Application of Fungicides and Microalgal Phenolic Extracts for the Direct Control of Fumonisin Contamination in Maize

Application of fungicides and microalgal phenolic extracts for the direct control of fumonisin contamination in maize

Priscila Tessmer Scaglioni[a*], Massimo Blandino[b], Valentina Scarpino[b], Debora Giordano[b], Giulio Testa[b], Eliana Badiale-Furlong[c]

[a] Centro de Ciências Químicas, Farmacêuticas e de Alimentos, Universidade Federal de Pelotas, 96160-000 Capão do Leão, RS, Brazil.

[b] Dipartimento di Scienze Agrarie, Forestali e Alimentari, Università degli Studi di Torino, Largo Braccini 2, 10095 Grugliasco (TO), Italy.

[c] Escola de Química e Alimentos, Universidade Federal do Rio Grande, 96203-900 Rio Grande, RS, Brazil.

*Corresponding author

Telephone number: +55 53 3275 7387

E-mail address: priscilascaglioni@gmail.com

ABSTRACT

Fungicides and, for the first time, microalgal phenolic extracts (MPE) from *Spirulina* sp. and *Nannochloropsis* sp. were applied on maize culture media and under field conditions to evaluate their ability to minimize *Fusarium* species development and fumonisin production. An *in vitro* assay against an *F. verticillioides* was carried out, using maize grains as culture medium. An open field experiment was carried out in North West Italy under naturally-infected conditions. The compared treatments were factorial combinations of: two insecticide applications (untreated control and pyrethroid against European Corn Borer), four antifungal compounds (untreated control, MPE from *Spirulina* sp. and *Nannochloropsis* sp., synthetic fungicide) and two timings of application of the antifungal compounds (maize flowering and milk stage). The MPE were capable to inhibit fumonisin production *in vitro* more efficiently than tebuconazole. Insecticide application reduced the infection by *Fusarium* species and subsequent fumonisin contamination. However, fumonisins in maize fields were not significantly controlled with both fungicide and MPE application.

KEY-WORDS: *Zea mays*; mycotoxins; *F. verticillioides*; *Nannochloropsis* sp.; *Spirulina* sp.; fungicide.

INTRODUCTION

Amongst the agriculturally important pathogens through the world, *Fusarium verticillioides* is the best known and widespread in maize, causing ear rot disease during cultivation and the production of fumonisins (FBs) in the grains.[1,2] The occurrence of these toxic compounds in maize grains at harvest is influenced by the environmental conditions during the growing season, but also by the agricultural practices applied during plant growth and maturation.[3,4] In particular, infestation with the European Corn Borer (ECB, *Ostrinia nubilalis*) plays an important role in promoting contamination with *Fusarium* species.[1,5] In countries where the cultivation of Bt hybrids is not allowed and particularly in the maize food chain, the direct control of ECB through the application of insecticides is one of the most important measures to evaluate the infection by these fungal species and the consequent production of fumonisins in maize grains.[6] Other practices that could minimize fumonisin occurrence in maize are related to early planting times and strategies to avoid stress to the crop. Their application, following an integrated approach, leads to a more effective and constant reduction of FB contaminations compared to the application of single practices.[3]

However, considering the health risk represented by fumonisin toxicity [7,8] and the economic losses, it is necessary to find new control solutions that could be inserted in order to integrate the available preventive control practices for minimizing the risk of fumonisin contamination.

The use of synthetic fungicides is the primary effective strategy to control fungal diseases in several crops. In particular, fungicide application is an important practice for reducing the overall risk of mycotoxin contamination in wheat grains.[9,10] However, a few studies have investigated the efficacy of fungicides against fumonisin producers

and the effectiveness of their application in reducing the content of these mycotoxins in maize fields.[11]

Because of an increased public concern regarding the negative effects of pesticides on human health and the environment,[12,13] the use of natural compounds with antifungal activity represents a potential important alternative to chemical methods for controlling the infection and development of toxigenic fungi.

Natural antimicrobials are sourced from animal, plant, and microbial origins as defense against pathogens by causing inefficiency or making them unviable. Some compounds with antimicrobial properties are able to promote protection against physical and chemical effects, reinforcing the defense against pathogens.[14] Naturally occurring antifungal compounds also act in the fungal and mycotoxigenic inhibition by affecting different defense mechanisms of the microbial metabolism. Phenolic compounds, proteins, and essential oils, among others, can inhibit cell wall components such as glycosamine, chitin, ergosterol, and mannoproteins, destroying the membrane integrity and impeding nutrient transport.[15-17] They can also inhibit protein and amino acid synthesis and the biosynthesis of sphingolipids to interfere in the transport of electrons, making the fungal cell integrity unfeasible.[18]

Microalgae are a diverse group within prokaryotes and eukaryotes and produce a wide variety of commercially interesting products such as lipids, oils, sugars, pigments, and many other bioactive compounds. In addition, some microalgae, such as *Spirulina* sp. or *Nannochloropsis* sp., are rich sources of natural antioxidants such as phenolic compounds and carotenoids.[19] The antifungal activity against strains of the *Fusarium* complex (*F. graminearum* and *F. meridionale*) by compounds extracted from *Spirulina* sp. has recently been shown,[17,20] but further studies are needed to optimize their formulation and further control their efficacy at larger scales.

The aim of this manuscript is to verify the possibility to apply a direct control strategy to fumonisin producers in maize through the application of synthetic fungicides and, for the first time, microalgal phenolic extracts (MPE) from the microalgae *Spirulina* sp. and *Nannochloropsis* sp. The efficacy of the compared compounds in controlling *F. verticillioides* and FB contamination was studied both *in vitro* media and in the field, taking into account different agronomical conditions.

