Cropping systems and plant-based biopesticides to reduce the risk of *Fusarium* mycotoxins in wheat

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Doctoral Thesis (DISS. ETH NO. 26729)

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A thesis submitted to attain the degree of

DOCTOR OF SCIENCES of ETH ZURICH

(Dr. sc. ETH Zurich)

presented by

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2020

Cover page

- Top: the drawings of maize, red clover, white mustard and wheat, from left to right, were sketched by Jonas Lehner, Agroscope.
- Middle: the photo of the wheat head with the characteristic disease symptoms of Fusarium head blight was taken on 26.06.2019 in the research facilities of Agroscope-Tänikon, 8356 Ettenhausen, Switzerland.
- Bottom: the coins of ancient Greece (Lucania - Metapontum, 325-280 BC; left: Demeter, goddess of agriculture; right: barley head) were retrieved from WildWinds (2020).

The research described in this doctoral dissertation was performed within the research group Ecological Plant Protection in Arable Crops, research division Plant Protection, Agroscope-Reckenholz, Zurich, Switzerland.

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Preface

The current doctoral dissertation was conducted in 2016-2020 at the group of Ecological Plant Protection in Arable Crops at Agroscope-Reckenholz in Zurich, Switzerland. The direct supervisor of my work was Dr. Susanne Vogelgsang. During my doctorate, I was enrolled in the group of Sustainable Agroecosystems at ETH Zurich in Switzerland, and Prof. Johan Six was my academic supervisor.

This dissertation would be impossible without the contribution of several people. First, I am particularly grateful to my supervisors, Susanne and Johan, who guided me throughout the entire thesis process with plentiful attention and expertise. Susanne was really supportive and was always providing me with essential feedback. She is an excellent tutor who gave me the opportunity to develop and implement my own research ideas. Moreover, she is a compassionate mentor with a strong interest in my overall well-being in her group. Johan, apart from the valuable guidance and feedback, offered me the great opportunity to be part of his group interacting with numerous researchers as well as participating in the research progress seminars. I would like to acknowledge Dr. Hans-Rudolf Forrer who served as a committee member for my doctoral thesis and gave me essential feedback during my work.

Special thanks to Andreas Kägi who contributed the most in the preparation and maintenance of the field experiments providing valuable feedback in several aspects of my research. Many thanks to the students that I supervised during this time and significantly contributed to my doctorate: Pascal Weber (BSc thesis), Anja Marty (MSc internship) and Nicole Togni (MSc thesis). Many thanks to other technical staff, students, interns and researchers who generously assisted me and provided inputs: Eveline Jenny, Irene Bänziger, Alejandro Gimeno, Tomke Musa, Torsten Schöneberg, Sibel Dugan, Fabian Hess, Jonas Lehner, Fabian Zahnd, Sofie Bolt, Vincent Jordon and Dante Alahiari. Also, I would like to thank my office mates Torsten Schöneberg, Karen Sullam and Lara Reinbacher for spending nice moments together. I would like to extend my gratitude to all members of the Ecological Plant Protection in Arable Crops group as well as the Sustainable Agroecosystems group. Moreover, I would like to thank the group of field services (‘Feldequipe’) of Agroscope in Reckenholz and Tänikon for the field operations and the advices on crop management practices.

Many thanks to Prof. Giuseppe Meca and his group at the University of Valencia, Spain, for the great collaboration towards the chapters I, II and IV of this dissertation. Many thanks to Dr. Felix Wettstein and Dr. Thomas Bucheli for their guidance and assistance regarding the mycotoxin extraction and analysis of the grain samples for chapter III. I would also like to thank Dr. Alexander Zorn for his valuable inputs regarding the economic analysis in chapter III.

Finally, I would especially like to thank my family and friends who gave me strength and encouragement to complete this work - ευχαριστώ για όλα. Special thanks to Dilaycim Cansever who supported me the most during this 'journey' - Çok teşekkürler.

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.

Research group Ecological Plant Protection in Arable Crops, Agroscope-Reckenholz, Zurich, Switzerland (18.06.2019).

Summary

Fusarium head blight (FHB) is a devastating fungal disease of cereals worldwide causing significant reductions in yield and, most importantly, severe grain contaminations with mycotoxins, such as deoxynivalenol (DON) and zearalenone (ZEN). In most parts of the world, *Fusarium graminearum* is the predominant species of the FHB complex in wheat. The fungus overwinters on infected crop residues, such as maize stalks and wheat straw. Hence, suitable crop rotation with non-host species and management of crop residues with plough are effective agronomic practices to control FHB. However, continuous ploughing has several drawbacks, such as increased risks of soil erosion and soil degradation. Recently, there is a movement towards reduced reliance on synthetic pesticides to minimise the negative impact on the environment and human health. The main objective of this doctoral dissertation was to explore the potential of cropping systems and plant-based biopesticides, botanicals, to reduce *Fusarium* infection and mycotoxin contamination in wheat in a maize-wheat rotation under reduced tillage practices.

First, we investigated the effect of botanicals on suppressing different stages of the life cycle of *F. graminearum in vitro*. Aqueous extracts of white mustard seed flours and milled Chinese galls at 2 % concentration reduced mycelium growth by up to 100 %, germination of conidia and ascospores by up to 100 % and 97 %, respectively, perithecia formation on maize stalks by up to 56 % and discharge of ascospores from perithecia by up to 77 %. Furthermore, we quantified the principal glucosinolate component sinalbin of the mustard-based botanicals, i.e. the precursor of p-hydroxybenzyl isothiocyanate, a compound known for its antimicrobial activity. Moreover, we showed that Chinese galls contain different gallotannins as well as gallic and tannic acids, compounds also known for their antimicrobial properties.

Secondly, we investigated prevention strategies to control FHB in a simulated maize-wheat rotation under no-tillage. Maize residues were artificially inoculated with *F. graminearum* and subsequently placed on the soil surface in field plots after wheat sowing. Botanicals or fresh mulch layers were applied onto *F. graminearum*-inoculated maize residues. Botanicals included aqueous extracts of white mustard seed flours and milled Chinese galls. Botanicals were more effective in the second experimental year than the first year reducing DON and ZEN contents in wheat grain by up to 42 % and 78 %, respectively. For the mulch layers, a novel cut-and-carry biofumigation method was used where 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, Indian mustard and berseem clover

consistently decreased mycotoxin contents in both years, i.e. DON by up to 50 %, 58 % and 56 %, and ZEN by up to 76 %, 71 % and 87 %, respectively.

Thirdly, we investigated the effect of maize-intercropping and cover cropping on FHB and mycotoxins in wheat under no-tillage and reduced tillage. In the first experimental year, the use of white mustard and Indian mustard as intercrops in maize decreased the DON content in grain of subsequent winter wheat by 58 % and 32 %, respectively, compared with sole maize and the effects were significant under both no-tillage and reduced tillage. However, maize-intercropping did not significantly reduce mycotoxins in wheat under very high disease pressure, which was the case in the second experimental year. Moreover, we showed that under no-tillage growing cover crops after silage maize, i.e. white mustard, Indian mustard and winter pea, effectively reduced FHB and mycotoxins in the following spring wheat, even to a similar extent as with deep ploughing of the maize debris. Nevertheless, we observed economic trade-offs of both maize-intercropping and cover cropping systems due to increased operating costs.

Fourthly, we investigated direct control strategies with mustard-based botanicals to combat *F. graminearum* using *in vitro* and *in planta* experimental systems. *In vitro*, the botanicals showed equal or higher efficacies in inhibiting mycelium growth than the fungicide treatment. Under controlled environment in the growth chamber, botanicals based on white mustard were as effective as the fungicide treatment in reducing DON in grain. The antifungal activity of the mustard may be attributed to its bioactive matrix containing isothiocyanates and phenolic acids. Nevertheless, under field conditions, only the application of fungicide significantly decreased FHB infection and DON in grain.

In conclusion, we shed light on alternative, environmentally sustainable crop protection strategies utilising innovative cropping systems and botanicals to reduce the risk of *Fusarium* mycotoxins in wheat. Cereal growers could benefit from the developed prevention strategies, i.e. cut-and-carry biofumigation, application of botanicals, cover cropping and intercropping, by decreasing the risk of mycotoxin contamination in harvest products and thus improving grain yield and quality. Innovation in cropping systems may lead to additional costs for growers, which results in conflicts between farm profitability and food safety goals. This conflict could be addressed by appropriate agricultural policies at both producer and consumer levels, fostering sustainable cultivation systems that contribute to safe food and feed.

Zusammenfassung

Die Ährenfusariose (AF) ist eine verheerende Pilzkrankheit in Getreide, welche weltweit zu grossen Ernteverlusten führt. Noch bedeutender ist die damit zusammenhängende Belastung von Körnern mit Mykotoxinen, wie zum Beispiel Deoxynivalenol (DON) und Zearalenon (ZEN). In den meisten Ländern der Welt ist Fusarium graminearum die vorherrschende Art innerhalb des AF-Komplexes in Weizen. Dieser Pilz überdauert in infizierten Ernterückständen von Mais und Weizen. Eine angepasste Fruchtfolge mit Nicht-Wirtspflanzen sowie die Bearbeitung der Ernterückstände mit dem Pflug sind daher wirksame Anbaumethoden zur Bekämpfung der AF. Wiederholtes Pflügen birgt jedoch mehrere Nachteile, wie zum Beispiel ein erhöhtes Risiko von Erosion und Reduktion der Bodenfruchtbarkeit. Zudem wird seit einigen Jahren angestrebt, den Einsatz von synthetischen Pflanzenschutzmitteln zu verringern, um deren negativen Auswirkungen auf die Umwelt und die menschliche Gesundheit zu vermindern. Das Hauptziel dieser Dissertation war die Untersuchung des Potenzials von Anbausystemen und Naturstoffen (Pflanzen-basierte Biopestizide) zur Reduktion von Fusarium-Infektionen und Mykotoxin-Belastungen in einer Mais-Weizen-Fruchtfolge ohne Pflugeinsatz.

