Field Crops Research 246 (2020) 107681

Contents lists available at ScienceDirect

Field Crops Research

journal homepage: www.elsevier.com/locate/fcr

Prevention of Fusarium head blight infection and mycotoxins in wheat with cut-and-carry biofumigation and botanicals

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ARTICLE INFO

Keywords:
Fusarium graminearum
Mycotoxin
Wheat
Mustard
Clover

ABSTRACT

Fusarium head blight (FHB) is a devastating fungal disease of wheat worldwide causing yield losses and grain contamination with mycotoxins that jeopardise food and feed safety. Field experiments using mulch layers or botanicals were conducted in two consecutive years to investigate prevention measures with the potential to suppress FHB and reduce mycotoxins in wheat. We simulated a system with high disease pressure, i.e. maize-wheat rotation under no-tillage, by applying maize residues artificially inoculated with *Fusarium graminearum* in field plots after wheat sowing. For mulch layers, a novel cut-and-carry biofumigation approach was employed. Cover crops grown in separate fields were harvested in autumn, chopped and applied directly onto the inoculated maize residues after wheat sowing. Mulch layers of white mustard (*Sinapis alba*), Indian mustard (*Brassica juncea*) or berseem clover (*Trifolium alexandrinum*) were applied. Botanicals included aqueous extracts of white mustard seed flours or milled Chinese galls and were applied to inoculated maize residues after wheat sowing in autumn or at wheat tillering in spring. Mulch layers of white mustard, Indian mustard or clover consistently suppressed *Fusarium* infection in both years and decreased mycotoxin contents in wheat grain, i.e. deoxynivalenol by up to 50 %, 58 % and 56 %, and zearalenone by up to 76 %, 71 % and 87 %, respectively. Botanicals were more effective in the second year, when the disease pressure was higher, reducing deoxynivalenol and zearalenone contents in grain by 22%–42% and 60%–78%, respectively. However, there were no clear differences between autumn and spring applications of botanicals on disease pressure and mycotoxin contamination. Mulch layer treatments improved grain yield up to 15 % compared with the positive control, while the botanicals had a minor impact on crop yield. Within the context of sustainable crop protection, cereal growers could benefit from the recommended prevention strategies by decreasing the risk of mycotoxin contamination in harvest products and thus improving grain yield and quality.

1. Introduction

Wheat (*Triticum* spp.) is one of the most important crops with over 220 million ha production area worldwide (Anonymous, 2017b) hosting a wide range of fungal diseases. Fusarium head blight (FHB) is a devastating fungal disease of wheat causing yield loss and grain contamination with mycotoxins, such as deoxynivalenol (DON) and zearalenone (ZEN), threatening human and animal health. Risks of exposure to DON are mainly related to intestinal, immune and brain systems, while ZEN is an estrogenic mycotoxin and considered to be toxic for liver, kidney and immune systems (Escrivá et al., 2015). In 2006, the European Commission established maximum limits of mycotoxins in unprocessed wheat for DON and ZEN (Anonymous, 2006). *Fusarium graminearum* (teleomorph *Gibberella zeae*) is commonly the predominant species of the FHB disease complex in wheat (Osborne and Stein, 2007) and one of the main producers of DON and ZEN (Bottalico and Perrone, 2002).

F. graminearum is an ascomycete that can develop both sexually and asexually and its life cycle has been thoroughly described (Trail, 2009). The overwintering mycelium grows saprophytically in crop debris and acts as inoculum source in the following spring by developing perithecia, which forcibly discharge ascospores, and macroconidia infecting wheat heads during anthesis. Therefore, wind-dispersed ascospores and rain-splashed macroconidia represent the two infection pathways of

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https://doi.org/10.1016/j.fcr.2019.107681
Received 25 July 2019; Received in revised form 12 November 2019; Accepted 12 November 2019

this fungal pathogen. Then, the fungus colonises the inflorescences resulting in shrunken or undeveloped kernels, which are frequently contaminated with mycotoxins depending on the disease severity. The preferable hosts of *F. graminearum* are small-grain cereals, maize and grasses (Parry et al., 1995), but it was also isolated from several non-gramineous weed species, belonging to important families such as Asteraceae and Solanaceae, which could serve as alternative hosts (Mourelos et al., 2014).

The highest risk of FHB infection and mycotoxin contamination in wheat and barley crops has been observed when maize was the previous crop and reduced or no-tillage practices were implemented (Blandino et al., 2012; Schöneberg et al., 2016; Vogelgsang et al., 2019). The survival rate of *F. graminearum* was reported to be enhanced with higher amounts of crop residues left on the soil surface (Leplat et al., 2013). Therefore, burying the residues of the previous crop with tillage is a common practice to control FHB (Shah et al., 2018). However, continuous ploughing has several drawbacks, such as increasing soil erosion risks and decreasing soil fertility (Triplett and Dick, 2008). In turn, reduced or no-tillage practices have been suggested during the last decades as a measure to preserve soil quality and mitigate soil degradation (Llewellyn et al., 2012; Busari et al., 2015). In Switzerland, several cantons provide direct payments to producers who adapt reduced or zero tillage, representing approximately 24 % of the total arable land without pastures in 2016 (Anonymous, 2017a).

The fungicide efficacy against FHB is frequently inconsistent. This could be due to the short time frame for application (i.e. anthesis period), heterogeneous anthesis within the same field as well as the limited number of available fungicides (all demethylation inhibitors) which increase the risk of resistance development (Wegulo et al., 2015). At the same time, ecological plant protection has gained more ground to reduce the potential negative environmental impact of synthetic pesticides. Thus, besides direct control measures to manage FHB, prevention strategies should be further explored in cereal-based rotations, such as treating the remaining residues from the previous crop in order to suppress the inoculum load of *F. graminearum*.

