# Impact of microalgal phenolic extracts on the control of *Fusarium graminearum* and deoxynivalenol contamination in wheat fields

# *Running title:* Microalgal phenolic extracts to control fungal contamination in wheat fields

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# ABSTRACT

In this study phenolic extracts of *Nannochloropsis* sp. and *Spirulina* sp. were tested in field experiments for their ability to control *Fusarium graminearum* development and limit deoxynivalenol (DON) contamination, in comparison with synthetic fungicides. Prothioconazole and prothioconazole+tebuconazole application resulted in control of *Fusarium* Head Blight (FHB) and foliar disease, leading to a significant increase in grain yield (+13%) and a reduction in DON content (-46%) compared to the untreated control. The application of MPE at wheat flowering reduced the severity of FHB compared with the control (-35% for *Spirulina* and -39% for *Nannochloropsis*). However, the MPE did not significantly enhance the grain yield and reduce the DON content in comparison to the control. In view of these results, the use of *Nannochloropsis* sp. and *Spirulina* sp. phenolic extracts in wheat fields as alternatives to conventional fungicides require to find solutions to empower their persistence and efficacy.

**Keywords:** *Giberella*; *Fusarium*; Mycotoxins; *Nannochloropsis* sp.; *Spirulina* sp.; Biological control.

# Introduction

*Fusarium* species that infect cereal crops are pathogenic throughout the whole lifecycle of the plants. *Fusarium* head blight (FHB) causes losses in crop competitiveness, as it has an impact on grain yield due to total or partial early senescence of the spikes. It can also affect grain safety and quality, due to the occurrence of mycotoxins in the grain, which limit the possibility of marketing affected products (1).

Several *Fusarium* species cause this disease in wheat. Among them, *Gibberella zeae* (anamorph *Fusarium graminearum*), which is a predominant and aggressive species worldwide and can cause the accumulation of toxic metabolites in the kernels, such as trichothecenes (2). These are harmful for humans and livestock.

The most common mycotoxin in wheat is deoxynivalenol (DON), which acts as a potent inhibitor of protein synthesis in eukaryotes (3). In humans, consumption of food contaminated with DON causes decreased leukocyte rates, nausea, vomiting, anorexia and convulsions (4,5). Therefore, strategies are employed during cultivation to manage fungal incidence and mitigate mycotoxin contamination.

Some effective measures including crop rotation, soil tillage and selection of resistant wheat lines have been used to control DON contamination during grain production (6). Among these strategies, the application of synthetic fungicides is relatively successful in controlling the development of the pathogen agent and mycotoxin production (7). Triazoles are the main class of fungicides registered for the control of FHB in wheat and are the most effective active substances applied during wheat anthesis to minimize DON contamination. However, care should be taken with regard to their use due to the risk of resistance development to these compounds. Total inefficacy of a fungicide can be caused by the polygenic resistances (e.g. DMI fungicides, triazoles), usually characterized by the accumulation of different mechanisms of adaptation over an extended period time. Fungal mutants selected in the laboratory for resistance to sterol biosynthesis inhibitors also showed resistance to other sterol biosynthesis inhibitors, a phenomenon termed cross resistance. Besides that, the longer and the more often an active ingredient is used, the more likely is the selection of partially or totally resistant microbial strains (8). Moreover, since EU policy is directed towards significant reductions in pesticide use in the short to medium term (according to Directive 2009/128), several triazole compounds may become unavailable in the near future because of their potential health risks (9).

This situation has encouraged a search for natural compounds that can protect wheat from *Fusarium* infection (10,11). Several studies have documented antifungal and antimycotoxigenic effects of phenolic compounds obtained from microalgae (12-17). However, the mode of action in inhibiting microorganism development and mycotoxin production, as well as the quantities required for effective control, call for further studies.

