.05$) fumonisin accumulation
43 (FB₁, FB₂ and FB₃) with regard to inoculated control (Fig. 1.). In the case of LG 30681
44 variety, the accumulation of these mycotoxins showed lower values than in the other
45 variety and it was not affected by treatments on seeds (Fig. 1.). Regarding the
46 proportion of different fumonisins detected, FB₁ had the highest concentrations, followed
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1 by FB₂ and finally FB₃ (Fig. 1.).

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4 The same 20 seedlings repetition⁻¹ were used to quantify fungus DNA (Fum 1 gene) as an
5
6 estimation of the *F. verticillioides* biomass. Significant differences ($P\leq 0.05$) for this
7
8 parameter were explained by effects of seed treatments and maize varieties (Table 1 and
9
10 Fig. 2). An interaction between variety and treatment was observed (Table 1). Significant
11
12 higher quantity of *F. verticillioides* DNA was observed in MAS 68 K seedling than in the
13
14 variety LG 30681 (Fig. 2.). Significant lower amount ($P\leq 0.05$) of fungal DNA was observed
15
16 in MAS 68 K variety treated with 0.01% of natural fertilizer (Fig. 2.). In LG 30681 seedlings
17
18 variety, the 3 doses reduced significantly ($P\leq 0.05$) the amount of fungal DNA measured
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20 (Fig. 2.). We note that in negative control seedlings, fungal DNA was not detected. It
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22 indicates that there had not been cross contamination during inoculation or incubation
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24 (Data not shown).
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30 **Plant height, dead plants percentage and weight of aerial biomass of inoculated** 31 **plants with *F. verticillioides***

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34 No interaction between variety and treatment was detected (Table 1), so the results for
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36 these parameters are represented as the means of the two varieties (Table 3).
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38
39 One month after the transplantation of the seedlings, the seed treatments and inoculation
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41 with *F. verticillioides* had not a significant effect on the plant height and weight of aerial
42
43 biomass of plants under control condition (Table 1 and Table 3). Nevertheless, we
44
45 observed that the dilutions 1% and 0,05% of seed treatments incremented significantly
46
47 ($P\leq 0.05$) the percentage of dead plants (Table 1 and Table 3). In both inoculated and non-
48
49 inoculated controls, dead plants were not observed (Table 3). The dilution 0.01% of seed
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51 treatment showed dead plants but this parameter was not significantly different from the
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53 inoculated and non-inoculated control (Table 1 and Table 3).
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1 No significant differences were observed among treatments for the weight of aerial biomass
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4 (Table 1 and Table 3).
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8 **Number of productive plants, plant height and yield**

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10 To determine the effect on seeds of natural fertilizer in a field experiment, we chose the
11 0.01% dilution applied on seed because it showed the best behaviour in the evaluated
12 parameters under controlled conditions (germination, fumonisin accumulation, plant death
13 and biomass weight aerial), compared with the effect of control seed on number of
14 productive plants, plant height and yield in the two maize varieties (MAS 68 K and LG
15 30681) under field conditions (Table 4 Table 5).
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23 Eighty-four days after sowing, the number of productive plants coming from treated seeds
24 was significantly lower ($P \leq 0.05$) than in non treated controls (Table 4 and Table 5).
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27 Three months after sowing, when the plants were flowering, the plant height was not
28 significantly different among different treatments (Table 4 and Table 5).
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31 The yield, estimated as kg ha^{-1} at the end of the season, was not significantly affected by
32 the seed treatment (Table 4 and Table 5).
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38 **DISCUSSION**