Zunächst prüften wir die *in vitro*-Wirkung von Naturstoffen auf die Unterdrückung verschiedener Stadien im Lebenszyklus von F. graminearum. Wässrige Extrakte aus gemahlenen Weisssenf-Samen und gemahlener Chinesischer Galle (2 %) reduzierten das Myzelwachstum um bis zu 100 %, die Keimung von Konidien und Askosporen um bis zu 100 % bzw. 97 %. Zudem wurde die Bildung von Perithezien auf Maisstängeln um bis zu 56 % und das Ausschleudern der Askosporen aus den Perithezien um bis zu 77 % reduziert. Zusätzlich quantifizierten wir in den Senf-basierten Naturstoffen den Gehalt von Sinalbin, dem Hauptbestandteil der Senfölglycoside (Glucosinolate). Sinalbin ist die Vorläufersubstanz von p-Hydroxybenzylisothiocyanat, ein Stoff, der für seine antimikrobielle Wirkung bekannt ist. Ebenso stellten wir fest, dass Chinesische Galle verschiedene Gallotannine, Gallussäuren und Tanninsäuren enthält. Auch diese Substanzen verfügen über antimikrobielle Effekte.

Zweitens untersuchten wir Vermeidungsstrategien zur Bekämpfung der AF in einer simulierten Mais-Weizen-Fruchtfolge ohne Bodenbearbeitung. Maisterntereste wurden künstlich mit F. graminearum inokuliert und nach der Weizenaussaat in Feldparzellen auf die Bodenoberfläche ausgebracht. Zur Behandlung der F. graminearum-infizierten Maisernte-reste wurden entweder Naturstoffe oder frisches Mulchmaterial verwendet. Die Naturstoffe bestanden aus wässrigen Extrakten von Weisssenf und Chinesischer Galle. Die Wirkung der Naturstoffe war im zweiten Versuchsjahr stärker als im ersten Jahr und reduzierte den DON- und den ZEN-Gehalt in Weizenkörnern um bis zu 42 % bzw. 78 %. Für die Mulchbehandlung

wurde eine neuartige «cut-and-carry» Biofumigationsmethode verwendet. Dazu wurden Gründüngerpflanzen in separaten Feldern angebaut, im Herbst geerntet, gehäckselt und nach der Weizensaat direkt auf die inokulierten Maisterntereste appliziert. Mulchmaterial aus Weisssenf, Braunsenf und Alexandrinerklee verringerten die Mykotoxingehalte in beiden Versuchsjahren bei DON um bis zu 50 %, 58 % bzw. 56 % und bei ZEN um bis zu 76 %, 71 % bzw. 87 %.

Drittens ermittelten wir die Wirkung von Mais-Untersaaten und Zwischenfrüchten auf den AF-Befall und die Mykotoxin-Belastung in Weizen sowohl ohne als auch mit reduzierter Bodenbearbeitung. Im Vergleich zu Maisanbau ohne Untersaat verringerten Untersaaten mit Weisssenf und Braunsenf im ersten Versuchsjahr den DON-Gehalt in den Körnern des nachfolgenden Weizens um bis zu 58 % bzw. 32 % und die Wirkungen waren bei beiden Bodenbearbeitungssystemen statistisch gesichert. Jedoch führten die Untersaatensysteme im zweiten Jahr mit sehr hohem Krankheitsdruck nicht zu einer signifikanten Mykotoxinreduktion. Der Anbau der Zwischenfrüchte Weisssenf, Braunsenf oder Wintererbse nach der Maisernte reduzierte den AF-Befall und die Mykotoxingehalte im direkt gesäten Sommerweizen effizient und die Wirkung war sogar vergleichbar mit dem tiefen Unterpflügen der Maisterntereste. Aufgrund der erhöhten Betriebskosten stellten wir jedoch bei den Anbausystemen mit Untersaaten und Zwischenfrüchten wirtschaftliche Nachteile fest.

Viertens überprüften wir sowohl *in vitro* als auch *in planta* die Wirkung von Senf-basierten Naturstoffen als direkte Bekämpfungsmassnahme gegen F. graminearum. *In vitro* hemmten die Naturstoffe das Myzelwachstums ebenbürtig oder sogar stärker als die Fungizid-Behandlung. Unter kontrollierten Bedingungen in Klimakammern reduzierte der Naturstoff Weisssenf den DON-Gehalt in Weizenkörnern ebenso stark wie das Fungizid. Die antifungale Wirkung ist vermutlich auf die bioaktive Matrix mit Isothiocyanaten und Phenolsäuren zurückzuführen. Unter Feldbedingungen konnte jedoch nur die Fungizidbehandlung die AF-Infektion und den DON-Gehalt signifikant verringern.

Abschliessend ist es uns gelungen, Aufschluss über alternative und ökologische Pflanzenschutz-Strategien zu erhalten, welche das Risiko von Fusarium-Mykotoxinen in Weizen mittels innovativer Anbausysteme und Naturstoffen verringern. Im Rahmen von nachhaltigen Pflanzenschutzmassnahmen könnten Getreideproduzenten von den hier entwickelten Vermeidungsstrategien profitieren, namentlich «cut-and-carry»-Biofumigation, Einsatz von Naturstoffen sowie der Anbau von Untersaaten oder Zwischenfrüchten. Dadurch wird das Risiko von Mykotoxinbelastungen in Ernteprodukten verringert, wodurch gleichzeitig der Ertrag und die Qualität verbessert wird. Da innovative Anbausysteme zusätzliche Kosten für die Produzenten verursachen, könnte ein Zielkonflikt zwischen der Wirtschaftlichkeit des

Betriebs und der Lebensmittelsicherheit entstehen. Dieser Konflikt könnte durch geeignete agrarpolitische Massnahmen auf Ebene der Produzenten und der Konsumenten bewältigt werden, um nachhaltige Anbausysteme zu fördern, die einen Beitrag zu sicheren Lebens- und Futtermitteln leisten.

1. General introduction

1.1. MycoKey project

The current doctoral dissertation was part of the MycoKey EU project, and more specifically of the work packages 'prevention in the field' and 'intervention strategies' (Fig. 1.1). The MycoKey project aimed at addressing mycotoxin contamination along the food and feed chain using a holistic approach. The main targeted crops were wheat (*Triticum* sp.), maize (*Zea mays*) and barley (*Hordeum vulgare*). It consisted of six research areas, i.e. toxigenic fungi and mycotoxin monitoring, prevention in the field, intervention and remediation strategies as well as information and communication technology solutions for mycotoxin management.

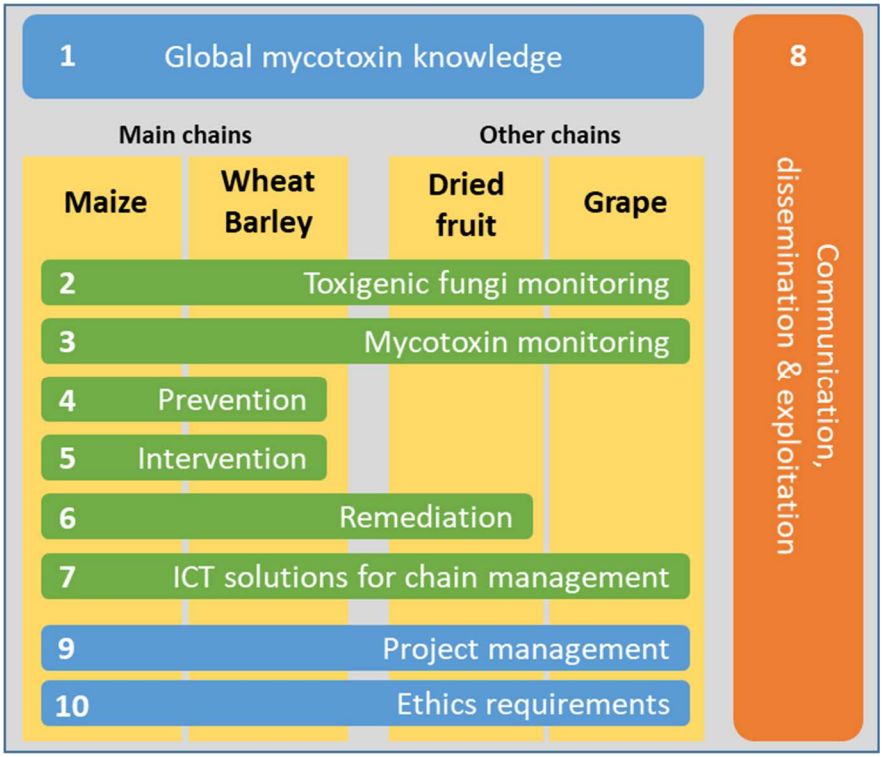

Fig. 1.1. Framework of the MycoKey project (adapted from MycoKey (2019)).

1.2. Fusarium head blight and mycotoxins in small-grain cereals

Fusarium head blight (FHB) is one of the most important cereal diseases worldwide causing not only significant reductions in grain yield but also severe contaminations of the harvested products with mycotoxins, jeopardising food and feed safety (Parry et al., 1995). Severe epidemics in the 1990s affected wheat growers in Europe, the United States, Canada, Argentina and China (Singh et al., 2016). For example, between 1998 and 2000, FHB caused

an economic loss of approximately $2.7 billion in the United States (Nganje et al., 2004). In most parts of the world, the predominant species of FHB in wheat is *Fusarium graminearum* (Osborne and Stein, 2007). Depending on the geographic region and the grain crop, less frequently observed FHB species are *F. poae*, *F. avenaceum*, *F. culmorum*, *F. cerealis* and the non-toxigenic *Microdochium* species (Bottalico and Perrone, 2002; Xu et al., 2005).

F. graminearum is an ascomycete with the ability to develop both asexually and sexually (teleomorph *Gibberella zeae*) producing macroconidia and ascospores, respectively (Fig. 1.2). The fungus overwinters on previously infected crop residues, such as maize stalks and wheat straw, as mycelium and/or through chlamydospores (Dweba et al., 2017). The fruiting bodies, named perithecia, are formed on the surface of the crop residues and contain ascospores. The latter are forcibly discharged from mature perithecia during spring when the weather conditions are favourable (i.e. warm and humid), which frequently coincides with the anthesis period of small-grain cereals. The ascospores reaching the cereal head with the aid of wind are responsible for the primary infection. A secondary infection pathway takes place with rain-splashed macroconidia, derived by phialides and clustered in cushion-shaped masses called sporodochia. Subsequently, the fungus colonises the florets and, depending on the disease severity, the kernels may shrink or do not develop at all. Temperature, relative humidity (RH) and light are crucial factors for the growth and development of the fungus, while the fungal survival is enhanced by higher amounts of crop residues (Leplat et al., 2013). The optimum temperature for perithecia production ranges from 15 to 28.5°C, and for production of ascospores, from 25 to 28°C (Gilbert and Tekauz, 2000). Although light is not a requirement for the discharge of ascospores, the rate of ascospore release was found to be 8 to 30 % higher under light conditions compared with complete darkness (Trail et al., 2002). The most favourable conditions for infection occur after at least a 24h-period of warm (~25°C) and humid (~100 % RH) weather.