The use of plant-based extracts, i.e. biopesticide botanicals, has recently been explored as an alternative solution to synthetic fungicides (da Cruz Cabral et al., 2013; Vogelgsang et al., 2013; Tian et al., 2016). In vitro studies showed that aqueous extracts of mustard-based botanicals and Chinese galls reduced mycelium growth and suppressed perithecia formation of *F. graminearum* on maize stalks (Drakopoulos et al., 2019). Moreover, application of Chinese galls aqueous extract to wheat heads during anthesis substantially reduced FHB severity and mycotoxin content in the field (Forrer et al., 2014). Mustard plants, which belong to the Brassicaceae family, are widely used as cover crops providing a broad range of benefits, such as biofumigation, weed control and soil preservation (Snapp et al., 2005). Biofumigation is a commonly used term for soil disinfection by the release of the glucosinolate-breakdown products, i.e. isothiocyanates, after biomass incorporation of mustard crops into the soil (Brown and Morra, 1997). Therefore, the application of fresh biomass from cover crops with potential antifungal activity, e.g. mustard, onto remaining crop residues could prevent diseases by suppressing the overwintering fungal inoculum. Exposure to isothiocyanates leads to negative effects on the growth of various fungal species including reduced oxygen consumption rate, intracellular accumulation of reactive oxygen species and mitochondrial depolarisation (Calmes et al., 2015).

The objective of this study was to assess prevention measures with potential to suppress *F. graminearum* and decrease mycotoxin contamination in wheat in a simulated maize-wheat rotation under no-tillage. Within this context, the effects of applying mulch layers or botanical extracts onto artificially inoculated maize residues with *F. graminearum* were investigated. We examined a cut-and-carry approach meaning that cover crops were cultivated in separate fields and transferred to the wheat crop in order to cover the maize residues.

2. Materials and methods

2.1. Experimental design and crop management

In 2016-2017 (year 1) and 2017-2018 (year 2), two field experiments were conducted at the research facilities of Agroscope-Reckenholz in Zurich, Switzerland. The field experiment was arranged in four blocks and experimental plots were randomised within each block. Treatments consisted of different mulch layer or botanical applications. All plots were divided in two subplots including two winter wheat (*Triticum aestivum*) varieties (Saatzucht Düdingen, Switzerland), i.e. var. Levis (maturity: medium-late; susceptibility to FHB: high) and var. Forel (maturity: medium-early; susceptibility to FHB: medium-high). The area of each subplot was 9 m^2 (6 × 1.5 m). Buffer plots with the above-mentioned wheat varieties and triticale var. Larossa (Saatzucht Düdingen, Switzerland) were cultivated in an area of 9 m^2 (6 × 1.5 m) for each species to prevent cross-contamination among the experimental plots. The field was ploughed before sowing, and winter wheat and triticale were sown using a rate of 350 grains m^{-2}. During wheat production, 141 kg nitrogen and 20 kg magnesium oxide were applied per ha. A herbicide mixture (Artist - 24 % flufenacet and 17.5 % metribuzin; Chekker - 12.5 % amidosulfuron and 1.25 % iodosulfuron-methyl-sodium; Bayer Crop Science, Germany) was applied at the end of tillering (BBCH 27–29), while an insecticide application (Karate Zeon - 9.43 % lambda-cyhalothrin; Syngenta Agro AG, Switzerland) against cereal leaf beetles occurred at head emergence (BBCH 57–59).

2.2. Semi-artificial infection of wheat with Fusarium graminearum

A maize-wheat rotation resulting in high FHB disease pressure was simulated by artificially inoculating maize stalks with conidial suspensions of *F. graminearum* as follows (Fig. 1 a–c): Silage maize (P8057; DuPont Pioneer, USA) was harvested from a field at Agroscope-Reckenholz (Zurich, Switzerland) leaving stalk pieces of 40–60 cm on the soil surface. The remaining stalks were brought to the lab, cut to 30 cm length each and dried at 30 °C with constant air flow for one week. The dried maize stalks were soaked in water for 48 h and subsequently drained to remove the excess water, placed in plastic bags and autoclaved twice at 121 °C for 15 min. Afterwards, the maize stalks were allowed to cool down to room temperature and stored at −20 °C until the day of inoculation with *F. graminearum*. One day prior to inoculation, the maize stalks were placed at room temperature in the dark to defreeze. Maize stalks were inoculated with conidial suspensions of *F. graminearum* containing 2×10^5 conidia ml^{-1} water solution and 0.0125 % Tween® 20 (Sigma-Aldrich, USA). A mixture of three fungal strains with equal amounts was used for the inoculation ('0410', CBS 121292, Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands; '2113', Research Group Crop Breeding and Genetic Resources, Plant Pathology, Agroscope, Nyon, Switzerland; '1145', Fungal Collection of Agroscope, Nyon, Switzerland; single-spore isolates from wheat in Switzerland; 15-acetyldeoxynivalenol genotypes). A total amount of 640 ml conidial solution was sprayed (nozzle XR TeeJet® 110 02 V P, TeeJet Technologies, USA; 1.5 bar pressure) directly inside each bag, containing 320 maize stalks, while mixing adequately to ensure a homogeneous inoculation. Afterwards, the maize stalks were incubated inside the open plastic bags at 18 °C and 80 % RH in the dark for six days. One day prior to field application, the maize stalks were acclimatised at 12 °C and 80 % for 24 h in the dark. On each wheat subplot, 40 maize stalks were homogenously distributed after wheat sowing in autumn by placing four stalks in ten parallel lines along the plot.

2.3. Application of mulch layers and botanicals

For mulch layers, a novel cut-and-carry biofumigation approach was employed. Mulch layers were applied onto the inoculated maize stalks (Fig. 1 d) in autumn and included fresh aboveground biomass of white

Fig. 1. Experimental set-up of the study: (a) maize field after harvest where stalks were collected, (b) inoculated maize stalk pieces with *Fusarium graminearum* conidial suspensions after incubation, (c) inoculated maize stalks on the soil surface of wheat plots after incubation, (d) applied mulch layers, (e) spraying application of botanicals.