MATERIALS AND METHODS

Microalgal biomass production and MPE obtainment

The sample of *Spirulina* sp. (LEB-18) was supplied by the Biochemical Engineering Laboratory at the Universidade Federal do Rio Grande (FURG), located in Rio Grande, RS, Brazil.[21]

The biomass of *Nannochloropsis* sp. (NANN-OCUL-1) was cultivated in the Phytoplankton and Marine Microorganism Laboratory at the FURG.[22]

The microalgae biomass samples were dried in tray dryers at 50°C for 5 h, ground up to 32 mesh, vacuum-packaged, and stored at 4°C until further analysis.

The free phenolic compounds were extracted and clarified.[16,17] Quantification and identification of phenolic acids in the extracts were performed using reference standards from Sigma-Aldrich, namely: caffeic, chlorogenic, p-coumaric, ferulic, gallic, p-hydroxybenzoic, protocatechuic, syringic, and vanillic acids, in a liquid chromatograph (Shimadzu, Tokyo, Japan, CLASS-M10A) coupled with a UV detector and a C18 reverse phase column (4.6 x 250 mm, 5 μm, Discovery, USA). The HPLC-UV operated at a flow rate of 0.7 mL min^{-1}, at 35°C, with a gradient isocratic solvent consisting of methanol and acidic water (acetic acid 1%) in a ratio of 20:80 (v/v) for 25 min. Detections were carried out at 280 nm for 15 min and at 320 nm for 25 min.[23]

Antifungal activity of MPE against *F. verticillioides* ***in vitro***

The *in vitro* experiment was conducted in Petri dishes containing different substrates for *F. verticillioides* development: agar and agar with maize kernels.

The maize kernels were previously autoclaved and used whole in sufficient quantities to cover the Petri dish surface (19 g).

In each experiment, 40 μg mL^{-1} of MPE were added, corresponding to the previously estimated EC_{50} value, this value was estimated through a linear regression relating the concentration of phenolic compounds found in different MPE volumes and the respective percentages of *Fusarium* halo inhibitions, equal to y=1.481x and y=1.170x using *Spirulina* sp. and *Nannochloropsis* sp., respectively (data not shown); subsequently, a mycelial disk of the *F. verticillioides* strain (1.1 mm diameter) was placed in the center of each plate.

The isolated fungus was obtained from experimental maize fields; identification was performed through morphological characteristics via optical light microscopy to comparison with the literature[24], the DNA extraction was performed with the kit Fungi/Yeast Genomic DNA Isolation (Norgen). COMPLETAR Fungal cultures were grown on *Spezieller Nährstoffarmer Agar* (SNA) at 25°C to induce sporulation and maintained at 4°C on SNA slants. The isolates were grown on potato dextrose agar (PDA) media for seven days to obtain mycelial discs for use as inocula for the *in vitro* experiments.

The control treatment was conducted with sterile water instead of MPE. An experiment with tebuconazole standard (Pestanal®) was also performed at 600 μg mL^{-1}. Petri dishes with the inoculum were incubated at 25°C and a light/dark photoperiod of 12-12 h. The efficacy of the treatments was evaluated daily for seven days by measuring

the diameter of the hyphae development orthogonally. All tests were performed in triplicate.

Fumonisin B_1 (FB_1) determination

In the *in vitro* experiment, FB_1 was determined using all the entire content of the Petri dishes. Extraction was performed using the QuEChERS method with 2 g of sample previously milled with 10 mL of distilled water and 10 mL of acidified acetonitrile 0.5% with acetic acid. The mixture was shaken on an orbital shaker at 300 rpm for 30 min. Salts were added to assist the extraction (4 g of $MgSO_4$ and 1 g of NaCl). After homogenization, the content was centrifuged at 3220 *g* at 20°C for 15 min; 5 mL of the supernatant were collected, 5 mL of hexane were added, and the mixture was vortexed for 1 min. After centrifugation at 3,220 *g* and 20°C for 1 min, the acetonitrile phase was collected (5 mL) and transferred to an amber flask; the contents were dried in a water bath at 50°C.[25,26]

Quantification was performed using a Liquid Chromatograph Alliance Separations model 2695 Waters (Milford, MA, USA), coupled with an automatic sampler, a quaternary pump, a degassing system, an MS Detector, Micromass® Four Micro™ API Waters equipped with an electrospray ionization (ESI) source, the Masslynx 4.0 Waters software data acquisition system and an Atlantis® analytical column HILIC silica 3.0 μm (50 × 4.6 mm id). The conditions of the mass spectrometer adapted for this mycotoxin detection were as follows: ionization source temperature at 110°C, desolvation gas temperature (N_2) of 500°C; desolvation gas flow rate of 500 L h^{-1}, and 50 L h^{-1} cone gas flow; the capillary voltage was 4 kV. The mobile phase was composed of ultrapure water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B), with a flow rate of 0.4 mL min^{-1} and a gradient elution (0–2 min: 5%

(A) and 95% (B); 2–5 min: 95% (A) and 5% (B); 5–10 min: 5% (A) and 95% (B)). The conditions of the fragmentation through the mass/charge (m / z) transition that related the precursor ion to the product ion was 722 > 334.1, used for quantification, with a positive ionization mode (ESI), a cone voltage of 50 V, and a collision energy of 40 eV. For quantification, a standard curve with the equation y = 3608.71x - 172.165 (linearity for 0.05 to 1.5 μg mL^{-1}) was used.

Application of MPE and fungicides in maize fields

An open field trial, was carried out in naturally infected conditions in the 2015 growing season at Carmagnola (44° 50’ N, 7° 40’ E; elevation 245 m), in North West Italy.

The agronomic techniques generally applied in the considered growing area were adopted. Briefly, the previous crop was maize, and the study was conducted using a hybrid that is suitable in the food chain (Pioneer P1547, FAO maturity class 600, 130 days relative to maturity). To prepare the proper seedbed, sowing was carried out in April 2nd after an autumn 0.3 m deep ploughing, followed by disk harrowing. Crop density was approximately 75,000 plants per hectare, and the experimental field received 250, 90, and 100 kg ha^{-1} of N, P, and K, respectively. Irrigation was carried out using a sprinkler, according to the conventional farm management system in force in the experimental area.