Upon infection of the inflorescences, several *Fusarium* species produce health-threatening secondary metabolites, named mycotoxins, such as deoxynivalenol (DON), zearalenone (ZEN) and nivalenol (NIV). The main producer of DON and ZEN is *F. graminearum* (Bottalico and Perrone, 2002), while NIV is closer related to infection by *F. poae* (Vogelgsang et al., 2019). The mycotoxin DON is a common contaminant of cereals associated with acute and chronic effects, such as vomiting, feed refusal and immune disorders (D'Mello et al., 1999). The toxicity of ZEN is linked to reproductive problems in animals and possibly in humans due to estrogenic effects (Marin et al., 2013). Therefore, the European Commission and other countries around the world have set maximum limits for DON and ZEN in human food and guidance levels in animal feed (Anonymous, 2006; Ferrigo et al., 2016). Nevertheless, special attention should

also be given to unregulated mycotoxins, such as NIV, diacetoxyscirpenol (DAS) and moniliformin (MON), since they have been suggested to cause adverse health effects. For example, feed contaminated with NIV or DAS resulted in reduced liver and body weight in broiler chicken, respectively (D'Mello et al., 1999). Moreover, MON, which is commonly produced by *F. avenaceum*, causes haematotoxicity and cardiotoxicity in humans and animals (Knutsen et al., 2018). Risk assessments should also target other emerging mycotoxins, such as enniatins, beauvericin and fusaric acid, which frequently occur in agricultural products posing potential health risks (Gruber-Dorninger et al., 2016).

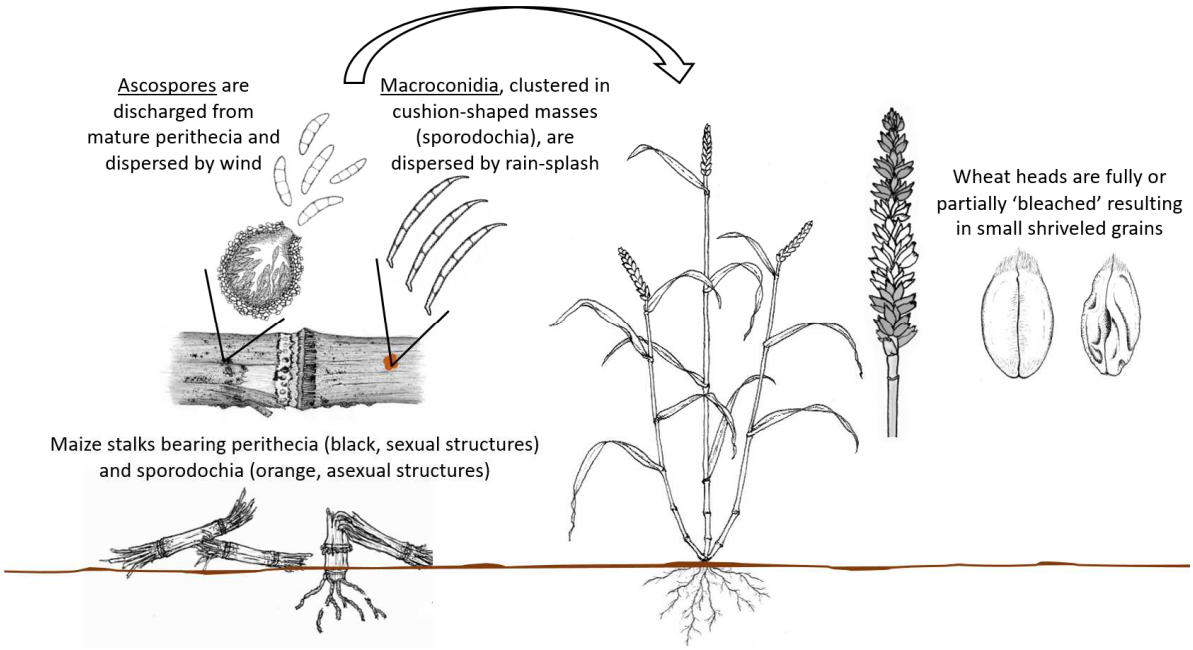

Fig. 1.2. Simplified life cycle of *Fusarium graminearum* (teleomorph *Gibberella zeae*) in a maize-wheat rotation. The individual components of this figure were drawn by Jonas Lehner, Agroscope.

1.3. Control of Fusarium head blight in small-grain cereals

Suitable crop rotation with non-host species and management of crop residues with deep ploughing are effective strategies to prevent FHB in small-grain cereals (Gilbert and Haber, 2013; Tian et al., 2016). An eight-year *Fusarium* and mycotoxin survey in wheat in Switzerland demonstrated that DON content was highest in grain samples from fields with the previous crop being maize cultivated under reduced tillage (Vogelgsang et al., 2019). Likewise, a two-year *Fusarium* mycotoxin survey in barley showed that the lowest incidence of *F. graminearum* and mycotoxin accumulation in grain occurred when the previous crop was non-cereal and when fields were ploughed before sowing (Schöneberg et al., 2016). However, cereal growers are commonly cultivating maize before small-grain cereals increasing the risk of FHB outbreaks and mycotoxin contamination (Vogelgsang et al., 2017).

Control of FHB with less susceptible cultivars and synthetic fungicides are additional crop protection measures among cereal growers. However, the majority of wheat cultivars still rate from medium to high susceptibility and the efficacy of fungicides is frequently inconsistent (Beres et al., 2018). The latter is mainly due to the short time frame for application (i.e. anthesis period), heterogeneous anthesis and development of resistant fungal strains (Wegulo et al., 2015). Although the triazole-based products are the most effective synthetic fungicides for the control of FHB, a meta-analysis of over 100 efficacy trials showed that the average reduction of DON in wheat ranged between 12 % and 52 %, depending on the triazole group (Paul et al., 2008). Moreover, strobilurin-based fungicides frequently increased FHB and DON content in grain (Forrer et al., 2000; Dubos et al., 2011; Vogelgsang et al., 2019).

Recently, there is a movement towards reduced reliance on synthetic pesticides to minimise the potential negative impact on the environment. For example, new pesticide registration procedures, such as the Food Quality Protection Act in the USA, have reduced the availability of synthetic pesticides (Dayan et al., 2009). Moreover, the EU has established regulations on maximum residue levels of pesticides in food and feed (Anonymous, 2005). Therefore, alternative crop protection strategies are needed to prevent FHB and mycotoxin contamination in small-grain cereals. Within this context, innovative cropping systems, such as intercropping and cover crops, as well as plant-based biopesticides could provide viable solutions for cereal growers to control FHB.

1.3.1. Cropping systems

Conventional tillage with mouldboard plough has historically been used for the management of crop residues, weed control, seedbed preparation and the reduction of disease inoculum. Nevertheless, intensive ploughing has several drawbacks, such as increased risks of soil erosion and soil degradation (Triplett and Dick, 2008). Non-inversion tillage practices within the broad term ‘conservation tillage’ have been extensively studied during the past decades as a more sustainable land management strategy aiming to preserve soil quality (Busari et al., 2015). However, in such systems, the cereal crop residues represent an important inoculum source for certain *Fusarium* species, infecting the subsequent cereal crop (Fig. 1.3). Although conventional tillage is generally considered as an effective agronomic practice to control FHB, it is not clear yet whether disease severity differs significantly between reduced and no-tillage (Gilbert and Fernando, 2004). For example, Dill-Macky and Jones (2000) found reduced FHB incidence and severity with mouldboard plough compared with chisel plough and no-tillage, but the differences between the two latter practices were not significant. An on-farm study on 14 zero tillage sites with a maize-wheat rotation showed that the risk of FHB infection and DON contamination was not adequately decreased through different mulching techniques

(Vogelgsang et al., 2011). Hence, reduced tillage or no-tillage systems should probably be complemented with other farming practices, such as intercropping and cover cropping, in order to prevent FHB infection and mycotoxins in small-grain cereals.