Fig. 2. Spore traps with *Fusarium* selective medium placed between two adjacent wheat varieties and adjusted to the same height as the flowering heads.

mustard (Mulch WM; *Sinapis alba*, Admiral; Feldsaaten Freudenberger, Germany), Indian mustard (Mulch IM; *Brassica juncea*, Vittasso; KWS, Germany) or berseem clover (Mulch Cl; *Trifolium alexandrinum*, Tabor; Jouffray-Drillaud, France). Cover crops were sown in mid-August in a neighbouring field at a rate of 20 kg ha^{-1}, 8 kg ha^{-1} and 30 kg ha^{-1}, respectively. After approximately three months (WM and Cl: anthesis; IM: vegetative stage), the aboveground biomass of the plants was collected, cut in 4–6 cm pieces with a sample chopper (Wintersteiger Hege 44, Austria) and 17 kg fresh biomass (equalling to 18.9 t ha^{-1}) was manually applied to each subplot, providing sufficient coverage of the inoculated maize stalks. The rationale behind this rate was to apply the same aboveground biomass that can usually be produced in the region of the study by the mustard crops. The same application rate was used for berseem clover to allow comparisons among the mulch layer treatments.

Botanicals were applied in the field to the inoculated maize stalks (Fig. 1 e) either in autumn or at wheat tillering (BBCH 27–29) in spring. The botanical treatments included Tillecur® (Ti; BIOFA, Germany), Pure Yellow Mustard (PYM; product 106, G.S. Dunn, Dry Mustard Millers, Canada) and Chinese galls (*Galla chinensis*; CG; origin Sichuan, China; purchased from Berg-Apotheke, Zurich, Switzerland). Ti and PYM are based on seed flours from white mustard, and CG was purchased in powder form. Detailed information about these botanicals as well as their biological activity in vitro against *F. graminearum* are provided in Drakopoulos et al. (2019). Botanical powders were suspended in deionised water at 4 % w v^{-1}, stirred for 2 h at room temperature and applied unfiltered with a backpack sprayer using a rate of 500 l ha^{-1} (four spraying nozzles TeeJet® TF-VP 3, TeeJet Technologies, USA; 2.5 bar pressure).

Maize stalks inoculated with *F. graminearum* but without any application of mulch layers or botanicals served as a positive control, while autoclaved (non-inoculated) stalks served as a negative control.

2.4. Measurements

2.4.1. Ascospore deposition and disease incidence

During wheat anthesis, spore traps (Fig. 2) with *Fusarium* selective medium containing pentachloronitrobenzene were placed in the field in order to catch the ascospores, which are discharged from perithecia. Details about the design of the spore traps and the selective medium can be found in Schöneberg et al. (2018) and Papavizas (1967), respectively. A volume of 42 ml medium was poured in each Petri dish (94 × 16 mm; Greiner Bio-One, Austria) using a filler (Mediajet, Integra Biosciences, Switzerland). One spore trap was placed one meter inside of each plot between the two adjacent wheat varieties (subplots) and adjusted to the same height as the flowering heads. Petri dishes were placed in the evening (6–7 pm) and collected in the following morning (9–10 am), allowing a period of 14–16 h for ascospore deposition. Upon collection, Petri dishes were incubated upside down at 18 °C in the dark for 3–5 days and then photos of the agar surface with the developed *Fusarium* colonies were taken (Canon EOS 750D, 60 mm EFS macro lens, Japan) for each dish. The colonies were counted manually using an image viewer (IrfanView version 4.38, Austria). For the placement of

the spore traps, days with low to moderate FHB infection risk were chosen since days with high infection risk would result in excessive number of colonies which does not allow quantification. To determine the daily risk level of infection for wheat, the Swiss forecasting system 'FusaProg' was used, providing regional weather-based FHB infection and field specific DON contamination risks (Musa et al., 2007). The sum of counted *Fusarium* colonies in spore traps from three time points during wheat anthesis was calculated for each treatment.

The disease incidence in the field was measured by counting the number of heads with typical FHB symptoms (i.e. fully or partially bleached heads). For each wheat subplot, ten heads from five different locations along both sides of the plot were observed resulting in 100 heads. Disease incidence was expressed as the percentage of symptomatic wheat heads.

2.4.2. Grain yield

Wheat was harvested using a plot combine harvester (Wintersteiger, Austria). The seed moisture content of a representative subsample was measured with a moisture tester (GAC 2100, Dickey-John, USA). Grain yield was measured with a balance and normalised to 12 % seed moisture content. For further analysis, a representative subsample of approximately 400 g was obtained from each wheat subplot by placing the grains on a flat surface and collecting approximately 40 g from 10 different spots. Grains were stored in plastic sealed containers at 10 °C.

2.4.3. DNA amount of Fusarium graminearum in wheat grain

A grain sub-subsample of 150 g was drawn using a riffle divider (Schieritz & Hauenstein AG, Switzerland) and ground with a mill (Cyclotec™ 1093; Foss Tecator, Sweden; 1 mm mesh size). Flours were stored at −20 °C until further processing. Polypropylene tubes (1.2 ml; BRAND®, Germany) were filled with 50 mg flour sample and DNA was extracted following the protocol of NucleoSpin® 96 Plant II Kit (Macherey-Nagel, Germany). Each sample was processed twice with one 3-mm tungsten bead in a TissueLyser II (Qiagen®, Hombrechtikon, Switzerland) for 30 s (frequency $20\,s^{-1}$) in lysis buffer (PL1). Quantitative PCR was performed as described in Schöneberg et al. (2018) by CFX96™ Real-Time PCR Detection System for in vitro diagnostics (C1000™ Thermal Cycler; Bio-Rad Laboratories, USA). The used qPCR method was originally developed by Brandfass and Karlovsky (2006) and adjusted to the available reaction mixtures and laboratory devices. The used plasmid contained a 284 bp fragment which is specific to *F. graminearum* (Nicholson et al., 1998). For each qPCR run, samples, standards and negative control were triplicated. Standards were spiked with DNA from healthy wheat (4 ng total DNA per reaction, volume 20 µl) such that the amount of total DNA was similar to that of the measured samples. The limit of quantification (LOQ) was 40 copies per reaction and the limit of detection one tenth of the LOQ. All samples contained *F. graminearum* DNA above the LOQ. To determine the amount of total DNA in the samples, the Fluorescent DNA Quantitation Kit (BIO-RAD, Switzerland) was used. The quantification was performed with a Cary Eclipse Fluorescence Spectrophotometer (Varian, Agilent Technologies, USA) based on the emitted fluorescence of a serially diluted DNA standard.