The compared treatments were factorial combinations of:

- two different strategies to control ECB larvae:
  - insecticide application at the milk stage (growth stage, GS75) [27]: lambda-cyhalothrin (pyrethroid) + chlorantraniliprole (diamide) mixture [Ampligo®, formulation: suspension concentrate, capsule suspension, Syngenta Crop

Protection S.p.A., Italy, applied at 0.015 and 0.030 kg of active ingredient (AI) ha^{-1}, respectively];

- untreated control.

- four compounds with antifungal activity applied to maize ears:
  - untreated control, sprayed with sterile water;
  - MPE of the microalgae *Nannochloropsis* sp. (0.070 kg ha^{-1});
  - MPE of the microalgae *Spirulina* sp. (0.062 kg ha^{-1});
  - fungicide mixture of prothioconazole + tebuconazole [Prosaro®, Bayer, Italy, emulsifiable concentrate formulation (EC), applied at 0.125 kg of each AI ha^{-1}].
- two application timings at different maize growth stages:
  - at maize flowering (GS 65, July 3rd)
  - at maize milk stage (GS 75, July 20th), according to the optimum timing for insecticide application.[3]

Application of the MPE was carried out at concentrations around 40 μg mL^{-1}, corresponding to the previously estimated EC_{50} value. Application of antifungal compounds was carried out by spraying 10 mL of solution for each primary ear, using a hand sprayer. The treatments were assigned to experimental units, using a completely randomized block design with three replicates. Each plot consisted of 10 consecutive plants presenting the same developing stage, separated by three untreated buffer rows on either side; inter-row distance was 0.75 m.

All ears were collected by hand from each plot at the end of the maturity (September 1st) at a grain moisture content between 23 and 27%, visually inspected for insect injuries and disease symptoms, and shelled using an electric sheller. The entire amount of grains (approximately 3 kg) collected from each plot was dried at 60°C for

three days and ground using a ZM 200 Ultra Centrifugal Mill (Retsch GmbH, Haan, Germany). The ground samples were stored at -25°C until mycotoxin analysis.

Entomological and mycological measurements

The ECB damage incidence was calculated as the percentage of ears per plots with kernel injuries or apical and basal tunnels in the cob due to larval activity. The ECB damage severity was calculated as the percentage of kernels per ear with injuries due to larval activity. A scale of 1 to 7 was used, in which each numerical value corresponded to a percentage interval of surfaces exhibiting visible kernel damage due to larval activity, according to the following schedule: 1 = no injuries, 2 = 1-5%, 3 = 6-10%, 4 = 11-20 %, 5 = 21-35%, 6 = 36-60%, 7 > 60%.[3]

Fungal ear rot incidence was calculated as percentage of ears per plot with symptoms, while fungal ear rot severity was calculated as kernel percentage per ear with symptoms. A scale of 1 to 7 was used, in which each numerical value corresponded to a percentage interval of surfaces exhibiting visible symptoms of the disease, according to the following schedule: 1 = no symptoms, 2 = 1-3 %, 3 = 4-10%, 4 = 11-25 %, 5 = 26-50%, 6 = 51-75%, 7 > 75%.[3] The ECB damage severity and ear rot severity scores were converted to percentages of ears exhibiting symptoms, and each score was replaced with the mid-point of the interval.

Fumonisin B_1 and B_2 concentrations in maize from the field experiment

Concentrations of FB_1 and FB_2 in maize samples from field cultivation were determined using 50 g of ground sample with 100 mL of a methanol:water solution (80:20 v/v), shaken for 20 min. After filtration through Whatman® nº1 paper, the samples were diluted with phosphate-buffered saline (PBS) for subsequent purification, using

immunoaffinity columns FUMtest (VICAM®). For this purpose, we used a 1 drop s^{-1} flow with a vacuum system; 5 mL of the extract were eluted through the column, after 2.5 mL of PBS, and the analyte was recovered with 2 mL of pure methanol and injected into the HPLC-MS/MS system, equipped with a Varian 212-LC chromatographic pump, a Varian column, Pursuit 5 C18 50 x 2.1 mm, a ProStar 410 autosampler, and a triple quadrupole mass spectrometer 310-MS.

The chromatographic run had a duration of 15 min (t_R FB_1 = 4.9 min; t_R FB_2 = 5.6 min), with acetonitrile and water acidified with acetic acid 0.1% as the mobile phase. The FBs were identified in a triple quadrupole mass spectrometer with the electrospray ionization source in the positive ion mode. The protonated FB_1 (722 m z^{-1}) molecule was fragmented into its product ions at 352 m z^{-1} (used for identification) and 334 m z^{-1} (used for quantification). For FB_2 (706 m z^{-1}), we used 318 m z^{-1} (used for identification) and 336 m z^{-1} (used for quantification).

Ergosterol content determination

The modified method was used for ergosterol determination[28] in samples from the *in vitro* and *in vivo* experiments. Briefly, 0.2 g of sample were mixed with 10 mL of methanol; the mixture was shaken on an orbital shaker at 200 rpm for 30 min (three times). The methanolic extract was then centrifuged at 3,200 g at 20°C for 10 min. Subsequently, it was heated under reflux for 30 min and cooled to 4°C. The refluxed material was subjected to four partitions with 20 mL of hexane. The hexane fraction was dried on a rotary evaporator at 60°C.

The residue was dissolved with methanol and determined via a chromatograph (Shimadzu, Tokyo, Japan, CLASSE-M10A) coupled with a UV detector and a C18 reverse phase column (4.6 x 250 mm, 5 μm, Discovery, USA). The HPLC-UV was

operated at 0.8 mL min^{-1} at 30°C, using a 100% methanol mobile phase for 17 min with detection at 282 nm. The ergosterol content was estimated by an ergosterol standard calibration curve with concentrations ranging from 0.05 to 2.0 μg mL^{-1}.[29]

Maize kernel phenolic acids

The phenolic acid content[23] of maize kernels at harvest was determined to check if the presence of these compounds in the grain might inhibit fungal growth and to compare this phenolic acid profile with the MPE one.