Many experiments have been conducted *in vitro* to estimate the antifungal activity of natural compounds (12-19), but there is little information regarding their viability for field application to control FHB and mycotoxin contamination and a lack of protocols for their use in the field. However, the application of synthetic fungicide at wheat flowering is a key agricultural practices not only to minimize the sanitary risk associated to FHB but also to control foliar disease, prolong the crop greenness during ripening and finally to enhance the grain yield (7). Thus the efficacy of MPE in controlling other wheat diseases as well as their negative impacts on grain yield should also be considered, in order to have a more applicative evaluation of the MPE in field in comparison to the conventional fungicide.

Septoria tritici blotch (STB), caused by the ascomycete *Mycosphaerella graminicola* (asexual stage *Zymoseptoria tritici*), is another important disease of wheat in temperate areas, characterized by necrotic lesions on leaves and stems that develop after infected cells collapse. STB infection can occur on leaves during the whole wheat life cycle, but its effect on the loss of productivity and grain quality is more important if environmental conditions, such as humidity and temperature, are favorable for fungal growth during the period from anthesis to grain formation (20,21) reducing the photosynthate available for grain-filling.

The aim of this study was to investigate the possibility of applying phenolic acid extracts from different microalgal genera to control FHB in wheat and thus minimize DON contamination. The efficacy of different natural compounds compared with the two selected synthetic fungicides was analyzed in open field experiments, where different growing seasons and agronomic conditions were taken into account.

# Materials and methods

# *Microalgal biomass production*

A sample of *Spirulina* sp. (“Laboratório de Engenharia Bioquímica”, LEB-18) was supplied by the Biochemical Engineering Laboratory at the Universidade Federal do Rio Grande (FURG), located in Rio Grande, RS, Brazil. The biomass was cultivated under agitation in fiberglass tanks with water from a lagoon called Lagoa Mangueira (33° 30’ 13” S and 53° 08’ 59” W), supplemented with 20% (v/v) Zarrouk medium, containing (g L-1): NaHCO3, 16.8; NaNO3, 2.5; K2HPO4, 0.5; K2SO4, 1.0; NaCl, 1.0; MgSO4·7H2O, 0.2; CaCl2, 0.04; FeSO4·7H2O, 0.01; EDTA, 0.08 and micronutrients. The microalgal biomass was separated by filtration after reaching a concentration of 1 g L-1 (22).

 Biomass of *Nannochloropsis* sp. (NANN-OCUL-1) was cultivated in the Phytoplankton and Marine Microorganism Laboratory at FURG, in f/2 medium, with nitrogen in the final concentration of 8.8·10-4 mol L-1 (1 mL L-1 of stock solutions with NaNO3 75 g L-1 and NH4Cl 47.1 g L-1) (23), at salinity 28 (Practical Salinity Units), at 20°C, with illumination at 40 μmol m-2 s-1 and a 12 h light/dark photoperiod (24).

 The microalgal biomass was dried in trays at 50°C for 5 h, ground up to a 32 mesh, vacuum packaged and stored frozen at -6°C until determinations and experiments were carried out.

***Phenolic acids determination***

The methanol-soluble phenolic compounds were extracted from microalgae with methanol at 1:6.6 w/v. The extracts were evaporated, dissolved in sterile distilled water, clarified (with barium hydroxide 0.1 M and zinc sulfate 5%) and vacuum filtered through a sterile membrane with a pore size of 0.45 µm (12,14,16,25)*.*

Identification of the phenolic acids in the extracts was performed using reference standards from Sigma-Aldrich, namely: caffeic, chlorogenic, p-coumaric, ferulic, gallic, p-hydroxybenzoic, protocatechuic, and syringic acids and vanillin. Aliquots of the extracts were previously dried under a nitrogen flow and dissolved in methanol:water (1:1). These phenolic acid solutions were injected into a liquid chromatography apparatus (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 was performed according toScaglioni et al. (25).

 The phenolic acid content in wheat grains was determined after harvesting by the same procedure, in order to see if the MPE application can lead to a different composition of endogenous phenolic acids of grain.