2.4.4. Quantification of mycotoxins in wheat grain

The mycotoxins DON and ZEN in wheat grain were quantified following the protocol of the ELISA kits for enzyme immunoassays (Celer® DON v3 Cod. MD100 and Celer® ZON Cod. MZ670; Tecna, Italy; 'ZON' in the kit represents ZEN). Flour samples of 5 g were drawn from the same sub-subsample that was used for DNA and extracted with 25 ml solution of 70 % methanol and 4 % sodium chloride (Sigma-Aldrich, USA) while shaken for 10 min in an orbital lab shaker at 250 rpm. Samples were then filtered through folded filter paper (Whatman®, Grade 595 ½, Sigma-Aldrich, USA), filtrates were pipetted in cluster tubes (Corning® 96 well PP 1.2 ml; Sigma-Aldrich, USA) and stored at 5 °C. Reference flours (Trilogy Analytical Laboratory, USA) with known DON and ZEN contents were extracted in the same way. If toxin concentrations were lower than the lowest standard, which was only the case for ZEN (LOQ < $0.01\,mg\,kg^{-1}$), the values were replaced by a constant value (x = LOQ ÷ 2). The absorbance microplate reader Sunrise™ (TECAN, Austria) was used for the ELISA measurements.

2.4.5. Climatic data

The climatic data were obtained from a nearby (< 500 m) weather station (MeteoSwiss, Federal Office of Meteorology and Climatology). Hourly data for temperature, relative humidity and precipitation were retrieved for the period from the beginning of anthesis (BBCH 61) until harvest (BBCH 92) of wheat. The average daily values were calculated for temperature and relative humidity and the daily sum for precipitation. For each experimental year, climatic data were categorised into three periods according to the wheat growth stages, i.e. anthesis (BBCH 61–69), seed watery ripe until early dough (BBCH 71–83) and soft dough until ripening (BBCH 85–92).

2.5. Data analysis

Analysis of variance was performed to test the main effects of treatment and wheat variety and their interactions within each experimental year as well as to test the year effect. Shapiro-Wilk and Brown-Forsythe tests were performed to verify the assumptions of normality and homogeneity of variances, respectively. If these assumptions were not fulfilled ($p < 0.05$), data transformations were performed. For the first year, the logarithmic transformation was used for *F. graminearum* DNA amount and ZEN content in grain, while the arcsine transformation was used for disease incidence. For the second year, only the data of the ZEN content were subjected to logarithmic transformation. Duncan's method was used for post hoc comparisons ($\alpha = 0.05$). The untransformed data are presented in tables and figures. Pearson's product-moment correlation was used to test the linear associations (r) among the examined response variables within each experimental year. Statistical analysis was performed with SigmaPlot (Systat Software Inc., USA) and Figs. 3 and 4 were prepared with Prism 5.0 (GraphPad Software Inc., USA).

3. Results

3.1. Ascospore deposition, disease incidence and Fusarium graminearumDNA amount in wheat grain

Overall, the mean ascospore deposition, disease incidence and DNA amount of *F. graminearum* in wheat grain was 2-, 6- and 2-fold higher, respectively, in the second year than in the first year ($p < 0.001$). In both years, there was no significant interaction ($p > 0.05$) between wheat variety and mulch layer or botanical treatments on ascospore deposition, disease incidence and DNA amount of *F. graminearum* in grain. Disease incidence and DNA amount of *F. graminearum* were higher in var. Levis than in var. Forel ($p < 0.001$). In both years, all mulch layers and Ti applied in spring decreased the ascospore deposition by 35%–48% compared with the plots containing untreated maize stalks inoculated with *F. graminearum*, i.e. positive control (Table 1, $p < 0.05$). In plots with non-inoculated maize stalks (i.e. negative control), the ascospore deposition was 92 % and 87 % lower compared with the positive control in the first year and second year, respectively ($p < 0.05$). In the first year, disease incidence was 58 %, 63 %, 50 % and 86 % lower for Mulch WM, Mulch Cl, Ti applied in spring and the negative control, respectively, compared with the positive control (Table 1, $p < 0.05$). In the second year, disease incidence was 18 %, 19 % and 80 % lower for Mulch IM, Mulch Cl and the negative control, respectively, compared with the positive control (Table 1, $p < 0.05$). In the first year, the treatments Mulch WM, Mulch Cl and Ti applied in spring decreased the amount of *F. graminearum* DNA in grain by 53 %, 46 % and 49 %, respectively, compared with the positive control (Table 1, $p < 0.05$). In the second year, mulch layers and botanicals reduced *F. graminearum* DNA