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40 Maize fumonisins contamination poses a serious risk to human and animal health. The
41 study of new techniques for its control, overall if they are respectful with the environment
42 and they are low cost, is a priority for the sustainable control of this serious problem. The
43 aim of this work has been the study of the influence of the priming state on maize plants,
44 caused by the application of Chamae in the seed state.
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51 Seed priming is a pre-sowing technique for regulating the germination process by
52 triggering the pre-germinative metabolism that is normally activated during the early phase
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1 of germination. It is proposed as a way to achieve faster and better seed germination.
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3 Among the various priming techniques, biopriming involves a soaking treatment with
4 beneficial microorganisms or bioactive molecules such as phytohormones ¹⁷. In most cases,
5 previous researches showed a greater rate of germination in treated seed ¹⁸. However, the
6 results obtained by various priming techniques in seeds are heterogeneous according to the
7 different species, the quality of the seed or the conditions of germination, among other
8 parameters ¹⁹.
9

10 Molecular techniques which are used for the improvement of *F. verticillioides* resistance and
11 fumonisins production are efficient in breeding programs. They allow quick selection of lines
12 in maize. However, the complex genetic basis of these characters and the great
13 environment effect in their phenotypic expression, make the location of QTLs and the
14 efficiency of marker-assisted selection (MAS) more difficult ²⁰.
15

16 In this sense, the seeds priming technique could improve or help the effect obtained
17 through breeding of resistant maize varieties, being interesting for studies on an
18 intergrated disease management combining both techniques.
19

20 In the present study, we used three different dilutions of Chamae, soaking of maize seeds
21 for 20 hours as priming treatment. Due to the very high percentages of germination rates of
22 controls, no improvement of this trait was observed. However, all the treatments decreased
23 the germination delay by at least one day, in comparison with the control plants. In addition
24 to the beneficial effect on germination, priming is also proposed as a way to produce
25 tolerant plants against various stresses ²¹ by the initiation in the plant of a "preparation"
26 state that does not confer resistance in itself but allows the acceleration of induction of the
27 defences, once the plant faces such stress. The plant is then able to respond better than
28 other plants that have not experienced it ²². Thus, an advantage of this technique is that it
29 does not entail the costs normally associated with the activation of an inducible
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1 defence response, since the defensive response is triggered only if the plant recognizes
2 some of these types of stress, and without major ecological or physiological effects ²³.
3
4 Systemic infection of maize by *F. verticillioides* could be through the seeds via fungal
5 conidia or mycelia that are carried inside the seeds or coming from inoculums that survives
6 in crop residues in the soil. This is an infection route of ears that have been proposed by
7 several authors ^{9,10}. *F. verticillioides* is also considered as a “root pathogen” responsible of
8 damping-off symptoms ²⁴. To determine whether plants might respond to priming agents at
9 the seed stage, in the present study, we infected seedlings of two maize varieties with
10 spores of a Spanish strain of *F. verticillioides*. We observed that the amount of DNA from
11 the fungus was significantly lowered with the different dilutions of Chamae applied on
12 seeds, with a different behaviour of the two maize varieties used. The amount of fungus
13 DNA was three-fold lower in the variety LG-30681; it is known to be more tolerant than
14 variety MAS 68 K. This lower colonization of *F. verticillioides* in a tolerant variety than in a
15 susceptible variety agree with the observation of Wu *et al.* ²⁴. The three-fold lower
16 development of *F. verticillioides* led to a more than ten-fold lower fumonisin
17 accumulation. These micotoxins are known to contribute to virulence in seedlings grown
18 from inoculated maize seeds ^{25,26}. The partial resistance to colonization at seedling and
19 limitation of fumonisin production might be a component of the claimed low susceptibility of
20 LG-30681 to Fusarium ear rot. The same results were observed also in adult plants and
21 not only at the seedling stage ²⁷.

22
23 Previous studies showed high level of variability between susceptible and resistant maize
24 lines for the respond to *F. verticillioides* infection. Although both presented similar
25 functional categories of respond genes to infection. Resistant lines had basic defence to
26 the fungus because genes were highly transcribed before infection. However, in
27 susceptible maize lines, the genes were induced after infection. These results suggest that
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1 plant basal genes in seed tissue could decrease colonization and fumonisin synthesis by the
2 fungus^{27, 28}.