## ***MPE and fungicides application in wheat fields***

Field experiments were carried out from 2014 to 2016 in two growing seasons at Buriasco (TO), north-west Italy (44° 54’ N, 7° 24’ E; altitude 262 m.), in a sandy-medium textured soil, classified as Typic Udifluvents (USDA classification), under naturally-infected field conditions.

Two adjacent experimental fields of winter wheat with high and low agronomic risks concerning FHB infection and DON contamination (related to the presence of previous crop residues on the soil), were prepared each year. Each year, the previous crop and the pre-previous crop were maize, according to a crop sequence maize-maize-wheat ordinary applied in the growing areas. The agronomic conditions compared were related to the tillage method:

- minimum tillage with double disk harrowing (15 cm depth), with the previous crop residues left on the soil surface, to maximize pathogen inoculum availability;

- autumn ploughing (30 cm depth) which incorporated the maize debris into the soil, followed by disk harrowing to prepare a proper seedbed.

In both agronomic conditions, different fungicides and natural extracts with antifungal activity were applied at early flowering, [growth stage (GS) 62], (26):

 an untreated control;

 MPE from *Spirulina* sp., applied at 0.016 kg ha-1;

 MPE from *Nannochloropsis* sp., applied at 0.016 kg ha-1;

 an azole fungicide, tebuconazole [Folicur® WG, Bayer, Italy, water dispersible granules formulation (WG), applied at 0.200 kg of active ingredient (AI) ha-1];

 an azole fungicide, prothioconazole [Proline®, Bayer, Italy, emulsifiable concentrate formulation (EC), applied at 0.200 kg of AI ha-1];

 an azole fungicide mixture containing prothioconazole and tebuconazole [Prosaro®, Bayer, Italy, emulsifiable concentrate formulation (EC), applied at 0.100 kg of each AI ha-1];

The MPE rate was estimated through linear regressions relating the concentrations of phenolic compounds found in different MPE volumes and the respective percentages of *Fusarium* halo inhibitions (y = 1.481*х* and y = 1.170*x* for *Spirulina* sp. and *Nannochloropsis* sp., respectively; data not shown).

The fungicides as well as the MPE were applied with a four-nozzle precision sprayer (T-Jeet 110/04) using a fine mist at a slow walk to ensure effective coverage (1 L for 10 m2). The delivery pressure at the nozzle was 324 kPa.

The treatments for each field condition were assigned to experimental units using a completely randomized block design with three replicates. The plot size was 6 x 2 m.

The normal agronomic techniques of the growing area were applied. Briefly, wheat cultivar used was in both growing season Altamira, medium susceptibility to FHB and *Septoria tritici* blotch disease (Limagrain Italia S.p.A., Busseto, PR, Italy). Planting was conducted in 12 cm wide rows at a seeding rate of 450 seeds m-2 in October 29 and 23, for 2014 and 2015, respectively. Weed control was conducted with isoproturon and diflufenican at wheat tillering (GS 31). A total of 150 kg N ha-1 was applied to the plots as a granular ammonium nitrate fertilizer, and split between GS 23 and 32. Harvest was carried out in July 1 and 5 for 2015 and 2016, respectively.

***FHB symptoms***

The incidence and severity of FHB was recorded for each plot by performing visual evaluations of the disease on the grains at the soft dough stage (GS 85). The incidence was calculated as the percentage of ears with symptoms of the disease, using 200 ears, randomly selected. The severity was calculated as the percentage of spikelet per ear with symptoms, being estimated on a scale from 0 to 7. Each numerical value corresponds to a percentage range of surfaces that exhibit visible symptoms of the disease (27), following the scheme: 1 = 0 – 5%, 2 = 6 – 15%, 3 = 16 – 30%; 4 = 31 – 50%, 5 = 51 – 75%, 6 = 76 – 90%, 7 = 91 – 100%.