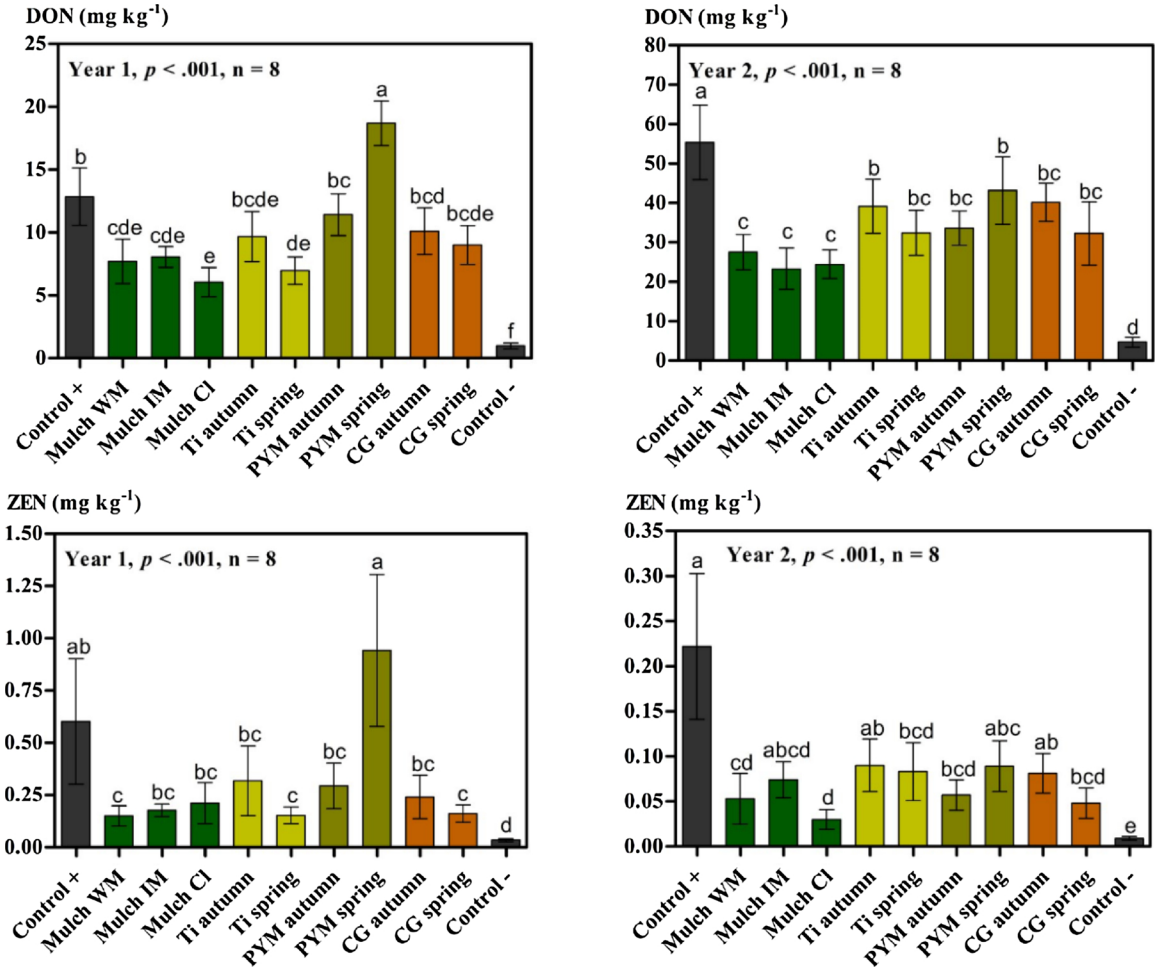

Fig. 3. Deoxynivalenol (DON, mg kg^{-1}) and zearalenone (ZEN, mg kg^{-1}) contents in wheat grain in year 1 (2016-2017) and year 2 (2017-2018) as affected by application of mulch layers or botanicals to maize stalks inoculated with *Fusarium graminearum*. Note the different scales of mycotoxin contents between year 1 and year 2. Control refers to positive/negative control, i.e. untreated inoculated/sterilised maize stalks; Mulch WM/IM/Cl refers to fresh mulch layers harvested from white mustard/Indian mustard/berseem cloverand applied in autumn; Ti/PYM/CG refers to Tillecur/Pure Yellow Mustard/Chinese galls botanical aqueous extracts applied in autumn or spring. Average data from two wheat varieties (var. Levis and var. Forel) are presented and error bars represent the standard error of the mean (n = 8). Different letters indicate significant differences according to Duncan's method for post hoc comparisons (α = 0.05).

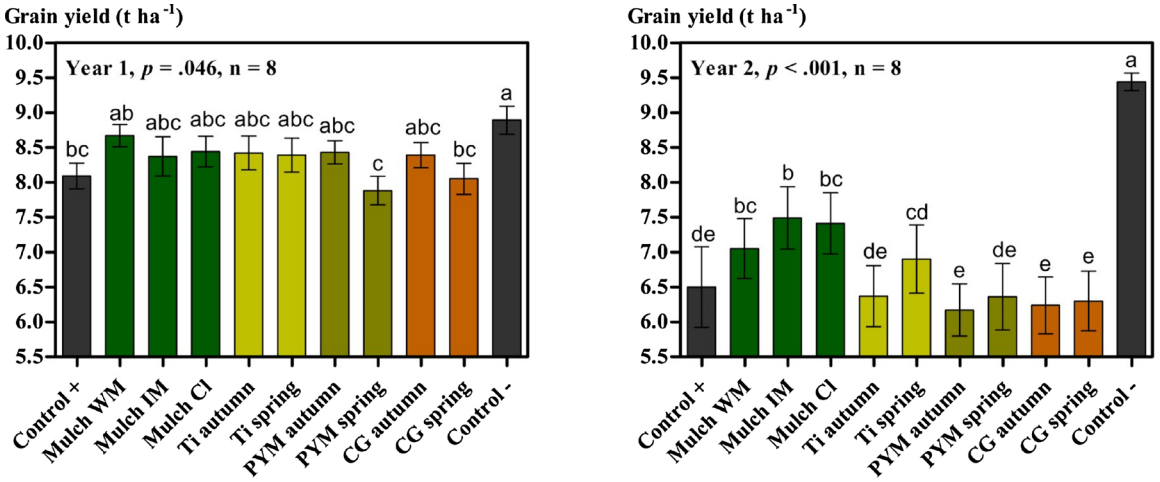

Fig. 4. Grain yield (t ha^{-1}) of wheat in year 1 (2016-2017) and year 2 (2017-2018) as affected by application of mulch layers or botanicals to maize stalks inoculated with *Fusarium graminearum*. Control refers to positive/negative control, i.e. untreated inoculated/non-inoculated maize stalks; Mulch WM/IM/Cl refers to fresh mulch layers harvested from white mustard/Indian mustard/berseem cloverand applied in autumn; Ti/PYM/CG refers to Tillecur/Pure Yellow Mustard/Chinese galls botanical aqueous extracts applied in autumn or spring. Average data from two wheat varieties (var. Levis and var. Forel) are presented and error bars represent the standard error of the mean (n = 8). Different letters indicate significant differences according to Duncan's method for post hoc comparisons (α = 0.05).