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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.*

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

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Myco toxin 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 myco toxin occurrence is to limit the biosynthesis by the fungus when the crop
is cultivated¹³.

1 After infection, *F. verticillioides* might develop systemic colonization of young plants. In the
2
3 present work, after planting of seedling in a horticulture substrate in pots, we obtained
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5 either plants that developed normally without symptoms or plants that died before they
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7 reached their first month of growth. Because of the possibility of plant colonization by the
8
9 fungus, we wanted to know if after inoculation in seedlings, this colonization of the stem
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11 had occurred when the plants were already 1 month old. Therefore, the extraction of DNA
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13 from the stem of the plants was carried out and the presence of *F. verticillioides*' DNA was
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15 studied by quantitative-PCR. Whereas no symptoms were observed on plants whatever
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17 the treatments and fungus, DNA was not detected by PCR in the stems of the inoculated
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19 plants after surface sterilisation (Data not showed). The aerial biomass was not significantly
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21 affected in the plants from treated seeds inoculated with *F. verticillioides*. According to the
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23 work of Howard ³² and Duncan and Howard ³³, *F. verticillioides* does not form penetrating
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25 structures that break the epidermis, which can hinder a systemic infection. Oren *et al.* ⁹
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27 observed asymptomatic systemic infection characterized by infection of only certain
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29 tissues, intercellular growth of a limited number of fungal hyphae, and reproduction of the
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31 fungus in a few cells without invasion of other cells. In the stems, they detected only few
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33 mycelium of *F. verticillioides* green fluorescent protein expressing transgenic isolate. We
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35 cannot exclude that such asymptomatic colonization occurred in the present experiment
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37 with levels of mycelium that were not detectable. In our experiment, necrotrophic symptoms
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39 were not observed in non-treated control whereas they were shown at the lowest dilutions
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41 or highest concentration of Chamae. The fungus probably used the nutrients from the
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43 fertilizer coating the seeds for its external development and then rotted mesocotyl and main
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45 root cells with massive production of fungal mycelium as was observed by Oren *et al.* ⁹ in
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47 physiologically weakened plants. In their study, this always led to stop the development
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49 of the plants and their death. This negative effect might be
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1 modulated under other circumstances of *F. verticillioides* inoculum potential and soil
2 environment, where the space could be occupied before by competitors.
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5 The defences induced by seed treatments may be associated to a plant energy cost that
6 could result into losses of productive plants, production or even plant size³⁴. Benefits from
7 induced resistance responses via the application of different products might be limited by
8 the inherent costs of defence³⁵. The costs associated with direct activation of defences
9 also could be associated with loss of yield or detrimental effects on other agronomic
10 factors of plants. Such costs were observed with direct activation of the defense system in
11 plants²¹, but not when a priming state was induced through application jasmonic acid on
12 seeds¹⁹. In the present work, the fertilizer applied on seed had a minimal impact on the
13 height and the aerial biomass weight of 1 month old plants and it did not affected final yield
14 (kg ha⁻¹), despite a lowering of the number of plant with standard height and regular ear
15 size (productive plants) in each repetition.
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31 **CONCLUSION**

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36 In conclusion, according to our results, provoking a priming state by applying the natural
37 fertilizer Chamae on maize seeds could improve germination and limit the colonization of
38 seedlings by *F. verticillioides* and its associated fumonisin accumulation. However,
39 depending on the dilution of fertilizer applied, further plant death was observed, and finally
40 the number of productive plants under field conditions was lowered, but without affecting
41 the final yield, suggesting a higher level of biomass production per plant. This situation can
42 be amended with an increase of planting density. The 0.01% dilution of the seed treatment
43 is the most recommended since it improved the germination, reduced the amount of
44 fungus DNA and fumonisins production by fungus, did not increment significantly
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1 death plants and did not reduce the yield. Therefore, the priming can be recommended as
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3 a low cost and effective technique in the control of the fumonisins production and the
4
5 development of *F. verticillioides* from seed infection, which does not affect the crop
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7 production.
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10 11 12 **ACKNOWLEDGEMENTS**