**Septoria tritici *blotch (STB) symptoms***

For each plot, the STB severity was evaluated on the leaves at the soft dough stage (GS 85). Leaf disease was classified into 6 classes (0 = 0%; 1 = 2%; 2 = 5%; 3 = 10%; 4 = 25%; 5 = 50%; 6 = > 50%) according to the visible symptoms (28). Fifteen flag leaves and 15 penultimate leaves, randomly selected, were used.

***Canopy greenness***

A hand-held optical sensing device, GreenSeekerTM® (Trimble©, Sunnyvale, California, USA), was used to measure the normalized difference vegetation index (NDVI) from flowering to the end of grain filling stage, in all plots. This method is not destructive and can be conducted in real time. The Green SeekerTM® device can be used to monitor active radiation to obtain reflectance data that is independent of solar illumination. It has its own consistent light emission source, photodiode detectors and interference filters for red and near infrared wavelengths at the 671 ± 6 nm and 780 ± 6 nm spectral bands, respectively (28); it provides the NDVI, which is calculated as follows (30):

$NDVI=\frac{RNIR-RRed}{RNIR+RRed}$ (1)

where RNIR is the near infrared radiation reflectance and RRed is the visible red radiation reflectance. The instrument was held approximately 80 cm above the canopy of each plot and its effective spatial resolution was 2 m2.

The NDVI values were proportional to the crop biomass and greenness. Since, in the present experiment, the measurements were conducted starting from wheat flowering, at which time the crop biomass reaches a maximum and remains almost stable until harvest, the NDVI values are proportional to the amount of total chlorophyll in the crop canopy and can be considered descriptors of the crop senescence process.

The Area Under Canopy Greenness Curve (AUCGC) was calculated for each treatment starting from the NDVI measurement for each observation date and using the following formula:

$AUCGC= \sum\_{i}^{n-1}\left\{\left[\left(R\_{i}+R\_{i+1}\right)/2\right] \left(t\_{i+1}- t\_{i}\right)\right\}$ (2)

where R is the NDVI value, t is the time of observation and n is the number of observations.

***Production and composition parameters of wheat grain***

The grain yields were obtained by harvesting the whole plot with a Walter Wintersteiger cereal plot combine-harvester. Grain moisture was analyzed using a Dickey-John GAC2100 grain analyzer (Auburn, IL, USA). The grain yield results were adjusted to a 13% moisture content. The harvested grains were mixed thoroughly and 4 kg grain samples were taken from each plot and were completely ground using a Retsch ZM 200 (Retsch GmbH, Haan, Germany), fitted with a 1 mm aperture sieve and the resulting wholemeal was analyzed for the DON content

***DON determination in field experiments samples***

A high-performance liquid chromatography apparatus coupled with a mass detector (HPLC-MS-MS) with a linearity range of 20 – 2000 μg kg-1 was used for the experimental field samples.

Twenty-five grams of ground sample was extracted with 100 mL of water for 30 min and the whole extract was collected after a filtration step. Columns based on immunoaffinity antibodies were used (DON test™ WB Columns VICAM) to clean up the extracts. First, each column was conditioned with 1 mL of deionized water, afterwards loaded with 1 mL of the extract from each sample at a rate of 1–2 drops s-1. The columns were washed with 2.5 mL of deionized water and finally the DON was eluted from the column with 2 mL of methanol.

Quantification was performed by injecting 10 μL of purified eluate into the HPLC-MS-MS system, which consisted of a Varian 212-LC chromatographic pump and a 310-LC-MS TQ mass spectrometer. A Varian Polaris C18-A reverse phase analytical column (100 x 2.00 mm, 3 μm) was used and the mobile phase was a mixture of methanol and water acidified with acetic acid 0.1% at a flow rate of 0.2 mL min-1. The chromatographic run had a duration of 13 min (tR DON = 4.1 min). With the use of a triple quadrupole, the DON was identified with the electrospray ionization source in the negative ion mode. The deprotonated DON (295 m z-1) molecule was fragmented into its product ions at 138 m z-1 (used for identification) and 265 m z-1 (used for quantification) (31).