Table 1
Ascospore deposition, disease incidence and DNA amount of *Fusarium graminearum* (FG) in wheat grain in year 1 (2016-2017) and year 2 (2017-2018) as affected by application of mulch layers or botanicals to FG-inoculated maize stalks. Positive/negative control refers to untreated inoculated/sterilised maize stalks; Mulch WM/IM/Cl refers to fresh mulch layers harvested from white mustard/Indian mustard/berseem clover and applied in autumn; Ti/PYM/CG refers to Tillecur/Pure Yellow Mustard/Chinese galls botanical aqueous extracts applied in autumn or spring. Average data from two wheat varieties (var. Levis and var. Forel) are presented and ± represents the standard error of the mean (n = 8).

	Ascospore deposition (×100 count of *Fusarium* colonies)	Disease incidence (% of symptomatic heads)	FG DNA amount in grain (×100 DNA copies ng total DNA^{-1})	Ascospore deposition (×100 count of *Fusarium* colonies)	Disease incidence (% of symptomatic heads)	FG DNA amount in grain (×100 DNA copies ng total DNA^{-1})
	Year 1 (2016–2017)			Year 2 (2017–2018)		
Positive control	21.7 ± 4.2 a	17.4 ± 4.2 ab	16.7 ± 4.1 ab	40.9 ± 7.1 a	84.1 ± 7.8 a	42.3 ± 7.5 a
Mulch WM	12.7 ± 2.2 b	7.4 ± 1.5 de	7.8 ± 1 c	24.3 ± 3.2 cd	78.4 ± 7.2 abc	24.6 ± 4.2 bc
Mulch IM	11.5 ± 2.4 b	10.8 ± 2 bcde	9.1 ± 0.6 bc	21.2 ± 1.7 d	68.8 ± 7.7 bc	19.5 ± 3.5 c
Mulch Cl	11.9 ± 0.7 b	6.5 ± 1.2 e	9.0 ± 1.9 c	24.0 ± 3.8 cd	68.3 ± 8.4 c	22.1 ± 3.8 bc
Ti autumn	16.4 ± 3.9 ab	13.6 ± 3.3 bcd	11.2 ± 2.5 bc	30.8 ± 5.4 abcd	80.3 ± 9.3 ab	29.2 ± 5.7 b
Ti spring	12.9 ± 1.4 b	8.8 ± 1.3 cde	8.4 ± 0.9 c	26.7 ± 4.6 bcd	74.9 ± 9.7 abc	27.7 ± 5.1 bc
PYM autumn	15.3 ± 1.8 ab	17.9 ± 2.7 ab	14.1 ± 1.6 ab	33.9 ± 6.6 abc	80.3 ± 5.6 ab	24.6 ± 3.7 bc
PYM spring	21.7 ± 1.3 a	21.6 ± 4.3 a	20.8 ± 3.5 a	36.6 ± 5.1 ab	84.6 ± 4.8 a	27.9 ± 4 bc
CG autumn	16.6 ± 2.2 ab	11.5 ± 2.7 bcde	11.0 ± 1.8 bc	32.7 ± 3.0 abc	82.5 ± 6 a	30.3 ± 3.5 b
CG spring	13.5 ± 1.3 b	12.0 ± 1.6 bcde	10.7 ± 0.9 bc	33.6 ± 6.7 abc	75.1 ± 7.8 abc	22.3 ± 4.3 bc
Negative control	1.8 ± 0.4 c	2.5 ± 0.5 f	1.5 ± 0.3 d	5.4 ± 0.8 e	17.1 ± 4.9 d	5.3 ± 1.3 d
p-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Different letters indicate significant differences according to Duncan's method for post hoc comparisons (α = 0.05).

amount in grain by 42%–54% and by 28%–47%, respectively, compared with the positive control (Table 1, $p < 0.05$).

3.2. Mycotoxins in wheat grain

Overall, the mean DON content in grain was 3.5-fold higher in the second year than in the first year(32.4 versus 9.2 mg kg^{-1}, $p < 0.001$). In both years, no significant interactions ($p > 0.05$) were detected for DON and ZEN contents in grain between wheat variety and mulch layer or botanical treatments. In both years, DON and ZEN contents were higher in grains from var. Levis than in those from var. Forel ($p < 0.001$). In the first year, DON was reduced by 40 %, 37 %, 53 % and 46 % with use of Mulch WM, Mulch IM, Mulch Cl and Ti applied in spring, respectively, compared with the positive control (Fig. 3, $p < 0.05$). In the second year, all mulch layers and botanicals decreased DON by 50%–58% and by 22%–42%, respectively, compared with the positive control (Fig. 3, $p < 0.05$). In the first year, use of Mulch WM, Ti and CG applied in spring decreased ZEN by 73%–75% compared with the positive control (Fig. 3, $p < 0.05$). In the second year, the treatments Mulch WM, Mulch Cl, Ti applied in spring, PYM applied in autumn and CG applied in spring reduced ZEN by 76 %, 87 %, 62 %, 74 % and 78 %, respectively, compared with the positive control (Fig. 3, $p < 0.05$).

3.3. Grain yield

Overall, the mean grain yield was lower in the second year than in the first year (6.9 versus 8.4 t ha^{-1}, $p < 0.001$). In both years, no significant interaction ($p > 0.05$) was detected on grain yield between wheat variety and mulch layer or botanical treatments. In both years, var. Forel resulted in higher yield than var. Levis (year 1: 8.54 versus 8.19 t ha^{-1}, $p = 0.005$; year 2: 7.80 versus 6.06 t ha^{-1}, $p < 0.001$). In the first year, the grain yield of most mulch layer or botanical treatments was between the positive control (8.09 t ha^{-1}) and the negative control (8.89 t ha^{-1}; Fig. 4, $p < 0.05$). In the second year, the mulch layer treatments resulted in 8%–15% higher grain yield compared with the positive control, while the highest yield was observed in the negative control (Fig. 4, $p < 0.05$).