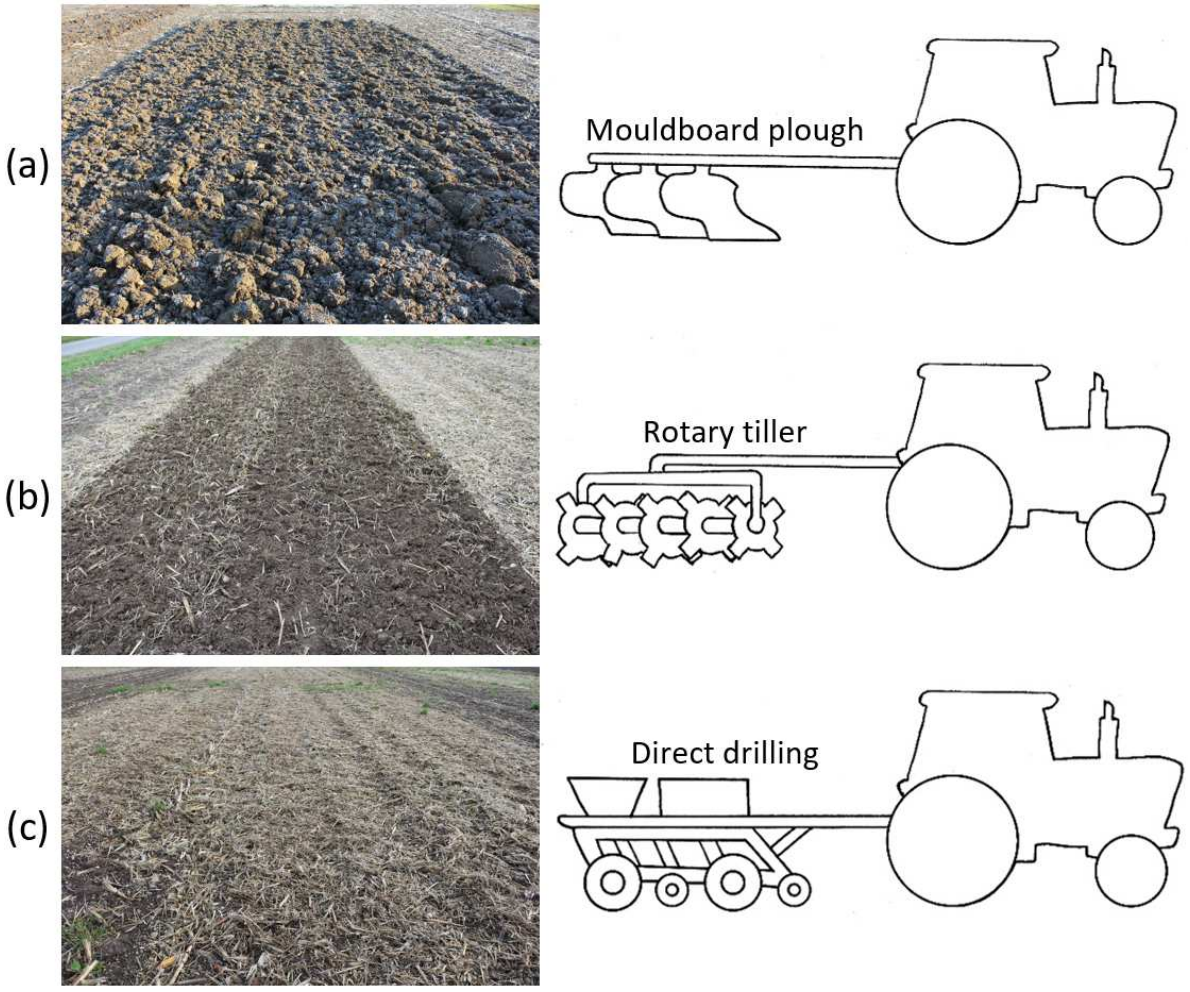

Fig. 1.3. Amount of remaining maize residues under different tillage systems: (a) no residues under conventional tillage with mouldboard plough; (b) intermediate amount of residues under reduced tillage with rotary tiller, adjusted to till the top 10-cm soil layer; (c) high amount of residues under no-tillage with mulching followed by direct drilling (pictures taken at Agroscope-Tänikon, 8356 Ettenhausen, Switzerland; drawings by Jonas Lehner, Agroscope).

Intercropping is a farming practice that involves two or more crop species, or genotypes, growing together (Fig. 1.4). Intercropping has numerous potential advantages as opposed to conventional sole cropping systems and could therefore support sustainable intensification of agroecosystems (Brooker et al., 2015). The increased on-farm biodiversity can improve the overall productivity and enhance ecosystem services through augmented resource-use efficiency. For example, synergistic effects occur when intercropped species with complementary traits interact positively resulting in increased overall productivity. Crop yields and nutrient acquisitions have been found higher for intercropped wheat, maize and soybean

compared with sole cropping of the same species, suggesting interspecific facilitation in terms of available nutrients during crop growth (Li et al., 2001). Other net benefits of intercropping are the attraction of pollinators and naturally occurring enemies of pests and pathogens, greater carbon assimilation and weed suppression. Experiments in organic fields across western Europe showed that weed biomass was 3-fold lower in pea-barley intercropping compared with pea as sole crops (Corre-Hellou et al., 2011). Mixed and row intercropping of bean with maize reduced common bacterial blight and rust diseases of bean compared with sole cropping (Fininsa, 1996). Thus, maize intercropping with plant species possessing antimicrobial properties, such as mustard containing glucosinolates, could provide solutions as a sustainable crop protection strategy against FHB in the subsequent wheat crop.

Fig. 1.4. Example of maize intercropping with red clover (left) and maize as a sole crop (right) in Agroscope-Tänikon, 8356 Ettenhausen, Switzerland.

Cover crops are commonly grown before or after cash crops in a rotation and have multiple benefits for agroecosystem services. These include suppression of pests and diseases, enhancement of inherent soil fertility, decreased soil erosion and weed suppression (Everts, 2002; Scholberg et al., 2010; Madsen et al., 2016). Cover crops can interfere with the disease cycle of certain pathogens, such as survival and propagation, and minimise disease development in subsequent crops. Several studies have shown that growing cover crops can reduce the incidence of soil-borne diseases through inducing soil suppressiveness (Scholberg et al., 2010). Mustard plants belonging to the Brassicaceae family, such as Indian or oriental mustard (*Brassica juncea*) and white mustard (*Sinapis alba*), are widely used cover crops with biofumigation properties (Brown and Morra, 1997; Weil and Kremen, 2007). Upon tissue disruption, the enzyme myrosinase hydrolyses the glucosinolates releasing the isothiocyanate (ITC) reactive compounds into the soil, which have potent antifungal activity (Fig. 1.5). For example, soil incorporation of *Brassica juncea* was an effective strategy to control Rhizoctonia root rot of sugar beet (Motisi et al., 2009), as well as powdery scab and common scab diseases in potato (Larkin and Griffin, 2007). Although most of the Brassicaceae species do not establish

symbiosis with vesicular-arbuscular mycorrhizal fungi (Glenn et al., 1988), it was earlier shown that control of *Pythium ultimum* in sugar beet was improved by an integrated application of *Trichoderma* species with *Brassica carinata* seed meal (Galletti et al., 2008). Management practices that disrupt cover crop-mediated benefits are frequent tillage and the application of herbicides and fungicides (Vukicevich et al., 2016). Wittwer et al. (2017) showed that especially under reduced tillage intensity or when production is converted to organic, the inclusion of cover crops is essential to maintain crop yields at a certain level. Thus, cover crops could suppress important residue-borne diseases, such as FHB in small-grain cereals, and enhance crop yields under conservation tillage practices.

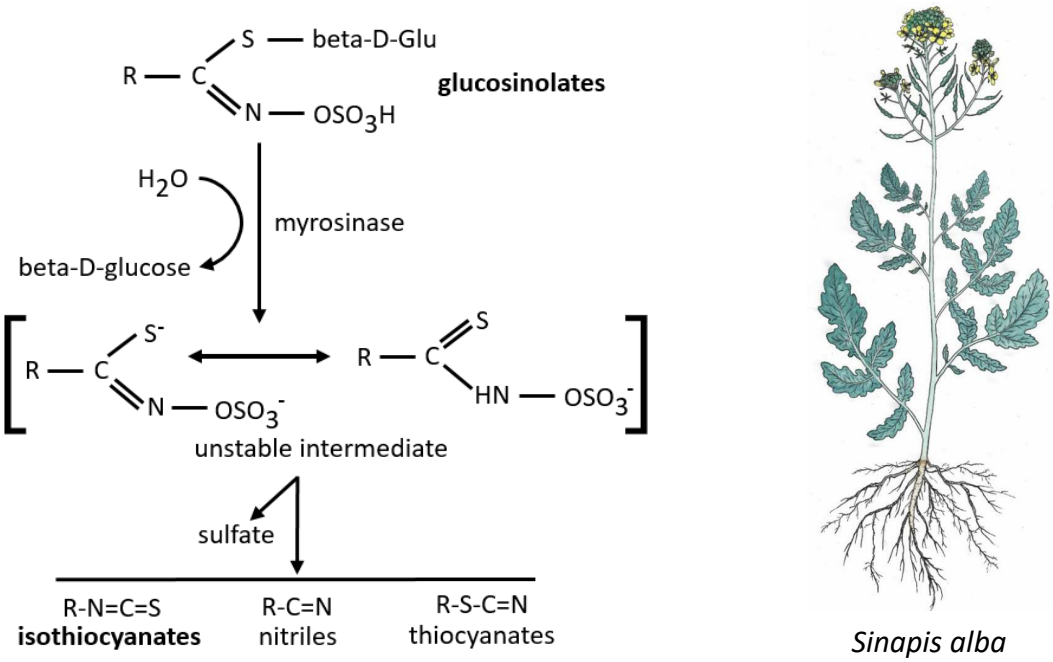

Fig. 1.5. Enzymatic hydrolysis of glucosinolates via myrosinase upon plant tissue disruption. Adapted from Zhou et al. (2012). The white mustard plant was drawn by Jonas Lehner, Agroscope.

Cut-and-carry green manures is an emerging fertilisation strategy where the aboveground biomass of crops with high nitrogen content, e.g. legumes or grass-clover mixtures, is collected and transported to another field to fertilise the cash crop. Cut-and-carry green manures are commonly used in organically managed fields when the use of organic fertilisers, e.g. farmyard manure, is limited. Moreover, following this fertilisation strategy, essential soil nutrients and organic matter from the cover crops remain within the farm boundaries instead of exporting them as sold fodder. Besides the benefits of fertilisation, the cut-and-carry approach should also be investigated for the potential to suppress residue-borne diseases. For instance, cut-and-carry biofumigation (Fig. 1.6) using the aboveground biomass of crops with antimicrobial properties, such as mustard, could suppress overwintering FHB inoculum.

Fig. 1.6. An example of cut-and-carry biofumigation in Agroscope-Reckenholz, 8046 Zurich, Switzerland; cut plant material from white mustard, which was grown in a separate field, provides thorough coverage of maize stalks in a wheat field with no-tillage.

1.3.2. Plant-based biopesticides

Within the context of minimising the impact of synthetic pesticides on the environment and the arising public concern about health associated risks, natural products have been promoted during the last decades. Plant-based biopesticides, i.e. botanicals, have been applied over millennia in ancient Egypt, China, Greece and India by farmers to protect crops against pests and diseases (Isman, 2006). Some examples are the essential oils from rosemary (*Rosmarinus officinale*), eucalyptus (*Eucalyptus globus*), clove (*Syzygium aromaticum*), thyme (*Thymus vulgaris*) and mint (*Mentha* species) which have been used for their fumigant and insecticidal activities (Isman and Machial, 2006). As the active ingredients of most botanical products are composed of plant secondary metabolites, they are usually degrading quicker than formulated synthetic agrochemicals, minimising the potential risks towards non-target organisms and the environment (Regnault-Roger and Philogène, 2008). On the other hand, the quick degradation of the active ingredients could negatively impact the control efficacy of plant-based biopesticides.