***Statistical analysis***

All of the recorded parameters were compared using ANOVA, in which the natural compound/fungicide applications and the soil tillage conditions were set as independent variables, while the growing season was a random factor. The normal distribution of residuals 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 the treatment means, using a 0.05 probability level. The ergosterol and DON contents were transformed using the y’ = ln(x+1) equation to normalize the residuals. The statistical package SPSS for Windows, Version 24.0 (SPSS Inc., Chicago) was used for the statistical analysis.

# Results

***Characterization of microalgal phenolic extracts (MPE)***

The phenolic fractions from both microalgae were quantified by HPLC-UV and confirmed by LC-ESI-MS/MS, the results indicated the predominance of chlorogenic acid (Table 1). However, although chlorogenic was the main phenolic extract, the phenolic acids present in the biomass of *Nannochloropsis* sp. were more diverse, comprising seven different compounds, whereas only four different phenolic acids were identified in *Spirulina* sp.

**Table 1.**

No significant differences were observed between the compared treatments for phenolic concentrations in wheat grains neither for MPE nor for the fungicide treatments, thus their average content in all collected wheat samples was reported in Table 1. This is promise behavior because showed that the treatments with the MPE or synthetic fungicides did not affect the secondary metabolism of wheat.

***Field experiments***

The effects of different antifungal treatments (synthetic or natural) applied at wheat flowering (GS 62) were evaluated at the field scale under different tillage conditions during two growing seasons (Table 2).

**Table 2.**

 The grain yield was slightly but significantly increased using ploughing as the tillage practice, compared with minimum tillage. A clear and strong increasing grain yield effect was observed with the application of fungicide at wheat flowering (GS 62). The most effective active ingredient was prothioconazole, either alone or in combination with tebuconazole. On the other hand, the MPE did not significantly enhance the grain yield compared to the control. The lack of a positive significant effect of these natural compounds on grain yield could be related to their lesser degree of control against the main observed foliar disease, *Septoria tritici* blotch, in comparison with the chemical fungicides. The results of canopy greenness during crop maturation, expressed as AUCGC, revealed that only the triazole fungicides were significantly able to protect the crop. The severity of FHB was significantly reduced by the natural extracts, and this effect was statistically comparable with that of tebuconazole. However, the prothioconazole, alone or in combination with tebuconazole, showed the strongest effect in the reduction of FHB.

 The microalgal extracts did not significantly reduce the DON mycotoxin content in comparison to the control. Similarly, but with a reduction of 25%, tebuconazole was also not able to significantly reduce DON contamination. On the other hand, prothioconazole applied alone or in combination with tebuconazole significantly reduced the DON content by 43% and 50% respectively compared with the control.

 None of the interactions between the treatments and tillage practices or treatments and years were significant.

**Discussion**

To date, some studies have focused on antioxidant secondary metabolites (phenolic compounds, carotenoids and tocopherols) in cereals as potential causal agents of varietal genetic resistance to *Fusarium* sp. and mycotoxin accumulation (32,33). Phenolic compounds derive from the phenylpropanoid pathway and are divided into two groups: flavonoid phenylpropanoids (flavones, flavonols, flavanones, flavanols, anthocyanins and chalcones) and non-flavonoid phenylpropanoids (stilbenes, lignans, and phenolic acids) (32). To the best of our knowledge this is the first study to evaluate the fungicidal capacity and the efficiency of MPE applied in the field for direct control of the two fungal pathogen diseases FHB and STB of wheat and DON contamination.

