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

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

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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.

45 **INTRODUCTION**

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

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

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

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

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

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

86 Microalgae are a diverse group within prokaryotes and eukaryotes and produce a
87 wide variety of commercially interesting products such as lipids, oils, sugars, pigments,
88 and many other bioactive compounds. In addition, some microalgae, such as *Spirulina*
89 sp. or *Nannochloropsis* sp., are rich sources of natural antioxidants such as phenolic
90 compounds and carotenoids.[19] The antifungal activity against strains of the *Fusarium*
91 complex (*F. graminearum* and *F. meridionale*) by compounds extracted from *Spirulina*
92 sp. has recently been shown,[17,20] but further studies are needed to optimize their
93 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]

119

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

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

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

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

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

140 The control treatment was conducted with sterile water instead of MPE. An
141 experiment with tebuconazole standard (Pestanal®) was also performed at 600 μg mL^{-1}.
142 Petri dishes with the inoculum were incubated at 25°C and a light/dark photoperiod of
143 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%

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

175

176 **Application of MPE and fungicides in maize fields**

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

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

188 The compared treatments were factorial combinations of:

189 ▪ two different strategies to control ECB larvae:

190 - insecticide application at the milk stage (growth stage, GS75) [27]: lambda-
191 cyhalothrin (pyrethroid) + chlorantraniliprole (diamide) mixture [Ampligo®,
192 formulation: suspension concentrate, capsule suspension, Syngenta Crop

193 Protection S.p.A., Italy, applied at 0.015 and 0.030 kg of active ingredient
194 (AI) ha^{-1}, respectively];
195 - untreated control.
196 ▪ four compounds with antifungal activity applied to maize ears:
197 - untreated control, sprayed with sterile water;
198 - MPE of the microalgae *Nannochloropsis* sp. (0.070 kg ha^{-1});
199 - MPE of the microalgae *Spirulina* sp. (0.062 kg ha^{-1});
200 - fungicide mixture of prothioconazole + tebuconazole [Prosaro®, Bayer,
201 Italy, emulsifiable concentrate formulation (EC), applied at 0.125 kg of each
202 AI ha^{-1}].
203 ▪ two application timings at different maize growth stages:
204 - at maize flowering (GS 65, July 3rd)
205 - at maize milk stage (GS 75, July 20th), according to the optimum timing for
206 insecticide application.[3]
207 Application of the MPE was carried out at concentrations around 40 µg mL^{-1},
208 corresponding to the previously estimated EC_{50} value. Application of antifungal
209 compounds was carried out by spraying 10 mL of solution for each primary ear, using a
210 hand sprayer. The treatments were assigned to experimental units, using a completely
211 randomized block design with three replicates. Each plot consisted of 10 consecutive
212 plants presenting the same developing stage, separated by three untreated buffer rows
213 on either side; inter-row distance was 0.75 m.
214 All ears were collected by hand from each plot at the end of the maturity
215 (September 1st) at a grain moisture content between 23 and 27%, visually inspected for
216 insect injuries and disease symptoms, and shelled using an electric sheller. The entire
217 amount of grains (approximately 3 kg) collected from each plot was dried at 60°C for

218 three days and ground using a ZM 200 Ultra Centrifugal Mill (Retsch GmbH, Haan,
219 Germany). The ground samples were stored at -25°C until mycotoxin analysis.
220
221 *Entomological and mycological measurements*
222 The ECB damage incidence was calculated as the percentage of ears per plots with
223 kernel injuries or apical and basal tunnels in the cob due to larval activity. The ECB
224 damage severity was calculated as the percentage of kernels per ear with injuries due to
225 larval activity. A scale of 1 to 7 was used, in which each numerical value corresponded
226 to a percentage interval of surfaces exhibiting visible kernel damage due to larval
227 activity, according to the following schedule: 1 = no injuries, 2 = 1-5%, 3 = 6-10%, 4 =
228 11-20 %, 5 = 21-35%, 6 = 36-60%, 7 > 60%.[3]
229 Fungal ear rot incidence was calculated as percentage of ears per plot with
230 symptoms, while fungal ear rot severity was calculated as kernel percentage per ear with
231 symptoms. A scale of 1 to 7 was used, in which each numerical value corresponded to a
232 percentage interval of surfaces exhibiting visible symptoms of the disease, according to
233 the following schedule: 1 = no symptoms, 2 = 1-3 %, 3 = 4-10%, 4 = 11-25 %, 5 = 26-
234 50%, 6 = 51-75%, 7 > 75%.[3] The ECB damage severity and ear rot severity scores were
235 converted to percentages of ears exhibiting symptoms, and each score was replaced with
236 the mid-point of the interval.
237
238 *Fumonisin B_1 and B_2 concentrations in maize from the field experiment*
239 Concentrations of FB_1 and FB_2 in maize samples from field cultivation were determined
240 using 50 g of ground sample with 100 mL of a methanol:water solution (80:20 v/v),
241 shaken for 20 min. After filtration through Whatman® n°1 paper, the samples were
242 diluted with phosphate-buffered saline (PBS) for subsequent purification, using

243 immunoaffinity columns FUMtest (VICAM®). For this purpose, we used a 1 drop s^{-1}
244 flow with a vacuum system; 5 mL of the extract were eluted through the column, after
245 2.5 mL of PBS, and the analyte was recovered with 2 mL of pure methanol and injected
246 into the HPLC-MS/MS system, equipped with a Varian 212-LC chromatographic pump,
247 a Varian column, Pursuit 5 C18 50 x 2.1 mm, a ProStar 410 autosampler, and a triple
248 quadrupole mass spectrometer 310-MS.