Botanicals from seed flour of white mustard (*Sinapis alba*) and oriental mustard (*Brassica juncea*) are characterised by their potent antimicrobial activity, which is mainly associated with the glucosinolate-derived ITC (Saladino et al., 2016). The oriental mustard contains elevated amounts of the glucosinolate sinigrin, which is hydrolysed into allyl ITC, while the white mustard mainly contains the glucosinolate sinalbin, which is hydrolysed into p-hydroxybenzyl ITC. Studies on mustard-based whole products or specific substances have mainly focused on food spoilage bacteria and fungi for potential uses as food preservatives. For instance, allyl ITC reduced the growth of the mycotoxigenic fungi *Aspergillus parasiticus* and *Penicillium expansum* (Manyes et al., 2015), while p-hydroxybenzyl ITC showed good efficacy against *Salmonella* sp. (David et al., 2013). Apart from the bioactive ITC compounds, Brassicas also contain phenolic acids with antimicrobial activity, such as ferulic and benzoic acids. Some of the suggested mode of actions include cell wall damage, disruption of plasma membrane and inhibition of isocitrate lyase enzyme activity (Teodoro et al., 2015). To the best of our knowledge, studies on the effect of mustard-based products on *F. graminearum* are rather limited to *in vitro* experiments (Sarwar et al., 1998; Ekanayake et al., 2006).

Chinese galls (*Galla chinensis*) is a traditional botanical used in human medicine with potent antimicrobial activity due to the elevated amounts of gallotannins as well as gallic and tannic acids (Tian et al., 2009a; Djakpo and Yao, 2010). Gallotannins interact with membrane proteins of microorganisms by means of hydrogen bonding through their hydroxyl groups causing changes in membrane permeability and cell destruction (Burt, 2004). Vogelgsang et al. (2013) demonstrated that Chinese galls fully inhibited mycelium growth and greatly reduced conidia germination of *Microdochium majus*, an important seed and soil-borne pathogen of winter cereals. Moreover, the authors demonstrated that Chinese galls applied to seeds improved plant emergence and increased yield of winter wheat (Vogelgsang et al., 2013). Moreover, other *in vitro* studies showed that Chinese galls greatly inhibited conidia germination and mycelium growth of *F. graminearum* (Forrer et al., 2014). However, we need to test the bioactivity of this botanical against other important fungal structures of *F. graminearum*, such as perithecia formation and ascospores germination.

1.4. Objectives

The main objective of the current doctoral thesis was to explore the potential of innovative cropping systems and plant-based biopesticides to reduce Fusarium head blight and mycotoxin contamination in wheat.

The specific aims explored in the different chapters were:

- Examine whether botanicals are able to suppress different stages of the life cycle of *Fusarium graminearum in vitro* [**chapter I**]. Depending on the control efficacy of the botanical treatments against different developmental stages of the fungus, explore their potential *in planta*.
- Investigate whether prevention strategies in the field can suppress *Fusarium* inoculum and reduce mycotoxins in wheat using cut-and-carry biofumigation and botanicals [**chapter II**]; intercropping and cover cropping [**chapter III**].
- Elucidate whether mustard-based botanicals are able to control FHB and reduce mycotoxins in wheat, both *in vitro* and *in planta* [**chapter IV**].

1.5. Research framework

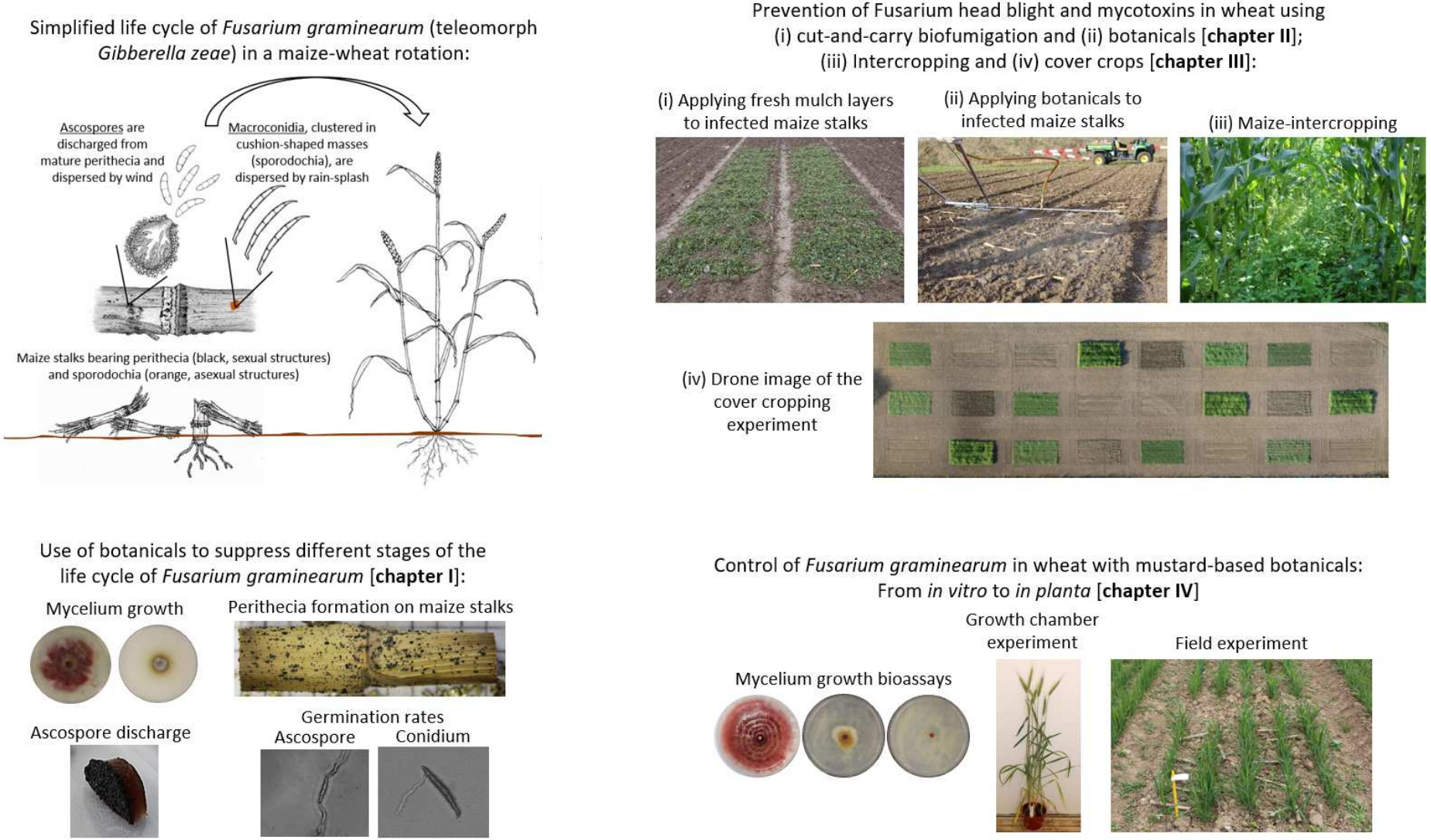

Fig. 1.7. Research framework summarising the experimental approaches of the current doctoral study.

1.6. Scientific publications

In the course of this doctoral study, the following manuscripts were prepared:

- Chapter I. "Use of botanicals to suppress different stages of the life cycle of *Fusarium graminearum*" (Drakopoulos et al., 2019).
- Chapter II. "Prevention of Fusarium head blight infection and mycotoxins in wheat with cut-and-carry biofumigation and botanicals" (Drakopoulos et al., 2020).
- Chapter III. "Innovative cropping systems to improve food safety in wheat: An agronomic and economic assessment" (Drakopoulos et al., in preparation).
- Chapter IV. "Control of *Fusarium graminearum* in wheat with mustard-based botanicals: from *in vitro* to *in planta*" (Drakopoulos et al., submitted).
- Co-authorship in the manuscript "From laboratory to the field: Biological control of *Fusarium graminearum* on infected maize crop residues" (Gimeno et al., 2020).

2. Chapter I. Use of botanicals to suppress different stages of the life cycle of *Fusarium graminearum*

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Abstract

Fusarium head blight (FHB) is one of the most important cereal diseases worldwide causing yield loss and contamination of harvested products with mycotoxins. *Fusarium graminearum* (FG) is one of the most common FHB causing-species in wheat and barley cropping systems. We assessed the ability of different botanical extracts to suppress essential stages of the fungal life cycle using three strains of FG (FG0410, FG2113, and FG1145). The botanicals included aqueous extracts from white mustard (*Sinapis alba*) seed flour (Pure Yellow Mustard (PYM) and Tillecur® (Ti)), as well as milled Chinese galls (CG). At 2 % concentration (w v^{-1}), PYM and Ti completely inhibited growth of mycelium of all FG strains, while at 1 %, CG reduced the growth by 65-83 %, depending on the strain. While PYM and Ti reduced the germination of both conidia and ascospores at 2 % w v^{-1}, CG was only effective in reducing conidia germination. Perithecia formation of FG0410, but not of FG2113 was suppressed by all botanicals. Moreover, application of botanicals on mature perithecia led to a 2- to 4-fold reduction in discharge of ascospores. Using liquid chromatography (LC) with diode array detection, we quantified the principal glucosinolate component of PYM and Ti as sinalbin. LC time-of-flight mass spectrometry was used to demonstrate that the bioactive matrix of CG contains different gallotannins as well as gallic and tannic acids. Possible antifungal mechanisms of the botanical matrices are discussed. The results of this study are promising and suggest that PYM, Ti and CG should be explored further for efficacy at managing FHB.

Keywords: biological control; botanicals; disease control; *Fusarium graminearum*; mycology

Introduction

Fusarium head blight (FHB) is a devastating cereal disease worldwide. FHB causes yield loss and can result in severe contaminations of grain with mycotoxins, jeopardizing food and feed safety (McMullen et al., 2012). Worldwide, *Fusarium graminearum* (FG) (teleomorph *Gibberella zeae*) is one of the most common causes of FHB in wheat and barley cropping systems (Osborne and Stein, 2007). FG produces the mycotoxins deoxynivalenol (DON) and zearalenone (ZEN), which can induce acute and chronic health issues in humans and animals when ingested through contaminated food or feed (Danicke and Brezina, 2013; Antonissen et al., 2014). Chronic effects of DON in animals can result in weight loss, anorexia, and increased susceptibility to facultative pathogens (Marin et al., 2013), while estrogenic effects in swine and cows have been associated with ZEN (D'Mello et al., 1999). Human exposure to DON mainly affects the function of the intestines, immune system, and brain (Escrivá et al., 2015).