 The identification of different phenolic acids in the biomass of microalgae is important in view of other studies showing that these compounds present toxicity towards *Fusarium* species, such as *F. graminearum*, *F. verticillioides*, *F. solani*, *F. culmorum*. The following compounds have been investigated: chlorogenic acid (34); gallic acid (32); protocatechuic acid (35); hydroxybenzoic acid (36); syringic acid (35,36); vanillic acid (35); and ferulic acid (35,36). Taking into account the results obtained in the present study the MPE were mainly composed by chlorogenic acid, which is recognized as a free antioxidant compound that could interfere with *Fusarium* spp. development (32). Given that the antioxidant activity and the inhibitory activity of hydrolases and oxide reductases from the phenolic extracts containing mainly chlorogenic acid was previously proven (25), this may constitute a natural defense mechanism of microalgae against fungal attacks, that fact suggest that the extract application against fungal contamination in the fields or along the grain beneficiation chain may be viable.

Studies indicate a down-regulation of the genes involved in DON biosynthesis by *F. graminearum* when ferulic acid is added to *in vitro* culture medium, which is in accordance with a transcriptional control exerted by phenolic acids (32,37). A few additional studies investigated the impact of natural compounds on diverse mycotoxins production but they led to opposite results, depending on the mycotoxin targeted. For example, while capsanthin (a major carotenoid in paprika) has been shown by Masood et al. (38) to inhibit aflatoxin production, another study demonstrated its lack of inhibitory effect on ochratoxin production (39). Concerning the phenolic extract of *Spirulina* (containing gallic, caffeic, salicylic and trans-cinnamic acids), a previous study showed an average inhibition of 73% on the NIV and DON production from four *Fusarium* isolates (*F. graminearum* and *F. meridionale*), while zearalenone production by the same isolates was not affected by the phenolic treatments (14). The varying inhibitory effect on different mycotoxins is due to the fact that each metabolic pathway requires the expression of carrier proteins and a network of regulatory genes. Trichothecene synthesis comprises sesquiterpene ring cyclization, catalyzed by the enzyme tricodiene synthase, followed by eight oxygenations and four esterifications. This sequence leads to the formation of basic structures such as DON, NIV and its acetylates (40).

 In *in vitro* experiments, using the medium containing wheat grains as substrate, to simulate the nutrient supply for *F. graminearum* under real conditions, the microalgae phenolic extracts present ability to inhibit trichothecenes (deoxynivalenol; nivalenol; 3-acetyl-deoxynivalenol; and 15-acetyl-deosynivalenol), the mean inhibition efficiency of trichothecenes production was 82 and 68% by the treatments with the phenolic extracts of *Nannochloropsis* sp. and *Spirulina* sp., respectively (16).

In the present study, at field scale, the microalgal phenolic extracts were able to reduce FHB symptoms compared to the untreated control, but their efficacy in controlling DON content was significantly lower than the one of the most efficient fungicides applied against FHB, the natural origin of the extracts can be make these more susceptible on field conditions, causing the reduction of their bioactivity.

Previously, similar behavior was found when, even though the MPE were capable of inhibiting fumonisin production *in vitro* more efficiently than tebuconazole, fumonisins in maize fields were not significantly controlled by either fungicide or MPE application (17).

 Fungicides containing triazoles as active ingredients are the most effective plant protection agents against FHB pathogens, they are systemic site-specific fungicides and therefore mainly used today in order to obtain reliable disease management (41). Historically, tebuconazole was one of the first active ingredients commercialized due to its high efficiency as a broad-spectrum fungicide. One of the more recent and effective active ingredients is prothioconazole (42). Similar to the results obtained in the present study, a multivariate meta-analysis (43) revealed that a mixture of prothioconazole + tebuconazole (applied at GS 62) exhibited the highest mean percentage control of FHB severity, followed by prothioconazole, with a mean reduction in DON content of 42%. However, quantitative azole resistance has been shown to develop in fungal pathogens, including *F. graminearum*, the main causal agent of FHB (43). Becher et al. (44) reported that an *in vitro* experiment tebuconazole-adapted *F. graminearum* isolates produced higher levels of nivalenol in grains. The resistance to triazoles probably occurred over time as a slow shift resulting in a decreased sensitivity to the triazole mode of action as de-methylation inhibitors (DMI) of sterol biosynthesis. Besides that, Scaglioni et al. (16) also demonstrated that the application of the fungicide tebuconazole resulted in an increase of DON concentration in the fungal biomass produced in *in vitro* study, it may be assumed that in the presence of certain concentrations of triazole fungicides, the fungal strains respond to this stress by increasing the production of secondary metabolites, such as the trichothecenes.