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Table 1. Analysis of Variance (ANOVA) for the studied parameters under controlled conditions: seed germination, fumonisins production (FB₁, FB₂ and FB₃), quantification of *F. verticillioides* 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)		
Source variation	df ^a	MS ^b	P	FB1	P	FB2	P	FB3	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

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Table 2. Effect of seed treatments on germination								
<i>Germination (%)</i>								
Varieties	LG-30681				MAS 68 K			
	Natural fertilizer dilutions (%)				Dilutions (%)			
Treatments	Control	1	0,01	0,005	Control	1	0,01	0,005
Natural fertilizer application	0 c	55.83 a	67.50 ab	65 a	0 c	83.33 ab	86.66 a	90 a
Different letters indicate significant differences according to LSD test ($P \leq 0.05$)								

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 ($P \leq 0.05$)					

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Table 4. Analysis of Variance (ANOVA) for the studied parameters under field conditions: number of productive plants, height plants and yield

Parameters								
Number of productive plants per block 84 days after sown								
Height plant (84 days after sown) in flowering								
Yield (Kg/Ha)								
Source variation	df ^a	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

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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 ($P \leq 0.05$)

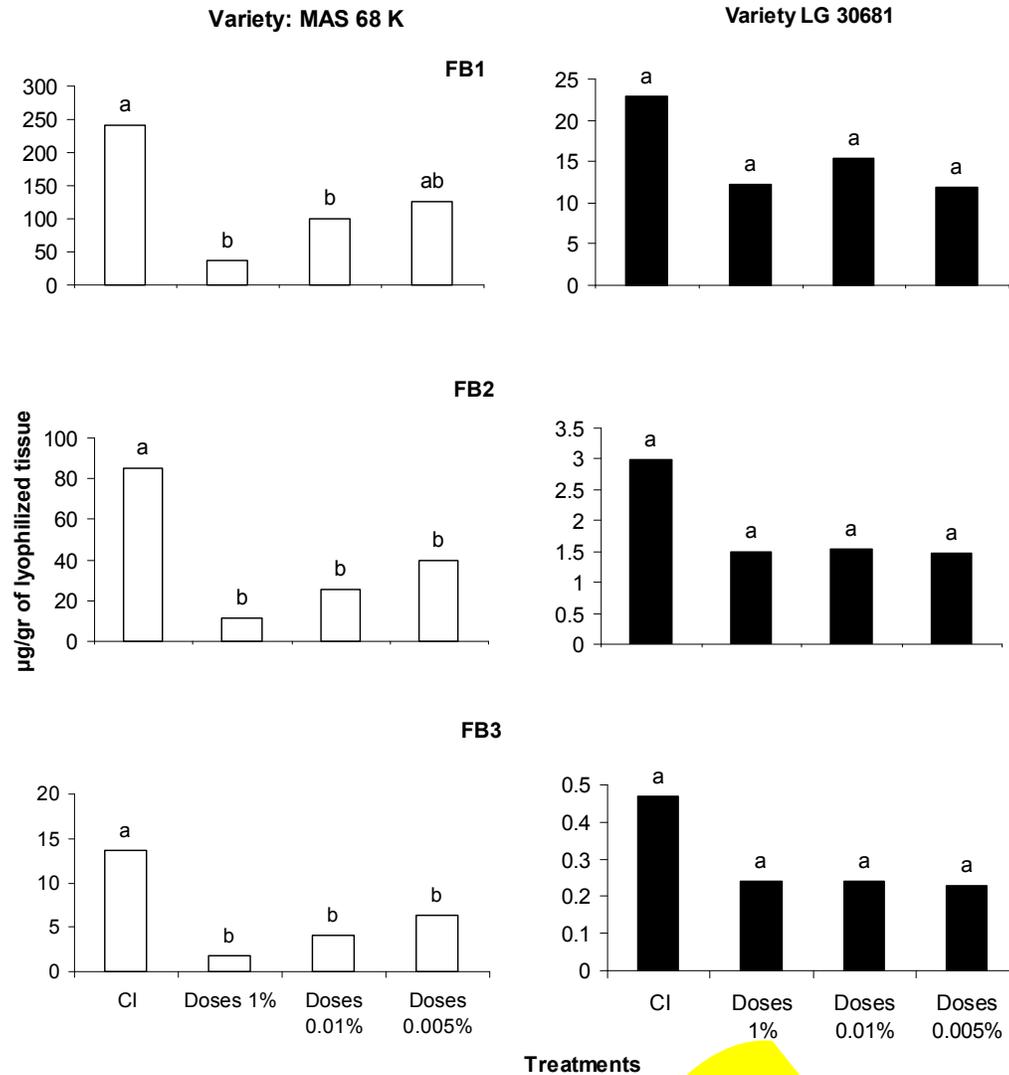
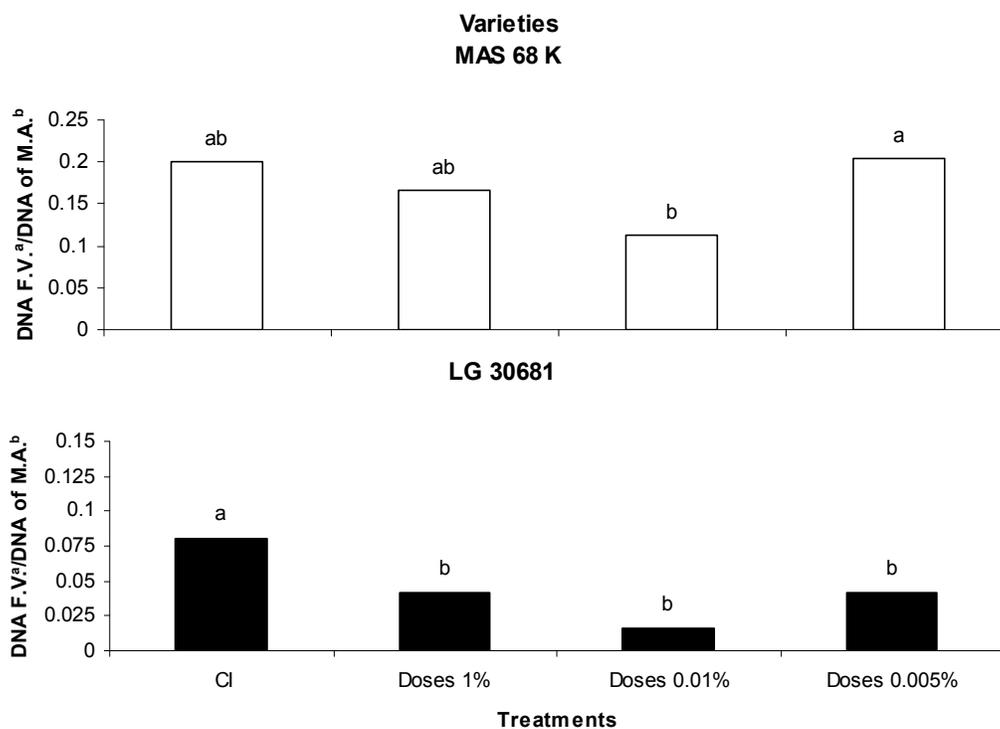


Figure 1. Accumulation of fumonisins: **FB₁**, **FB₂** and **FB₃**, 8 days after inoculation by *Fusarium verticillioides* in seedlings of maize, previously treated with different doses of a natural fertilizer on seeds of 2 varieties (MAS 68 K and LG 30681). CI: control inoculated plants and non-treated 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 ($P \leq 0.05$).



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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 ($P \leq 0.05$).