The life cycle of FG has been thoroughly studied and described (Parry et al., 1995; Trail, 2009; Dweba et al., 2017). Briefly, FG is an ascomycete with the ability to reproduce both asexually and sexually through conidia and ascospores, respectively. The fungus frequently overwinters on infected crop residues, such as maize stalks, mainly as mycelium. In spring, ascospores are forcibly discharged from mature perithecia when weather conditions are favourable (i.e. warm and humid) and infect wheat heads, mostly during anthesis. In contrast, conidia are dispersed on cereal heads and other plant residues by rain-splash. Subsequently, the fungus colonizes the florets and, depending on the severity of infection, kernels develop poorly or not at all. Field management of FHB caused by FG can be categorized into cultural, chemical and biological methods (McMullen et al., 2012; Shah et al., 2018). Cultural strategies mainly consist of optimized crop rotations with non-host crops, tillage practices to bury the crop residues, and use of less susceptible cultivars (Blandino et al., 2012; Gilbert and Haber, 2013). Chemical and biological strategies include direct control using fungicides (Amarasinghe et al., 2013) or biocontrol agents (Palazzini et al., 2007; Zhao et al., 2014; Palazzini et al., 2017), respectively.

Within the context of minimising the use of conventional crop protection products and to improve food and feed safety there is increasing interest in the use of natural, more environmentally-friendly plant-based compounds (i.e. botanicals) to control fungal diseases (Sellam et al., 2007; Dayan et al., 2009). Plants belonging to the Brassicaceae family contain glucosinolates, which are secondary metabolites that are hydrolysed by the enzyme myrosinase into three groups of substances: nitriles, thiocyanates, and isothiocyanates (ITCs) (Zhang and Talalay, 1994). The effects of the latter have been investigated extensively and

shown to have antimicrobial, herbicidal, antioxidant, and anticancer activity (Vig et al., 2009; Hyldgaard et al., 2012; Romeo et al., 2018). For example, extracts from white mustard (*Sinapis alba*) seed meal reduced the growth of several fungal species belonging to the genera *Fusarium, Aspergillus*, and *Penicillium* and this effect was attributed to p-hydroxybenzyl ITC (p-HBITC) (Quiles et al., 2018). Chinese galls (*Galla chinensis*), a traditional herb rich in gallotannins as well as gallic and tannic acids, are used in human medicine and have also been shown to display antimicrobial activities (Tian et al., 2009a; Djakpo and Yao, 2010).

To the best of our knowledge, there have been no studies on the effects of botanicals on different stages of the life cycle of FG. Thus, to better understand the effect of botanicals on critical stages of the life cycle of FG, we tested two mustard-based botanicals and extracts of Chinese galls using *in vitro* bioassays. Firstly, we measured the impacts of the botanicals on mycelium growth, conidia and ascospore germination, development of perithecia on maize stalks, and ascospore discharge from mature perithecia (Fig. I.1.). Secondly, we analysed the chemical composition of the mustard-based botanicals and the Chinese galls using liquid chromatography (LC) coupled to diode array detection or LC time-of-flight mass spectrometry, respectively.

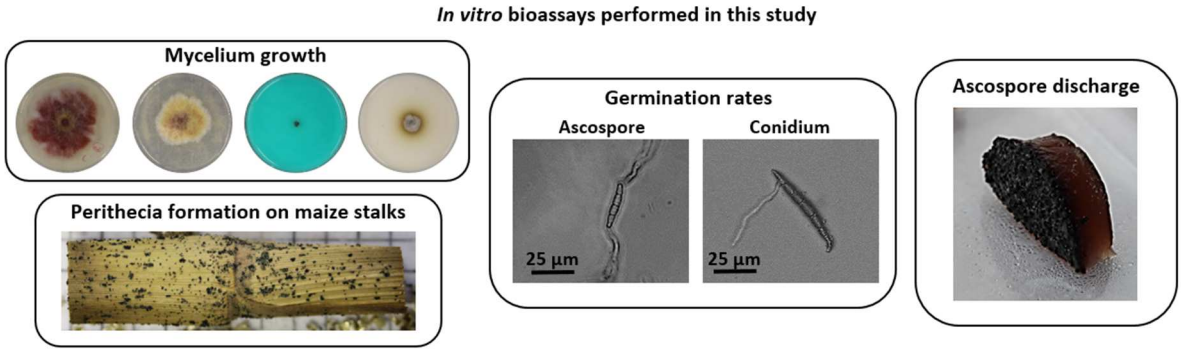

Fig. I.1. Components of the life cycle of *Fusarium graminearum* targeted to assess the suppressive effect of different botanicals using *in vitro* bioassays.

Materials and methods

Fungal strains and botanicals

The fungal strains FG0410 (CBS 121292; Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands), FG2113 (Research Group Crop Breeding and Genetic Resources, Plant pathology, Agroscope, Nyon, Switzerland), and FG1145 (Fungal Collection of Agroscope, Nyon, Switzerland) were isolated from wheat in Switzerland in 2004, 2011, and 1992, respectively. All strains are single-conidial isolates and were identified as 15-acetyldeoxynivalenol genotypes following the methods as described in Pasquali et al. (2011) for FG0410 and in Quarta et al. (2006) for FG2113 and FG1145. The botanicals Tillecur® (Ti; BIOFA, Germany), Pure Yellow Mustard (PYM; product 106, G. S. Dunn, Dry Mustard Millers, Canada), and Chinese galls (CG; origin Sichuan, P. R. China; purchased from Berg-Apotheke, Zurich, Switzerland) were used in this study and were purchased in a powder form. In organic agriculture, Ti is applied as seed treatment in cereals to protect against common bunt (*Tilletia caries*, synonym *T. tritici*). Ti and PYM are based on white mustard seed flour. Among other uses, CG is a raw material for industrial production of tannic acid and a detailed description of this botanical is provided by Tian et al. (2009b). Preliminary experiments were performed in order to determine the final protocols and the range in botanical concentrations for the bioassays.

Mycelium growth and pH

Ti and PYM were tested at 0.5 %, 1 %, and 2 % w v^{-1} and CG at 0.5 % and 1 % w v^{-1}. Potato dextrose agar (PDA; Oxoid Ltd, Basingstoke, UK) was prepared in Schott flasks and autoclaved at 121°C for 15 min. Flasks were cooled to 50°C before adding 0.1 g $liter^{-1}$ streptomycin sulphate (Sigma-Aldrich, St. Louis, MI, USA) and the desired quantities of botanicals (as powder). Subsequently, 20 ml of each PDA / botanical mixture was poured into Petri dishes (94 × 16 mm; Greiner Bio-One, Austria). There were four replicates of each treatment and a control on PDA without botanicals. Mycelial plugs of 0.5 cm diameter were cut using a cork borer from the margins of 7 to 14 days old FG colonies, grown at 18°C with a photoperiod of 12 h under near-ultraviolet light, and placed in the center of each dish with the mycelial side facing the agar. Dishes were incubated in the dark at 20°C and 80 % relative humidity (RH). After 6 to 8 days, when the fastest growing fungal colony had covered 90 % of the surface, the colony diameter was measured with a ruler. The bioassay was conducted twice for all strains. The pH of the botanicals was measured after suspending in deionized water (dH_2O) and after incorporation in PDA with a pH-meter (Hanna Instruments Inc., USA).

Conidia germination

Fresh cultures of FG0410, FG2113, and FG1145 were cultured at 18°C and a 12 h photoperiod under near-ultraviolet light. Conidial suspensions of each strain were prepared by flushing 7 to 10 days old cultures with dH_2O. The surface of the colonies was agitated using a Drigalski spatula and conidial suspensions adjusted to 2×10^5 conidia ml^{-1} using a hemocytometer (Thoma Counting Chamber, Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). Botanical treatments were tested at 0.5 %, 1 %, and 2 % w v^{-1}. Botanical powders were suspended in sterile dH_2O and stirred with a magnet for 2 h at room temperature. A microscope slide (76 × 26 × 1 mm; Paul Marienfeld GmbH & Co. KG, Germany) was placed in a Petri dish on a moistened filter paper (Ø 8.5 cm, Nr. 591, Schleicher & Schuell BioScience GmbH, Dassel, Germany). Four agar plugs, each of 1 cm diameter (20 g agar agar pulver (ERNE Surface SA, Avenches, Switzerland), amended with 0.1 g streptomycin sulphate (Sigma-Aldrich, USA) per L dH_2O), were placed on the microscope slide and served as replicates. A 15 µL aliquot of each botanical suspension was pipetted onto each plug. For the control treatment, sterile dH_2O was used. The surfaces of the agar plugs were allowed to dry and a 15 µL aliquot of the conidial suspension was pipetted onto each plug. Closed dishes were incubated in the dark at 14°C and 70 % RH for 18 h. Conidia were killed by applying 15 µL fungicide (0.19 % Pronto® Plus, Bayer Schweiz AG, Zollikofen, Switzerland) per agar plug. Germination rates were assessed with the aid of a light microscope (× 400 magnification) by determining the ratio of germinated and non-germinated spores in a sample of 40 conidia per plug in 4 to 6 fields of vision. A conidium was defined as germinated when the germination tube was longer than its width. The bioassay was conducted twice for all strains.

Ascospore germination

Production of perithecia from strains FG0410 and FG2113 was performed following a modified protocol (Klittich and Leslie, 1988; Manstretta et al., 2016) using carrot agar as the culture medium. The strain FG1145 was not included due to its very low germination rate. Fresh organic carrots (400 g) were washed, diced, and autoclaved for 15 min at 121°C in 400 ml dH_2O. Autoclaved carrots were pureed, mixed with an additional 500 ml dH_2O and 20 g agar and autoclaved again. After cooling to 50°C, 0.1 g $liter^{-1}$ streptomycin sulphate (Sigma-Aldrich, USA) was added. Aliquots of 12 ml of the medium were dispensed into small Petri dishes (60 × 15 mm). Petri dishes were inoculated with the desired strain of FG by placing a 0.5 cm diameter agar plug in the center of each dish. Agar plugs were obtained from 5 to 7 days old cultures of FG growing on PDA incubated at 18°C with a 12 h photoperiod under near-ultraviolet light. After inoculation, Petri dishes were incubated in the dark at 18°C for 4 to 6 days until mycelium had reached the outer edge of the dish. Subsequently, the aerial

mycelium was gently removed under sterile conditions and 1 ml of 2.5 % Tween®20 (Sigma-Aldrich, USA) was uniformly spread on the agar surface with a Drigalski spatula. The agar surface was allowed to dry before closing the dishes, which were incubated at 18°C with a 12 h photoperiod under near-ultraviolet light. After 1 to 2 weeks, ascospore discharge began from mature perithecia and were dispersed onto the inner lid surface of the Petri dishes (Schöneberg et al., 2015). Ascospores were collected by washing the inner surface of each lid twice with 1 ml sterile dH_2O and adjusting the suspension to 3×10^5 ascospores ml^{-1}. Botanicals were tested at 0.5 %, 1 %, and 2 % w v^{-1} and the ascospore germination assessment was performed following the same protocol as for conidia. The bioassay was conducted twice for each strain.