The MPE natural compounds did not protect the crop from other diseases, such as *Septoria tritici* blotch, resulting in a lower capacity to enhance canopy greenness during ripening and therefore subsequently achieved a lower grain yield. The lower efficacy of the MPE in the field indicates possible problems related to the persistence of these natural compounds, as once extracted, they are exposed to environmental conditions and can be quickly degraded. In particular, in order to protect against foliar disease, it is necessary to maintain activity of MPE for several weeks during maturation. Among the possible solutions for enhancing the persistence of phenolic acids and their efficacy in the field, encapsulating these extracts could be a promising approach to enhance the persistence of phenolic acids and their efficacy in field, Pagnussatt et al. (15) conclude that the encapsulated phenols do not suffer oxidative or hydrolytic degradation and thus remain stable and active for a longer period (compared to the compounds in the “free form”), a fact that makes this system an interesting option to control the release of the active compound.

Moreover, using the extracts in combination with chemical active substances might enable a reduction in the applied dose of synthetic fungicides. The option of combining MPE with conventional fungicides at lower rate could lead to new perspectives in controlling mycotoxin in wheat through a synergistic approach, at the same time mitigating the potential environmental impact and the development of resistance to the conventional active ingredients.

In short, the data collected clearly underline that a natural compound with antifungal activity could only be a real alternative to a chemical fungicide if it is able to guarantee a similar grain yield advantage and capacity to minimize the risk of the mycotoxin occurrence. Thus, as carried out in the present study, the evaluation of alternative direct control strategies requires a holistic approach, which takes into account simultaneously not only the target pathogen, but also other main crop diseases and both their quantitative and qualitative consequences.

Furthermore,the results of these experiments obtained under naturally-infected field conditions confirm the already known and significant link between agronomic practices, such as the previous crop residue management through tillage, and DON contamination. Thus, in order to manage DON contamination in wheat, the proposed direct wheat protection solutions have to carefully consider the interaction of the entire cropping system and inserted within integrated multiple strategies.

**Conclusion**

The efficacy of MPE against FHB and DON contamination in field was at present not comparable to the one of the most efficient fungicides. Moreover, there was no efficacy against other diseases, such as STB, and a sufficient return of investment in term of grain yield. In order to offer new solutions to the market for the direct control of FHB agent, eco-friendly and alternatives to conventional fungicide, this research highlight the need to increase the persistence of MPE, able to enhance the overall protective efficacy of these natural compounds in field.

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**Declaration of interest statement**

Authors have no conflict of interest.

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

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Phenolic compound | *Spirulina* sp.(µg g-1) | *Nannochloropsis* sp.(µg g-1) | Wheat grains *a*(µg g-1) |
|  | Chlorogenic acid | 585.2 | 489.5 | 0.2 |
|  | Gallic acid | 1.7 | 86.6 | 1.1 |
|  | Protocatechuic acid | 16.3 | 27.0 | 1.0 |
|  | Hydroxybenzoic acid | 24.6 | 1.4 | - |
|  | Syringic acid | - | 7.6 | 1.7 |
|  | Vanillic acid | - | 3.4 | - |
|  | Ferulic acid | - | 0.3 | 39.7 |
|  | Coumaric acid | - | - | 0.4 |
|  | Caffeic acid | - | - | 0.3 |

*a* The reported values for the phenolic compounds in wheat grains at harvest are the means related to the different compared treatments.

**Table 2**. Effect of different treatments and tillage practices on the wheat grain yield, area under canopy greenness curve (AUCGC), *Septoria tritici* blotch (STB) severity, *Fusarium* Head Blight (FHB) severity and deoxynivalenol (DON) contamination. Field experiments have been conducted in North West Italy during the 2014-2016 period.