Statistical analysis

An analysis of variance (ANOVA) was conducted to evaluate the effect of the MPE and the fungicide on the following factors: ergosterol concentration and FB_1 content (*in vitro* experiments), ECB incidence and severity, fungal ear rot incidence and severity, ergosterol and FBs contents (field experiment), using a completely randomized block design with the following independent variables: treatment with antifungal compounds and the medium used (*in vitro* experiments) and the combination of antifungal compounds, the timing of application and the insecticide treatment (field experiment). The residual normal distribution was verified using the Kolmogorov-Smirnov test, while variance homogeneity was verified using the Levene test; multiple comparison tests were performed according to the Ryan-Einot-Gabriel-Welsch F test on treatment means. The transformations used to normalize the residuals were: $y' = \ln(x + 1)$ for the ergosterol and FBs contents; it was not necessary to transform the ECB incidence and severity, and the fungal ear rot incidence and severity data. All statistical analyses were performed using the software package SPSS for Windows, version 24.0 (SPSS Inc., Chicago).

RESULTS

The phenolic extract from *Spirulina* sp. amounted to 627.8 μg g^{-1}, of which 93% was chlorogenic acid, with 0.3% gallic, 2.6% protocatechuic and 3.9% hydroxybenzoic acids. For *Nannochloropsis* sp., the extracts contained 615.8 μg g^{-1} phenolic compounds, of which 76% was chlorogenic acid, followed by gallic (13.5%), protocatechuic (4.2%), hydroxybenzoic (4.2%), syringic (1.2%), vanillic (0.5%) and ferulic (0.1%) acids (Table 1).

The phenolic acid concentrations in maize grains from the experimental field at harvest were also determined, because the presence of these compounds in the grain might inhibit fungal growth.[16,20] No significant differences were observed between the compared treatments for phenolic concentrations in grains, thus their average content in all collected maize samples was reported in Table 1. Phenolic content was 62 and 71 μg g^{-1} for the untreated control and the insecticide application, respectively; while their concentrations was 68.6, 66.4, 64.2 and 67.2 μg g^{-1} for untreated control, *Spirulina*, *Nannochloropsis* and tebuconazole + prothioconazole treatments, respectively. Among them, considering all the analyzed samples, the frequency of each acid determinate was: chlorogenic (100%); protocatechuic (100%); ferulic (97%); vanillic (94%); hydroxybenzoic (94%); coumaric (91%); syringic (82%); caffeic (81%); and gallic (28%).

Table 1.

Figure 1 shows the development of the fungal halo when submitted to the different *in vitro* treatments. Tebuconazole had a better inhibition effect (81% after 168 h of incubation) in the experiment with agar, while both MPE reduced the halo development by 29% at the end of the period. Tebuconazole presented a greater

tendency to inhibit the halo development at the end of incubation period when dried maize was the substrate (72% of halo inhibition), while both MPE presented a slight tendency towards this inhibition (36% with *Nannochloropsis* sp. and 18% with *Spirulina* sp.).

Figure 1.

Ergosterol, as an indicator of fungal biomass evolution on the culture media, showed a behavior similar to the observed effect on radial development (Table 2). The MPE and the synthetic fungicide reduced ergosterol contents in both mediums; however, tebuconazole was most efficient.

Table 2.

As shown in Table 2, the addition of tebuconazole resulted in a significant reduction of FB_1 compared to the control, whereas the MPE led to a further significant reduction of the FB content.

The *in vivo* experiment was conducted in maize field in North West Italy during the 2015 growing season in order to evaluate the effects of the factorial combination of: the insecticide application to control ECB, the main vector of *Fusarium verticillioides*, and the direct control of fungal infection through antifungal compounds (MPE or synthetic fungicide) at different timings.

Table 3.

The insecticide application resulted in a significant and positive role in minimizing ECB incidence and severity, ergosterol content, fungal ear rot incidence and severity and FB contamination (Table 3).

No significant differences were overall recorded between antifungal treatments (natural or synthetic) and the untreated control for ECB and fungal ear rot symptoms and FB contamination, considering both applications at flowering and milk stage.

Conversely, as far as the ergosterol content is concerned, although the antifungal treatments (natural or synthetic) did not significantly differ from the untreated control, they differentiated from each other in controlling this parameter with a greater efficacy of the synthetic fungicide.

With the exception of the ergosterol content, the interactions between the considered factors were never significant.

DISCUSSION

The data collected in the *in vitro* experiment suggest that the MPE presented a higher antimycotoxigenic capacity, while tebuconazole had a greater capacity to inhibit fungal development.

The synthetic fungicide reduces the multiplication of fungal biomass, inhibits compounds acting on the primary metabolism of nutrient production reactions, the production of membranes or cell walls, respiratory activity, and cell differentiation.[30] Consequently, this might lead to the production of secondary metabolites, such as mycotoxins as a response to growth medium stress.[12,13,15]

The inhibition zones of the colonies, the cell wall, the membrane constituents (ergosterol and glucosamine), and the alterations in enzyme activity with reduced biomolecular synthesis are indicators of cell multiplication inhibition. Few of these effects are evaluated in terms of mycotoxin production by toxigenic species. Therefore, information on the alteration of these metabolic pathways is fundamental to any recommendation for the use the extracts to prevent or inhibit microbial contamination in the food chain.[17,25]

The antifungal and antimycotoxigenic capacity of natural extracts has already been proven in other studies against other species of *Fusarium*, mainly against the *F. graminearum* complex, originating from cereals other than maize. Pagnussatt et al.[16] showed that the use of *Spirulina* LEB-18 phenolic compounds can inhibit fungal multiplication, especially in terms of the inactivation of enzymatic systems (amylase and protease) of 12 toxigenic strains of *Fusarium graminearum* isolated from barley and wheat. Heidtmann et al.[13] tested natural antifungals (γ-oryzanol, phenolic extract of neem seeds and rice bran) against three toxigenic strains of *F. graminearum* isolated from wheat, rice, and barley. Fungal growth was effectively inhibited, especially via the inactivation of the enzymatic systems of *F. graminearum* and the natural antifungals inhibiting deoxynivalenol production.