Perithecia formation on maize stalks

Maize stalks were collected from a field at the Agroscope-Reckenholz research station in Zurich, Switzerland. Stalks were dried at 30°C for 5 days and cut in 5 cm pieces, which included one node in the midpoint. Each stalk piece was cut in half longitudinally. Subsequently, stalks were immersed overnight in dH_2O, autoclaved twice for 15 min at 121°C and cooled to room temperature. The bioassay was conducted with strains FG0410 and FG2113 (FG1145 was not included due to its inability to produce sufficient perithecia). Autoclaved stalks were inoculated with a suspension containing 2×10^5 conidia ml^{-1} and 0.0125 % Tween®20 which was stirred for 5 min. Botanical powders were dissolved in 500 ml sterile dH_2O (2 % w v^{-1}) and stirred for 2 h. After inoculation, stalks were immersed in the solution containing the botanicals and stirred for 2 min. For the water control, stalks were immersed in sterile dH_2O and stirred for 2 min. For the control, stalks were not immersed in either water or botanical solution. Following the treatment application, two stalks were placed into a Petri dish (140 × 20 mm), containing 20 g water-saturated autoclaved vermiculite (ISOLA Vermiculite AG, Bözen, Switzerland), and incubated at 18°C with a 12 h photoperiod under near-ultraviolet light for approximately 3 weeks until perithecia were formed. One Petri dish represented one replicate and each treatment included six replicates. Images of stalk sections were captured with a digital camera (Canon EOS 750D) using a 60 mm EFS macro lens. Perithecia were counted along the full length of the stalk following a 1 cm wide transect centered on the middle of the longitudinally cut half stalk. Average values of perithecia on both stalk sections per Petri dish were calculated for each replicate. The bioassay was conducted twice for each strain.

Ascospore discharge

The ascospore discharge bioassay protocol of Trail et al. (2002) was modified to suit the requirements of the FG strains that were used. The bioassay was conducted with strains FG0410 and FG2113. Perithecia were produced on carrot agar using the protocol described in the previous section. When perithecia were mature, carrot agar plugs (1 cm diameter) were removed with a cork borer and the agar cores split longitudinally using a surgical blade ("half-plugs"). Four half-plugs were placed flat side down on the surface of a microscope slide (76 × 26 × 1 mm). Botanical powders were suspended in sterile dH_2O (2 % w v^{-1}) and stirred for 2 h at room temperature. Aliquots of 20 µl botanical suspension or sterile dH_2O for the control were applied directly on the upper semicircle side of each half-plug. The microscope slides were placed on moistened filter paper in transparent plastic boxes (80 × 50 × 25 mm). One box with four half-plugs represented one replicate, with six replicates of each treatment. Closed boxes were incubated at 20°C for 24 h under continuous near-ultraviolet light. Half-plugs were removed and the discharged ascospores were collected by washing each slide with 1 ml sterile dH_2O using a pipette and transferring the solution into 2-ml Eppendorf tubes. Spores were quantified with a hemocytometer. The bioassay was conducted twice.

Glucosinolate extraction and determination in mustards

Glucosinolates from PYM and Ti were extracted using a modified method from Prestera et al. (1996). Both matrices were subjected to an aqueous extraction and analysed using liquid chromatography with diode array detection (LC-DAD). Twenty grams of each botanical powder were placed into a 50 ml glass tube and autoclaved at 115°C for 15 min to inactivate the enzyme myrosinase. Samples together with 200 mL of boiling distilled water were added to a 500-ml Erlenmeyer flask and the mixture was stirred for 10 min at 350 rpm. Mixtures were allowed to cool down to room temperature, centrifuged at 2500 rpm for 5 min at 4°C and filtered through Whatman no. 4 filter paper into 50 ml screw-capped tubes. The extracts were filtered again through a 0.22 µm filter. Separation and quantification of glucosinolates were performed using a Shimadzu LC system (Shimadzu, Kyoto, Japan), equipped with a Gemini C18 column (4.6 × 150 mm i.d. 5 mm; Phenomenex, Torrance, California, USA). Elution was carried out isocratically for 20 min at a flow rate of 1 ml min^{-1}, using 20 % (v v^{-1}) acetonitrile and 80 % water with 0.02 M tetrabutylammonium hydrogen sulfate (pH 5.5). The injection volume was set to 20 µL. A UV detector was used to measure the absorbance at 227 nm in order to verify and quantify the presence of sinalbin at a reference retention time of 1.83 min.

Separation and identification of gallotannins in Chinese galls

The powder from the Chinese galls was suspended in water at 4 % w v^{-1} and vortexed for 1 min. The mixture was centrifuged for 10 min (3000 rpm) and the supernatant was transferred to a 15 ml plastic tube. The extract was filtered with a 0.22 µm Whatman filter into a LC amber vial. An Agilent 1200-LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with vacuum degasser, autosampler, and binary pump was used for the chromatographic determination. The column was a Gemini NX-C18 (2 mm Å~ 110 mm, particle size 3 µm; Phenomenex, USA). Mobile phases consisting of 0.1 % formic acid as solvent system A and acetonitrile as solvent system B were used with the following gradient elution: 0 min, 5 % B; 30 min, 95 % B; 35 min, 95 % B; 40 min, 5 % B. The column was equilibrated for 3 min prior to each analysis. The sample volume injection was 20 µL and the flow rate was 0.3 ml min^{-1}. MS analysis was carried out using a 6540 Agilent Ultra-High-Definition Accurate-Mass q-TOF-MS coupled to the HPLC, equipped with an Agilent Dual Jet Stream electrospray ionization (Dual AJS ESI) interface in negative ionization mode. The conditions were as follows: drying gas flow (N_2), 12 liter min^{-1}; nebulizer pressure, 50 psi; gas drying temperature, 350°C; capillary voltage, 3500 V; fragmentor voltage, and scan range were 200 V and m/z 50-3000, respectively. Automatic MS/MS experiments were carried out using collision energy values of 0, 20, and 40 eV. Integration and data elaboration were performed using the Mass Hunter Workstation software (Agilent Technologies, USA).

Statistical analysis

Data were analysed with the statistical software SigmaPlot 13.0 (Systat Software Inc., San Jose, CA, USA) and Figs were prepared with Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA). To check for normality and homogeneity of variances, data were subjected to Shapiro-Wilk and Brown-Forsythe tests, respectively. When both tests passed ($P > 0.05$), a parametric one-way analysis of variance (ANOVA) was conducted. Otherwise, the non-parametric Kruskal-Wallis one-way ANOVA on ranks was performed. All pairwise comparisons among treatments were conducted using the Student-Newman-Keuls method with an α value of 0.05. For each study, the effect of the interaction “experiment run” × “treatment” was tested before pooling the data from the two runs of each bioassay. The interactions were not significant for most variables.

Results

Mycelium growth and pH

PYM fully inhibited mycelium growth of FG0410 and FG2113 at 1 % w v^{-1} ($P < 0.001$), while Ti resulted in a full inhibition at 2 % w v^{-1} ($P < 0.001$; Fig. I.2). Compared with the control, CG at 1 % w v^{-1} suppressed mycelium growth of FG0410 by 82 % ($P = 0.009$) and FG2113 by 83 % ($P < 0.001$) (Fig. I.2). At 0.5 % w v^{-1}, Ti and PYM increased mycelium growth of FG1145 ($P < 0.001$ and $P = 0.005$, respectively). However, full inhibition was observed at 2 % w v^{-1} ($P < 0.001$; Fig. I.2). CG at 1 % w v^{-1} reduced mycelium growth of FG1145 by 65 % compared with control ($P < 0.001$; Fig. I.2). The pH of PDA in dH_2O was 5.6. The pH of Ti and PYM suspensions in dH_2O and after PDA incorporation ranged from 5.0 to 5.5, while with CG lower pH values (3.9 to 4.5) were observed (Supplementary Table SI.1).

Conidia germination

All botanicals at 2 % w v^{-1} inhibited germination of most conidia of all three FG strains ($P < 0.001$; Fig. I.3). At 0.5 % w v^{-1}, CG substantially decreased conidia germination (FG0410 by 89 %, FG2113 by 88 %, and FG1145 by 43 %) compared with the control ($P < 0.001$), while Ti and PYM were more effective at ≥ 1 % w v^{-1} ($P < 0.001$, Fig. I.3).

Ascospore germination

Ti and PYM at ≥ 1 % w v^{-1} inhibited germination of most ascospores of FG0410 and FG2113 ($P < 0.001$; Fig. I.4). At all concentrations tested, CG decreased ascospore germination of FG0410 only slightly compared with the control (4 to 8 %; $P < 0.01$), and no effect was observed with FG2113 (Fig. I.4). All pairwise comparisons among treatments with the corresponding *P* values for the bioassays of mycelium growth, conidia germination and ascospore germination are provided in Supplementary Table SI.2.

Perithecia formation on maize stalks

Ti, PYM, and CG at 2 % w v^{-1} suppressed perithecia formation of FG0410 on maize stalks by 48 %, 56 %, and 53 %, respectively, compared with the water control ($P < 0.001$), while the effects on FG2113 were not significant ($P > 0.05$; Fig. I.5). For both FG0410 and FG2113, there was no difference in the number of perithecia on maize stalks between the control and water control ($P > 0.05$; Fig. I.5).

Ascospore discharge

The number of ascospores discharged from mature perithecia was reduced with application of Ti, PYM, and CG at 2 % w v^{-1} (Fig. I.6). With FG0410, 77 %, 59 %, and 58 % fewer ascospores (P < 0.01), and with FG2113, 70 %, 54 %, and 62 % fewer ascospores (P < 0.001) were discharged when mature perithecia were treated with Ti, PYM, or CG, respectively (Fig. I.6).