249 The chromatographic run had a duration of 15 min (t_R FB_1 = 4.9 min;
250 t_R FB_2 = 5.6 min), with acetonitrile and water acidified with acetic acid 0.1% as the
251 mobile phase. The FBs were identified in a triple quadrupole mass spectrometer with
252 the electrospray ionization source in the positive ion mode. The protonated FB_1
253 (722 m z^{-1}) molecule was fragmented into its product ions at 352 m z^{-1} (used for
254 identification) and 334 m z^{-1} (used for quantification). For FB_2 (706 m z^{-1}), we used
255 318 m z^{-1} (used for identification) and 336 m z^{-1} (used for quantification).

256

257 **Ergosterol content determination**

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

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

268 operated at 0.8 mL min^{-1} at 30°C, using a 100% methanol mobile phase for 17 min with
269 detection at 282 nm. The ergosterol content was estimated by an ergosterol standard
270 calibration curve with concentrations ranging from 0.05 to 2.0 µg mL^{-1}.[29]
271

272 **Maize kernel phenolic acids**

273 The phenolic acid content[23] of maize kernels at harvest was determined to check if the
274 presence of these compounds in the grain might inhibit fungal growth and to compare
275 this phenolic acid profile with the MPE one.
276

277 **Statistical analysis**

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

293

294 **RESULTS**

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

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

313 **Table 1.**

314 Figure 1 shows the development of the fungal halo when submitted to the
315 different *in vitro* treatments. Tebuconazole had a better inhibition effect (81% after
316 168 h of incubation) in the experiment with agar, while both MPE reduced the halo
317 development by 29% at the end of the period. Tebuconazole presented a greater

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

322 **Figure 1.**

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

327 **Table 2.**

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

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

336 **Table 3.**

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

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

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

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

349

350

351 **DISCUSSION**

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

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

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

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

378 Beekrum et al.[31] reported the effect of ferulic acid on the growth and FB
379 production of *F. verticillioides*. These authors compared several natural phenolic
380 compounds such as chlorophorin, iroko and maakianin, caffeic acid, ferulic acid,
381 benzoic acid, and vanillic acid, and observed that benzoic acid and ferulic acid had no
382 effect on fungal growth, while, with the exception of benzoic acid, all the other
383 compounds reduced FB_1 production by 88–94%. Although 1 $\mu g\ mL^{-1}$ of ferulic acid
384 (5.15×10^{-6} mol L^{-1}) did not inhibit *F. verticillioides* growth, it reduced FB_1 production
385 by 90%.

386 Ferrochio et al.[32] verified that the application of ferulic acid at concentrations
387 ≥ 0.02 mol L^{-1} could be an effective post-harvest strategy to control the growth of *F.*
388 *verticillioides* and *F. proliferatum* and to reduce FB production.

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

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

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

398 **Table 4.**

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

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

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

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

417 timings for FB producers. *Fusarium verticillioides* and *F. proliferatum* could infect
418 maize kernels through silks and through kernel damage caused by insects. In temperate
419 maize cultivation areas, ECB injuries are most frequently associated pathway for *F.*
420 *verticillioides* infection, and the ECB activity could concern three to four months
421 between the beginning of ripening and harvest.[3] Moreover, a systemic infection of
422 plants is also possible, since the fungus could be present in seedlings, leaf sheaths, and
423 stalk tissue, without causing noticeable symptoms. The absence of a precise and defined
424 infection event makes it more difficult to identify the best timing of application of a
425 substance with fungicidal activity, in particular when its persistence is limited.
426
427 In conclusion, this study underlines the difficulties to apply direct strategy to
428 control the development of FB producers in maize production; unlike in other crops
429 such as wheat. Thus, at present, the adequate use of preventive agricultural practices and
430 the control of insect injuries, according to an integrated approach, still remains the most
431 effective strategy to minimize the risk for FB contamination in maize. However, further
432 studies are needed to evaluate the role of MPE as part of the strategy to prevent FB
433 contamination. The optimization of microalgae cultivation may favor the MPE
434 production, increasing the availability of these compounds in the total biomass of these
435 organisms. Another alternative for future studies is the encapsulation of phenolic
436 compounds into carrier systems, such as liposomes. This strategy can prevent their
437 degradation by metabolic processes, preserving and prolonging their antifungal and
438 antimycotoxigenic properties, besides it may facilitate their penetration into different
439 plant tissues.
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450

451 **NOTES**

452 The authors declare no competing financial interest.

453

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581 **Figure captions**

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583 **Fig. 1.** Radial development of the mycelial discs of the *F. verticillioides* submitted to
584 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. (µg g^{-1})	*Nannochloropsis* sp. (µg g^{-1})	Maize kernels [a] (µg 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 (μg 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.