Beekrum et al.[31] reported the effect of ferulic acid on the growth and FB production of *F. verticillioides*. These authors compared several natural phenolic compounds such as chlorophorin, iroko and maakianin, caffeic acid, ferulic acid, benzoic acid, and vanillic acid, and observed that benzoic acid and ferulic acid had no effect on fungal growth, while, with the exception of benzoic acid, all the other compounds reduced FB_1 production by 88–94%. Although 1 $\mu g\ mL^{-1}$ of ferulic acid (5.15×10^{-6} mol L^{-1}) did not inhibit *F. verticillioides* growth, it reduced FB_1 production by 90%.

Ferrochio et al.[32] verified that the application of ferulic acid at concentrations ≥ 0.02 mol L^{-1} could be an effective post-harvest strategy to control the growth of *F. verticillioides* and *F. proliferatum* and to reduce FB production.

The cited studies have shown that there is no linear response to ferulic acid use in terms of growth inhibition and FB production. This was also confirmed in the present study, where natural extracts inhibited fungal development less efficiently, but showed a

greater reduction of FB production compared to tebuconazole, which had the opposite effect.

Another important aspect to consider is the difference between the doses applied. In the *in vitro* experiments, the fungicide dose was 14 times higher than that of the MPE (Table 4), but the reduction of FB production was higher when natural extracts were used.

Table 4.

Although the compared fungicides and natural compounds showed antifungal activities *in vitro* against FB producers, the direct control of FBs in the maize field was not effective, also considering different application timings.

To the best of the author´s knowledge, no studies have yet reported a significant reduction of the infection level of FB producers after the application of fungicides. Folcher et al.[33] and Mazzoni et al.[34] reported that the addition of a fungicide (tebuconazole or tebuconazole + prothioconazole) to an insecticidal treatment at the flowering stage did not significantly reduce the FB concentration in maize grains compared to insecticide application alone. On the other hand, both the previously cited studies underline the important role of insecticide application against ECB in reducing the FB content.

The lack of direct control of FB contamination in maize could be related to a series of factors, such as the higher plant biomass of the crop compared to wheat or other crops and the difficulty of reaching the ear, which is covered by the husk and placed under several leaves. The overcoming of these constraints probably requires the application of higher dosages of active substances than those applied on small cereals.

Moreover, the limited efficacy of the direct control in maize could be related to the long period of maturation and the possibility of different infection pathways and

timings for FB producers. *Fusarium verticillioides* and *F. proliferatum* could infect maize kernels through silks and through kernel damage caused by insects. In temperate maize cultivation areas, ECB injuries are most frequently associated pathway for *F. verticillioides* infection, and the ECB activity could concern three to four months between the beginning of ripening and harvest.[3] Moreover, a systemic infection of plants is also possible, since the fungus could be present in seedlings, leaf sheaths, and stalk tissue, without causing noticeable symptoms. The absence of a precise and defined infection event makes it more difficult to identify the best timing of application of a substance with fungicidal activity, in particular when its persistence is limited.

In conclusion, this study underlines the difficulties to apply direct strategy to control the development of FB producers in maize production; unlike in other crops such as wheat. Thus, at present, the adequate use of preventive agricultural practices and the control of insect injuries, according to an integrated approach, still remains the most effective strategy to minimize the risk for FB contamination in maize. However, further studies are needed to evaluate the role of MPE as part of the strategy to prevent FB contamination. The optimization of microalgae cultivation may favor the MPE production, increasing the availability of these compounds in the total biomass of these organisms. Another alternative for future studies is the encapsulation of phenolic compounds into carrier systems, such as liposomes. This strategy can prevent their degradation by metabolic processes, preserving and prolonging their antifungal and antimycotoxigenic properties, besides it may facilitate their penetration into different plant tissues.

ACKNOWLEDGEMENT

The authors would like to thank Francesca Vanara for her precious help and cooperation in the laboratory work.

FUNDING

The authors are grateful to *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) for supporting this study through the *Programa Institucional de Bolsas de Doutorado Sanduíche no Exterior (PDSE)* (BEX 2384/15-9).

NOTES

The authors declare no competing financial interest.

REFERENCES

1. Kvas, M.; Marasas, W. F. O.; Wingfield, B. D.; Wingfield, M. J.; Steenkamp, E. T. Diversity and evolution of *Fusarium* species in the *Gibberella fujikuroi* complex. *Fungal Divers.* **2009**, *34*, 1-21.

2. Ogara, I. M.; Zarafi, A. B.; Alabi, O.; Banwo, O.; Ezekiel, C. N.; Warth, B.; Sulyok, M.; Krska, R. Mycotoxin patterns in ear rot infected maize: A comprehensive case study in Nigeria. *Food Control* **2017**, *73*, 1159-1168.

3. Blandino, M.; Reyneri, A.; Vanara, F.; Pascale, M.; Haidukowski, M.; Campagna, C. Management of fumonisin contamination in maize kernels through the timing of insecticide application against European corn borer. *Food Addit. Contam.* **2009**, *26*, 1501-1514.

4. Saladini, M.; Blandino, M.; Reyneri, A.; Alma, A. The impact of insecticide treatments on *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) and their influence on the mycotoxin contamination of maize kernels. *Pest Manag. Sci.* **2008**, *64*, 1170-1178.

5. Sobek, E. A.; Munkvold, G. P. European corn borer (Lepidoptera: Pyralidae) larvae as vectors of *Fusarium moniliforme*, causing kernel rot and symptomless throughdouble infection of maize kernels. *J. Econ. Entomol.* **1999**, *92*, 503-509.