3.4. Correlations among examined response variables

The Pearson product-moment correlation study revealed moderate to strong correlations among the examined response variables in both years (all $p < 0.001$, Table 2). Positive correlations were found between ascospore deposition and disease incidence (r = 0.63 and 0.73). Strong associations were observed between DNA amount of *F.*

Table 2
Pearson's product-moment correlation coefficient (r) for the examined response variables in year 1 (n = 88; above the diagonal boxes) and year 2 (n = 88; below the diagonal boxes). The response variables included ascospore deposition, disease incidence, *Fusarium graminearum* (FG) DNA amount in grain, deoxynivalenol (DON) and zearalenone (ZEN) contents in grain and grain yield. All *p*-values < 0.001.

	Ascospore deposition	Disease incidence	FG DNA amount	DON content	ZEN content	Grain yield
Ascospore deposition		.63	.62	.71	.41	-.35
Disease incidence	.73		.80	.80	.74	-.42
FG DNA amount	.71	.83		.87	.88	-.39
DON content	.75	.81	.89		.73	-.39
ZEN content	.40	.48	.73	.64		-.37
Grain yield	-.68	-.89	-.82	-.83	-.57	

graminearum and DON (r = 0.87 and 0.89) and ZEN (r = 0.88 and 0.73) contents in grain. Grain yield was negatively correlated with all FHB related parameters (r = -0.35 to -0.89) with stronger correlations in the second year than in the first year.

4. Discussion

In the current study, we simulated a system with high FHB pressure within a no-tillage maize-wheat rotation by artificially inoculating maize stalks with *F. graminearum*. To develop a sustainable control strategy, the use of botanicals and cut-and-carry biofumigation with mulch layers to suppress the disease and reduce mycotoxin contamination in wheat grain was investigated. The strong associations between DNA amount of *F. graminearum* in grain and mycotoxin contents (DON and ZEN) in grain confirm that *F. graminearum* was the main FHB-causing species in our study.

The effectiveness of mulch layers from white mustard, Indian mustard and berseem clover to prevent FHB infection and reduce mycotoxin content in grain was consistent throughout the experimental years. Mustard crops are frequently cultivated as green manures for biofumigation (Matthiessen and Kirkegaard, 2006). Following plant tissue disruption, the enzyme myrosinase catalyses the hydrolysis of glucosinolates (GSL) and, subsequently, isothiocyanate (ITC) reactive compounds are released. Soil incorporation of Indian mustard was an effective strategy to control Rhizoctonia root rot of sugar beet (Motisi et al., 2009) as well as to reduce powdery scab and common scab diseases in potato (Larkin and Griffin, 2007). In our study, we investigated a cut-and-carry biofumigation approach where mustard crops were grown at another field plot and transferred to the main crop (i.e. wheat) covering the inoculated maize residues after wheat sowing. Over the last decades, ITC compounds have been reported as the most biologically active substances of mustard and were thus considered as broad-spectrum biocides against soil-borne pathogens, pests and weeds (Brown and Morra, 1997; Rosa and Rodrigues, 1999). The main ITCs present in shoots of field grown Indian mustard at anthesis are 2-propenyl- (or allyl-) and 3-butenyl-ITCs, while for white mustard are p-hydroxybenzyl- and benzyl-ITCs (Kirkegaard and Sarwar, 1998). When 11 GSLs and their hydrolysis products (ITCs) were tested in vitro against *F. culmorum*, it was reported that native GSLs had no fungitoxicity, whereas several ITCs including the ones derived from sinalbin and sinigrin, which are present in white mustard and Indian mustard, respectively, significantly reduced fungal growth (Manici et al., 1997). Moreover, in vitro tests showed that 2-propenyl-, 3-butenyl- and benzyl-ITCs decreased colony diameter of *F. graminearum* (Sarwar et al., 1998). Smolinska et al. (2003) performed bioassays using sealed containers and found that propenyl-, ethyl-, benzyl- and phenethyl-ITCs were fungitoxic to conidia and chlamydospores of four *F. oxysporum* isolates. In another study, the radial growth of *F. sambucinum* was negatively correlated with allyl-ITC concentrations emitted from *Brassica* leaf tissue (Mayton et al., 1996). In our study, maize stalks were fully covered with mulch layers for several weeks, possibly leading to an environment in which the bioactive volatile ITCs of mustard suppressed the survival and growth of *F. graminearum* on the maize residues. Gimsing et al. (2007) found that the degradation of benzyl GSL in soil followed logistic kinetics with half-life ranging from 0.7–9.1 days depending on the soil type. Gimsing and Kirkegaard (2006) indicated that a significant proportion of plant GSL can persist un-hydrolysed in the soil for several days after incorporation. However, to the best of our knowledge, there have been no studies on degradation and release rates of GSLs and ITCs, respectively, between an applied mulch layer of mustard and crop residues.

The phytochemical profile of clover indicates the presence of a broad spectrum of biologically active substances such as flavonoids, phenolic acids, clovamides and saponins (Oleszek et al., 2007; Sabudak and Guler, 2009; Kolodziejczyk-Czepas, 2012). Leaf extracts from berseem clover had antibacterial activity against both gram-positive and gram-negative bacteria (Khan et al., 2012). Another study compared the free radical scavenging and antioxidant properties of six clover species and demonstrated that extracts from aerial parts of berseem clover had the highest antiradical and antioxidant activity (Kolodziejczyk-Czepas et al., 2014). The current study provides for the first time evidence for the biological activity of berseem clover against *F. graminearum*. Besides the prevention of FHB infection in wheat, an additional benefit of using clover as mulch layer could be the substantial nitrogen input into the system. In organic agriculture, an emerging fertilisation strategy is the use of cut-and-carry fertilisers, i.e. growing crops with high nitrogen content, such as legumes, in one site and then transporting the harvested aboveground biomass to the cash crop (Drakopoulos et al., 2016; Sorensen and Grevsen, 2016). Following this approach, organic producers are less dependent on farmyard manure as fertiliser input, which is often not easily available if nearby farms are stockless. Moreover, keeping leguminous crops instead of selling them as fodder would reduce the exportation of organic matter and essential macro- and micro-nutrients from the farm boundaries.