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Factor |  | Source of variation |   | Grain yield |   | AUCGC  |   | STB severity *a*  |   | FHB severity *bc* |   | DON *c* |
|  |   |  (t ha-1) |   |   | (%) |   | (%) |   | T | N (µg kg-1) |
| Year |   | 2015 |   | 7.1 |   | 17.3 |   | 24.2 |   | 1.7 |   | 7.1 | 1821 |
|   | 2016 |   | 7.3 |   | 19.5 |   | 37.9 |   | 4.2 |   | 5.7 | 445 |
| Tillage practices |   | Ploughing |   | 7.3 a |   | 17.8 a |  | 31.9 a |   | 2.1 a |   | 6.4 a | 1026 |
|   | Minimum Tillage |   | 7.1 b |   | 18.8 a |  | 28.7 a |   | 3.6 a |   | 6.6 a | 1372 |
|   |   |   |   |  |  |  |  |  |  |  |  |  |  |
|   |   | *P* (F) |  | 0.014 |   | 0.201 |   | 0.573 |   | 0.433 |   | 0.740 |  |
|   |   | sem *d* |   | 0.1 |   | 0.2 |   | 3.6 |   | 0.8 |   | 0.6 |  |
| Treatment |  | Untreated control |   | 6.7 c |  | 17.5 c |  | 43.1 a |  | 5.1 a |  | 7.1 a | 1532 |
|  | *Spirulina* sp. |   | 6.9 bc |  | 17.5 c |  | 38.2 a |  | 3.3 b |  | 7.1 a | 1479 |
|  | *Nannochloropsis* sp. |   | 6.9 bc |  | 17.4 c |  | 39.3 a |  | 3.1 b |  | 7.0 ab | 1336 |
|   | Tebuconazole |   | 7.3 ab |  | 18.3 b |  | 28.9 b |  | 3.1 b |  | 6.7 abc | 1155 |
|  | Prothioconazole |   | 7.7 a |  | 19.4 a |  | 17.6 c |  | 1.1 c |  | 5.7 bc | 874 |
|   | Tebuconazole + Prothiocolazole |   | 7.5 a |  | 19.2 a |  | 17.7 c |  | 1.3 c |  | 5.5 c | 767 |
|   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
|   |   | *P* (F)  |   | 0.024 |   | 0.006 |   | 0.041 |   | 0.033 |   | 0.001 |   |
|   |   | sem *d* |   | 0.6 |   | 0.5 |   | 11.5 |   | 1.5 |   | 0.3 |   |
| Treatment \* Tillage |   | *P* (F) |   | 0.524 |   | 0.122 |   | 0.445 |   | 0.159 |   | 0.789 |   |
| Treatment \* Year |   | *P* (F) |   | 0.118 |   | 0.193 |   | 0.037 |   | 0.193 |   | 0.975 |   |
| Tillage \* Year |   | *P* (F) |   | 0.970 |   | 0.058 |   | 0.030 |   | 0.029 |   | 0.024 |   |
| Treatment \* Tillage \* Year |  | *P* (F) |   | 0.350 |   | 0.813 |   | 0.109 |   | 0.358 |   | 0.439 |  |

Reported data for year and for tillage are the average of 36 replications (6 treatments X 2 soil management X 3 repetitions for year; 6 treatments X 2 years X 3 repetitions), while data for treatment are the average of 12 replications (2 years X 2 soil management X 3 repetitions). Means followed by different letters are significantly different (the level of significance is shown in the table).; a STB severity was calculated as the percentage of leaf surface with symptoms of disease at the dough stage (GS 85), based on 3 replications of 2 leaves from 15 different plants.; b FHB severity was calculated as the percentage of spikelets per ear with FHB damage, considering 200 ears per sample; c The DON contamination means reported are transformed [ T; y’= ln (x + 1)] and not transformed (N) values. dsem = standard error of mean