6. Blandino, M.; Scarpino, V.; Vanara, F.; Sulyok, M.; Krska, R.; Reyneri, A. The Role of the European Corn Borer (*Ostrinia Nubilalis*) on contamination of maize with thirteen *Fusarium* mycotoxins. *Food Addit. Contam. Part A* **2015**, *32*(4), 533-543.

7. Albonico, M.; Schutz, L. F.; Caloni, F.; Cortinovis, C.; Spicer, L. J. *In vitro* effects of the *Fusarium* mycotoxins fumonisin B_1 and beauvericin on bovine granulosa cell proliferation and steroid production. *Toxicon* **2017**, *128*, 38-45.

8. Fernández-Blanco, C.; Frizzell, C.; Shannon, M.; Ruiz, M. J.; Connolly, L. An *in vitro* investigation on the cytotoxic and nuclear receptor transcriptional activity of the mycotoxins fumonisin B_1 and beauvericin. *Toxicol. Lett.* **2016**, *257*, 1-10.

9. Scarpino, V.; Reyneri, A.; Sulyok, M.; Krska, R.; Blandino, M. Effect of fungicide application to control *Fusarium* head blinght and *Fusarium* and *Alternaria* mycotoxins in winter wheat (*Triticum aestivum* L.). *World Mycotoxin J.* **2015**, *8(4)*, 499-510.

10. Blandino, M.; Reyneri, A. Effect of fungicide and foliar fertilizer application to winter wheat at anthesis on flag leaf senescence, grain yield, flour bread-making quality and DON contamination. *Eur. J. Agron.* **2009**, *30*, 275-282.

11. Rensburg, B. J.; Mc Laren, N. W.; Schoeman, A.; Flett, B. C. The effects of cultivar and prophylactic fungicide spray for leaf diseases on colonization of maize ears by fumonisin producing *Fusarium* spp. and fumonisin synthesis in South Africa. *Crop Prot.* **2016**, *79*, 56-63.

12. Dors, G. C.; Primel, E. G.; Fagundes C. A.; Mariot, C. H. P.; Badiale-Furlong E. Distribution of pesticides in rice grain and rice bran. *J. Braz. Chem. Soc.* **2011**, *22*, 1921-1930.

13. Heidtmann-Bemvenuti, R.; Tralamazza, S. M.; Ferreira, C. F. J.; Corrêa, B.; Badiale-Furlong, E. Effect of natural compounds on *Fusarium graminearum* complex. *J. Sci. Food Agric.* **2016**, *96(12)*, 3998-4008.

14. Scaglioni, P. T.; Badiale-Furlong, E. Can microalgae at as source of preservatives in food chain? J. *Food Sci. Eng.* **2017**, *7*, 283-296.

15. Oliveira, M. S.; Badiale-Furlong, E. Screening of antifungal and antimycotoxigenic activity of plant phenolic extracts. *World Mycotoxin J.* **2008**, *1(2)*, 139-46.

16. Souza, M. M.; Prieto, L.; Ribeiro, A. C.; Souza, T. D.; Badiale-Furlong, E. Assesment of the antifungal activity of *Spirulina platensis* phenolic extract against *Aspergillus flavus*. *Ciênc. Agrotec.* **2011**, *35*, 1050-1058.

17. Pagnussatt, F. A.; Del Ponte, E. M.; Garda-Buffon, J.; Badiale-Furlong, E. Inhibition of *Fusarium graminearum* growth and mycotoxin production by phenolic extract from *Spirulina* sp. *Pestic. Biochem. Physiol.* **2014**, *108*, 21-26.

18. Marino, M.; Bersani, C.; Comi. G. Impedance measurements to study the antimicrobial activity of essential oils from Lamiaceae and Compositae. *Int. J. Food Microbiol.* **2001**, *67*, 187-95.

19. Plaza, M.; Herrero, M.; Cifuentes, A.; Ibáñez, E. Innovative natural functional ingredients from microalgae. *J. Agric. Food Chem.* **2009**, *57*, 7159-7170.

20. Pagnussatt, F. A.; De Lima, V. R.; Dora, C. L.; Costa, J. A. V.; Putaux, J. L.; Badiale-Furlong, E. Assessment of the encapsulation effect of phenolic compounds from *Spirulina* sp. LEB-18 on their aintifusarium activities. *Food Chem.* **2016**, *211*, 616-623.

21. Morais, M. G.; Costa, J. A. V. Isolation and selection of microalgae from coal fired thermoelectric power plant for biofixation of carbon dioxide. *Energy Convers. Manag.* **2007**, *48*, 2169-2173.

22. Borges, L.; Morón-Villarreyes, J. A.; D'oca, M. G. M.; Abreu, P. C. Effects of flocculants on lipid extraction and fatty acid composition of the microalgae

Nannochloropsis oculata and *Thalassiosira weissflogii*. *Biomass Bioenergy* **2011**, *35*, 4449-4454.

23. Scaglioni, P. T.; De Souza, T. D.; Schmidt, C. G.; Badiale-Furlong, E. Availability of free and bound phenolic compounds in rice after hydrothermal treatment. *J. Cereal Sci.* **2014**, *60*, 526-532.

24. Leslie, J. F.; Summerell, B. A. Species Description, In The *Fusarium* laboratory manual, Blackwell Publishing, Iowa:USA, 2006; 388 pp.

25. Anastassiades, M.; Lehotay, S. J.; Štajnbaher, D.; Schenck, F. J. Fast and easy multiresidue method employng acetonitrile extraction/partitioning and "dispersive solid-phase" for the determination of pesticide residues in produce**.** *J. AOAC Int.* **2003**, *83*, 412-431.