Another possible explanation for the effectiveness of the mulch layers could be the changes in microbial communities posing indirect antagonistic effects. For example, in some cases, the suppressive effect of mustard crops against pests and diseases was not related to GSL or ITC concentrations in plant tissues, but was attributed to changes in microbial populations due to incorporation of organic matter (Matthiessen and Kirkegaard, 2006). In our study, microbes possibly migrated and colonised the maize stalks during or after the decomposition process of the mulch layers, representing an important antagonistic force that competed with *F. graminearum* suppressing its development and spread. However, mulch layers did not physically inhibit the release of ascospores from perithecia, since the mulch material had been already decomposed before wheat anthesis. In addition to the promising cut-and-carry biofumigation approach, it would be highly valuable to explore the potential of wheat-intercropping systems with 'living mulches' to suppress the release of ascospores from infected crop residues.

Compared with the mulch layers, the effectiveness of the botanicals (Tillecur, Pure Yellow Mustard and Chinese galls) at 4 % w v^{-1} to prevent FHB under field conditions was limited and inconsistent between the experimental years. However, a previous study demonstrated that the use of the same botanicals suppressed different stages of the life cycle of *Fusarium graminearum* in vitro (Drakopoulos et al., 2019). The authors showed that the use of 2 % w v^{-1} botanical aqueous extracts inhibited mycelium growth, germination of conidia and ascospores, perithecia development and ascospore discharge from mature perithecia. In the current field study, only Tillecur applied in spring reduced DON and ZEN contents in grain in both years, while Pure Yellow Mustard and Chinese galls were more effective in the second year when disease pressure was higher. This inconsistent efficacy points out the necessity of an effective formulation strategy to improve the stability and therefore activity of the botanicals. Furthemore, our findings are less conclusive regarding application time of the botanicals, since no clear differences were observed between autumn and spring applications to the inoculated maize stalks.

The mulch layer treatments improved grain yield consistently in both years, whereas the effects of botanicals on yield were minor compared with the untreated infected plots (i.e. positive control). However, the highest grain yields were obtained in the plots with sterilised maize stalks (i.e. negative control). The mean grain yield across the whole field was lower in the second year than the first year. This could be due to the prevailing climatic conditions (Table 3) from beginning of anthesis (BBCH 61) to ripening (BBCH 92) of the wheat crop resulting in higher FHB disease pressure and mycotoxin contamination of grain. During the growth stages BBCH 61–69 and 71–83, the relative humidity was higher in the second year compared with the first year. Moreover, the total precipitation during BBCH 61–83 was

Table 3
Average daily temperature (°C), average daily relative humidity (%) and sum of precipitation (mm) over three distinct periods, i.e. anthesis (BBCH 61–69), seed watery ripe until early dough (BBCH 71–83) and soft dough until ripening (BBCH 85–92), of the wheat crop in 2017 (year 1) and 2018 (year 2).

	Temperature (°C)		Relative humidity (%)		Precipitation (mm)	
BBCH	Year 1	Year 2	Year 1	Year 2	Year 1	Year 2
61–69	18	18	69	72	41	24
71–83	23	19	63	77	25	64
85–92	20	19	69	65	102	49

22 mm higher in the second year compared with the first year. Therefore, the differences in relative humidity and precipitation during these periods could explain the higher disease pressure resulting in increased DON content in the second year. In addition, a study by Culler et al. (2007) showed that DON concentrations were lower after mist irrigation between hard dough (BBCH 87) and harvest (BBCH 92), suggesting leaching of DON. This finding is in parallel to our study since the precipitation at BBCH 85–92 was 53 mm higher in the first year compared with the second year. In contrast, ZEN content was 4-fold higher in the first year than in the second year (298 versus 76 µg kg^{-1}, $p < 0.001$). ZEN is strongly dependent on post-anthesis rainfall with greater level of rainfall and delayed harvest causing higher ZEN concentrations in grain (Edwards, 2011). Furthermore, ZEN content can remain at very low levels in the absence of moisture during late growth stages despite severe FHB infections (Kharbikar et al., 2015). This is in agreement with our study where the sum of precipitation at BBCH 85–92 was 102 mm versus 49 mm for the first and second year, respectively. Although grain yield was negatively correlated with all FHB related parameters in both years, stronger correlations were found in the second year showing that yield is better associated with FHB parameters in years with higher disease pressure.

The positive relationships between ascospore deposition and disease incidence as well as between ascospore deposition and DON content in grain suggest that the use of spore traps during anthesis is an excellent tool to predict FHB infection and DON contamination risks. In plots with sterilised maize stalks (i.e. negative control), the ascospore deposition was remarkably lower compared with the positive control in both years. This indicates that the experimental set-up using buffer plots contributed to the low level of cross-contamination between plots.

5. Conclusions

We showed for the first time that in a simulated system with high FHB disease pressure, the prevention of FHB and mycotoxins in wheat was feasible by applying mulch layers from white mustard, Indian mustard or berseem clover grown in separate fields, i.e. a cut-and-carry biofumigation, onto infected maize residues. An additional important outcome of this study is that the experimental set-up was appropriate with minimum cross-contamination between plots. Thus, further antifungal mulch layers from cover crops that are commonly grown in specific agronomic regions could be evaluated following this approach. However, extension agents should encourage policy makers to support farmers economically in order to use cut-and-carry biofumigation. The efficacy of the studied botanicals was not consistent in both years suggesting that these products would have to be applied several times and/or formulated to prolong their bioactivity. Within the context of sustainable crop protection, cereal growers could benefit from the recommended prevention strategies by decreasing the risk of mycotoxin contamination in harvest products while ensuring grain yield and quality.

Funding

This work was supported by the MycoKey project "Integrated and innovative key actions for mycotoxin management in the food and feed chain" funded by the Horizon 2020 Research and Innovation Programme under the grant agreement No. 678781; and the Swiss State Secretariat for Education, Research and Innovation.

Data statement

The data of this study are available upon request.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgments

The authors warmly thank Irene Bänziger for excellent technical assistance as well as interns and students who contributed to this study (Anja Marty, Pascal Weber, Fabian Hess and Sibel Dugan). Also, we would like to acknowledge G.S. Dunn (Dry Mustard Millers, Canada) for providing us with the Pure Yellow Mustard flour.

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