26. Desmarchelier, J. M. O.; Tella, P.; Gremaud, W. S.; Mottier, P. Development and Comparison of Two Multiresidue Methods for the Analysis of 17 Mycotoxins in Cereals by Liquid Chromatography Electrospray Ionization Tandem Mass Spectromety. *J. Agric. Food Chem.* **2010**, *58*, 7510-7519.

27. Lancashire, P. D.; Bleiholder, H.; Longelüddcke, P.; Stauss, R.; Van Den Boom, T.; Weber, E.; Witzenberger, A. An uniform decimal code for growth stages of crops and weeds. *Ann. Appl. Biol.* **1991**, *119*, 561-601.

28. Gutarowska, B.; Zakowska, Z. Mathematical models of mycelium growth and ergosterol synthesis in stationary mould culture. *Lett. Appl. Microbiol.* **2009**, *48*, 605-610.

29. Freitas, R. F.; Schrack, E. C.; He, Q.; Silliman, B. R.; Furlong, E. B.; Telles, A. C.; Costa, C. S. B. Consumer control of the establishment of marsh foundation plants in intertidal mudflats. *Mar. Ecol. Prog. Ser.* **2016**, *547*, 79-89.

30. Castro, H. G.; Ferreira, F. A.; Silva, D. J. H.; Mosquim, P. R. Metabólitos primários e secundários, In Contribuição ao estudo das plantas medicinais: Metabólitos secundários, Edition nº 2.; Visconde do Rio Branco, 2004; 133 pp.

31. Beekrum, S.; Govinden, R.; Padayachee, T.; Odhav, B. Naturally occurring phenols: a detoxification strategy for fumonisin B_1. *Food Addit. Contam.* **2003**, *20*, 490-493.

32. Ferrochio, L.; Cendoya, E.; Farnochi, M. C.; Massad, W.; Ramirez, M. L. Evaluation of ability of ferulic acid to control growth and fumonisin production of *Fusarium verticillioides* and *Fusarium proliferatum* on maize based media. *Int. J. Food Microbiol.* **2013**, *167*, 215-220.

33. Folcher, L.; Jarry, M.; Weissenberger, A.; Gérault, F.; Eychenne, N.; Delos, M.; Regnault- Roger, C. Comparative activity of agrochemical treatments on mycotoxin levels with regard to corn borers and *Fusarium* mycoflora in maize (*Zea mays* L.) fields. *Crop Prot.* **2009**, *28*, 302-308.

34. Mazzoni, E.; Scandolara, A.; Giorni, P.; Pietri, A.; Battilani, P. Field control of *Fusarium* ear rot, *Ostrinia nubilalis* (Hübner), and fumonisin in maize kernels. *Pest Manag. Sci***. 2011**, *67*, 458-465.

Figure captions

Fig. 1. Radial development of the mycelial discs of the *F. verticillioides* submitted to different treatments.

Table 1. Average phenolic composition from *Spirulina* sp., *Nannochloropsis* sp. and maize kernels from the experimental field at harvest.

Phenolic compound	*Spirulina* sp. ($\mu g\ g^{-1}$)	*Nannochloropsis* sp. ($\mu g\ g^{-1}$)	Maize kernels [a] ($\mu g\ g^{-1}$)
Chlorogenic acid	585.2	489.5	4.7
Gallic acid	1.7	86.6	4.0
Protocatechuic acid	16.3	27.0	19.3
Hydroxybenzoic acid	24.6	1.4	5.3
Syringic acid	-	7.6	2.6
Vanillic acid	-	3.4	0.4
Ferulic acid	-	0.3	28.1
Coumaric acid	-	-	2.8
Caffeic acid	-	-	2.6

[a] The reported values for the phenolic compounds in maize kernels at harvest are the means related to the different compared treatments.

Table 2. Effect of different mediums and treatments applied in culture medium containing *F. verticillioides* and maize grains on the ergosterol concentration and fumonisin B_1 contamination after 168 h of incubation.

Medium	Antifungal Treatment	Ergosterol [b]		Fumonisin B_1 [b]	
		T	N ($ng\ g^{-1}$)	T	N ($\mu g\ kg^{-1}$)
Agar	Untreated control	8.3 a	3924.1	6.8 a	926.4
	Spirulina	6.5 b	637.9	3.8 c	41.7
	Nannochloropsis	6.5 b	693.0	3.9 c	49.4
	Tebuconazole	3.9 c	50.0	5.2 b	181.7
	P (F)	< 0.001		< 0.001	
	sem [a]	0.5		0.4	
Agar and dried maize kernels	Untreated control	8.5 a	5027.1	7.3 a	1423.9
	Spirulina	7.2 b	1305.0	4.3 c	70.5
	Nannochloropsis	7.1 b	1166.0	4.2 c	71.9
	Tebuconazole	4.7 c	109.1	6.5 b	670.0
	P (F)	< 0.001		< 0.001	
	sem [a]	0.5		0.4	

For each medium, means followed by different letters are significantly different (the level of significance is shown in the table). [a] sem = standard error of mean.
[b] Means reported for ergosterol and Fumonisin B_1 are transformed values: [T; y'=ln(x+1)]; and not transformed (N) values.

Table 3. Effect of different treatments on the maize fungal ear rot incidence and severity, European Corn Borer (ECB) incidence and severity, ergosterol content and fumonisin (FBs) contamination. Field experiment has been conducted in North West Italy in the 2015 growing season.

Factor	Source of variation	ECB incidence [b]	ECB severity [c]	Fungal ear rot incidence [d]	Fungal ear rot severity [e]	Ergosterol [f]		FBs [g]	
		(%)	(%)	(%)	(%)	T	N ($ng\ g^{-1}$)	T	N ($\mu g\ kg^{-1}$)
Insecticide	Untreated control	90.0 a	15.6 a	86.9 a	7.9 a	2.9 a	28.8	7.6 a	3768
	Lambda-Cyhalothrin + Chlorantraniliprole	43.3 b	3.7 b	41.9 b	2.1 b	2.2 b	10.9	6.7 b	1427
	P (F)	< 0.001	< 0.001	< 0.001	< 0.001	0.009		0.041	
	SEM [a]	33.0	8.3	31.5	4.2	0.5		0.6	
Timing of antifungal application	Flowering stage	65.8 a	10.0 a	63.5 a	5.0 a	2.3 a	17.2	7.0 a	2679
	Milk stage	67.5 a	9.3 a	65.3 a	5.0 a	2.8 a	22.6	7.3 a	2516
	P (F)	0.738	0.654	0.803	0.971	0.054		0.357	
	SEM [a]	1.1	0.4	0.8	0.02	0.3		0.3	
Antifungal	Untreated control	66.6 a	9.0 a	64.9 a	4.9 a	2.6 ab	20.9	7.6 a	3028
	Spirulina	67.3 a	11.2 a	67.2 a	4.9 a	3.1 a	22.2	7.2 a	2204
	Nannochloropsis	67.8 a	9.2 a	65.4 a	5.2 a	2.8 a	27.7	6.9 a	2571
	Prothioconazole + Tebuconazole	64.8 a	9.0 a	60.2 a	5.0 a	1.8 b	8.8	7.0 a	2587
	P (F)	0.448	0.790	0.598	0.626	0.005		0.871	
	SEM [a]	7.4	1.4	6.3	1.2	1.0		0.4	
Insecticide X Timing	*P* (F)	0.795	0.925	0.812	0.798	0.996		0.558	
Insecticide X Antifungal	*P* (F)	0.645	0.096	0.145	0.433	0.617		0.931	
Timing X Antifungal	*P* (F)	0.903	0.990	0.775	0.972	0.134		0.258	
Insecticide X Timing X Antifungal	*P* (F)	0.813	0.984	0.945	0.926	0.048		0.245	

Reported data for insecticide and timing of antifungal application are the average of 24 replications (4 antifungal X 2 timing or insecticide X 3 repetitions), while data for antifungal are the average of 12 replications (2 insecticide X 2 timing X 3 repetitions). Means followed by different letters are significantly different (the level of significance is shown in the table). [a] SEM = standard error of mean. [b] ECB incidence was calculated as the percentage of ears with symptoms, based on 3 replications of 10 ears each. [c] ECB severity was calculated as the mean percentage

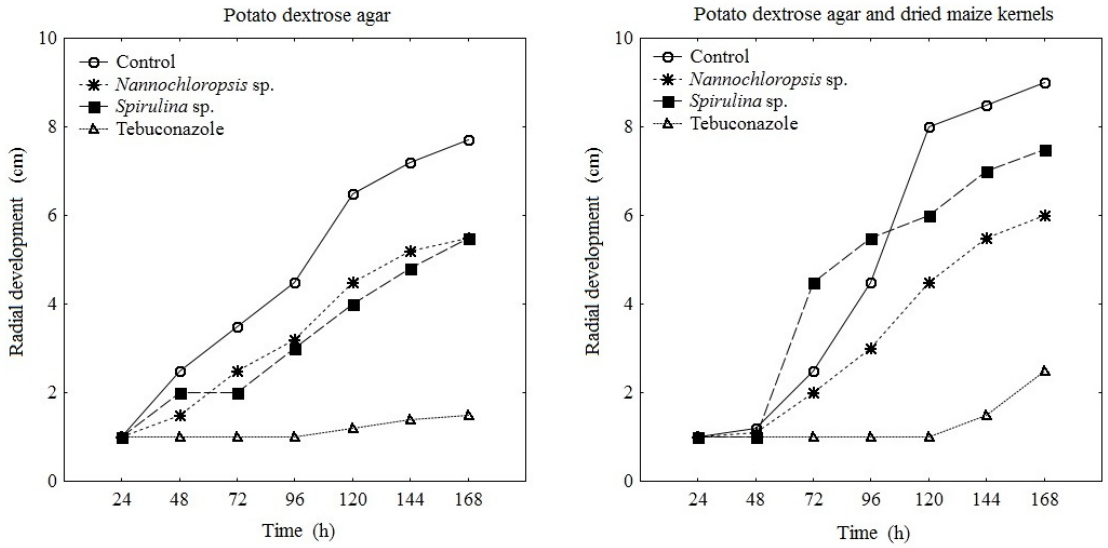
of kernels with symptoms per ear, based on 3 replications of 10 ears each. [d] Fungal ear rot incidence was calculated as the percentage of ears with symptoms, based on 3 replications of 10 ears each. [e] Fungal ear rot was calculated as the mean percentage of kernels with symptoms per ear, based on 3 replications of 10 ears each. [f] The ergosterol content means reported are transformed [T; y'= ln(x+1)] and not transformed (N) values. [g] The FBs (sum of Fumonisin B_1 and B_2) contamination means reported are transformed [T; y'=ln(x+1)] and not transformed (N) values.

Table 4. Dose efficiency data of the MPE (*Nannochloropsis* sp., *Spirulina* sp.) and fungicide applied in *in vitro*.

Antifungal compound	Dose ($\mu g\ g^{-1}$)	Average inhibition efficiency (%)[a]
MPE from *Nannochloropsis* sp. ($45.2\ \mu g\ mL^{-1}$)	4.8	95
Spirulina sp. ($40.0\ \mu g\ mL^{-1}$)	4.2	95
Fungicide ($600\ \mu g\ mL^{-1}$) [b]	63.2	64

[a] Value estimated by the average of all reductions in fumonisin concentration found for each extract (n = 6). [b] Tebuconazole Pestanal®

Figure 1.


Potato dextrose agar
Control
Nannochloropsis sp.
Spirulina sp.
Tebuconazole
Radial development (cm)
Time (h)
0
2
4
6
8
10
24
48
72
96
120
144
168
Potato dextrose agar and dried maize kernels
Control
Nannochloropsis sp.
Spirulina sp.
Tebuconazole
Radial development (cm)
Time (h)