Glucosinolate determination in mustards and gallotannin identification in Chinese galls

Glucosinolates present in PYM and Ti were analysed to determine the total quantity that could be converted into ITCs through the action of the enzyme myrosinase. The principal glucosinolate present in PYM and Ti was sinalbin, the powders having 57.4 and 56.4 g kg^{-1}, respectively. Analysis of gallotannins present in CG, using LC-TOF-MS, demonstrated that this bioactive matrix contained 14 different compounds, i.e. tannic acid (54 %), gallic acid (19 %), and 12 different gallotannins (Table I.1). Most of the gallotannins in CG had fragmentation profiles from the loss of one or more galloyl groups (152 mass units) and/or the loss of gallic acid (170 mass units). Some isomers of 4-galloylglucopyranose (GG) and 5-GG gave the fragmentation of digallic acid (m/z = 321), which indicates that not all the galloyl groups of these isomers are directly attached to the glucose core. Some of them might be attached to another galloyl group via a *meta*-depside bond. The molecular weight (MW) of the identified compounds ranged from low MW compounds, such as gallic acid with a MW of 169.0560, to organic compounds with a MW ranging from 635.1750 to 1243.2302 (molecules identified as 3-, 4-, 5-, 6-, and 7-GG). In addition, other compounds with high MW (i.e. 12-, 14-, and 15-GG) ranging from 2004.3014 to 2460.3391 were identified.

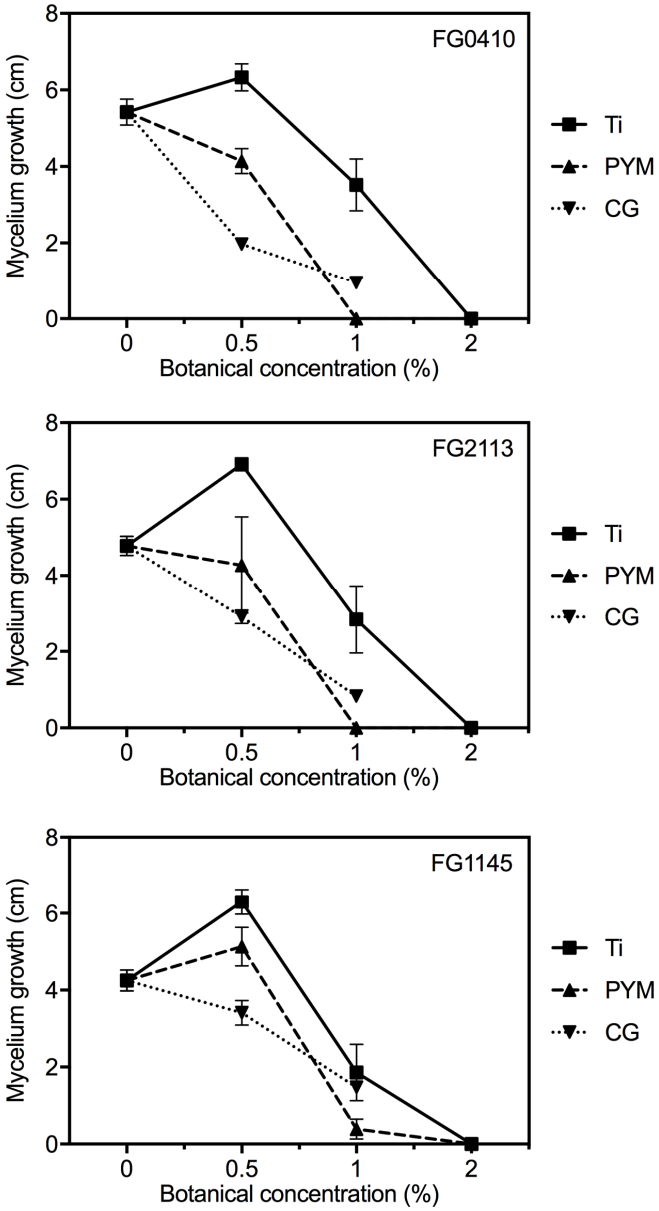

Fig. I.2. Mycelium growth (cm) of three strains of *Fusarium graminearum* (FG0410, FG2113, and FG1145) as affected by different concentrations (w v^{-1}) of the botanicals Tillecur® (Ti), Pure Yellow Mustard (PYM), and Chinese galls (CG). The control is indicated as 0 % botanical concentration. Average values from two experiments are presented and bars indicate the standard error of the mean (n = 8). The results from a Kruskal-Wallis test were significant for FG0410 (χ^2 = 66.0, df = 8, P < 0.001), FG2113 (χ^2 = 53.2, df = 8, P < 0.001), and FG1145 (χ^2 = 59.9, df = 8, P < 0.001). All pairwise comparisons were performed using a Student-Newman-Keuls test (α = 0.05).

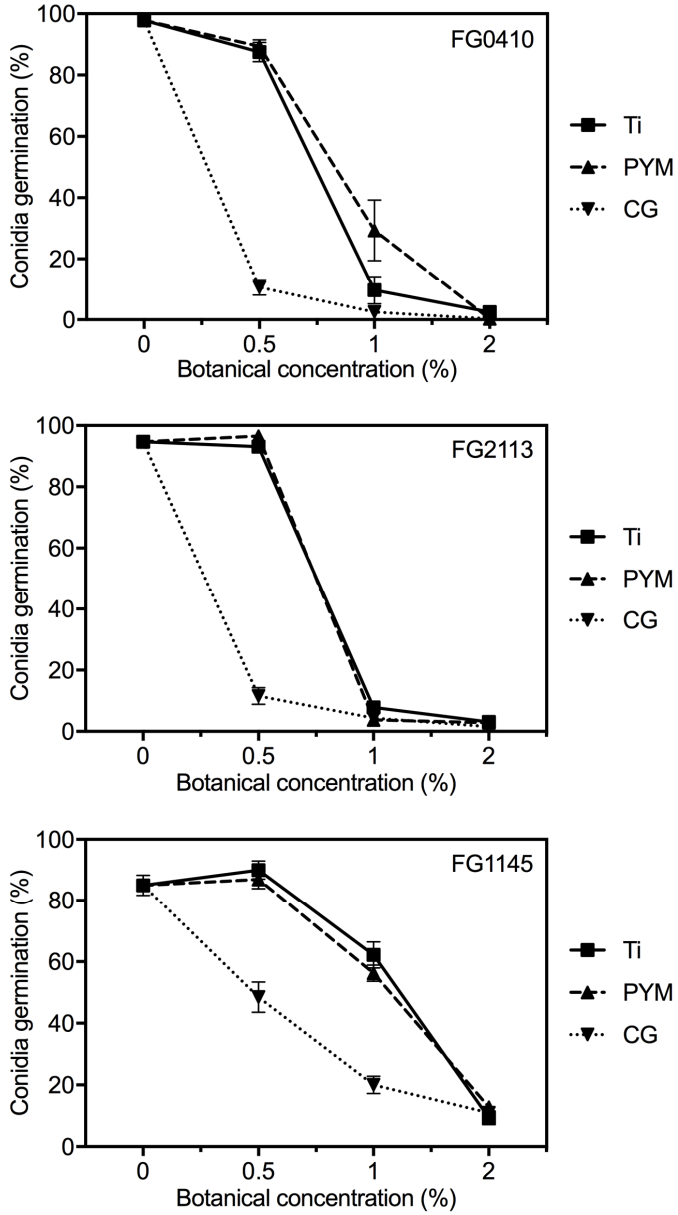

Fig. I.3. Conidia germination (%) of three strains of *Fusarium graminearum* (FG0410, FG2113, and FG1145) as affected by different concentrations (w v^{-1}) of the botanicals Tillecur® (Ti), Pure Yellow Mustard (PYM), and Chinese galls (CG). The control is indicated as 0 % botanical concentration. Average values from two experiments are presented and bars indicate the standard error of the mean (n = 8). The results from a Kruskal-Wallis test were significant for FG0410 (χ^2 = 64.0, df = 9, P < 0.001). The results from a one-way ANOVA were significant for FG2113 ($F_{9,70}$ = 781.3, P < 0.001), and FG1145 ($F_{9,70}$ = 112.4, P < 0.001). All pairwise comparisons were performed using a Student-Newman-Keuls test (α = 0.05).

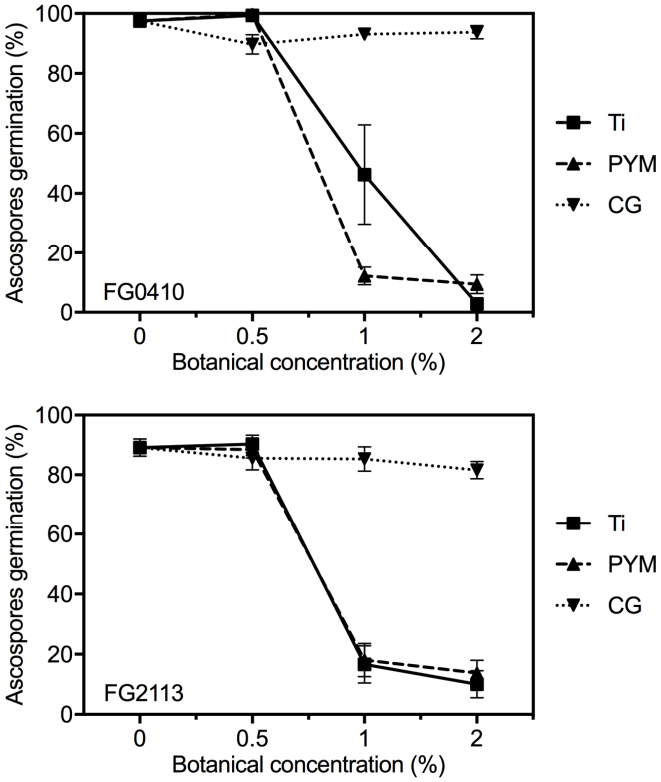

Fig. I.4. Ascospore germination (%) of two strains of *Fusarium graminearum* (FG0410 and FG2113) as affected by different concentrations (w v^{-1}) of the botanicals Tillecur® (Ti), Pure Yellow Mustard (PYM), and Chinese galls (CG). The control is indicated as 0 % botanical concentration. Average values from two experiments are presented and bars indicate the standard error of the mean (n = 8). The results from a Kruskal-Wallis test were significant for FG0410 (χ^2 = 62.7, df = 9, P < 0.001) and FG2113 (χ^2 = 59.1, df = 9, P < 0.001). All pairwise comparisons were performed using a Student-Newman-Keuls test (α = 0.05).

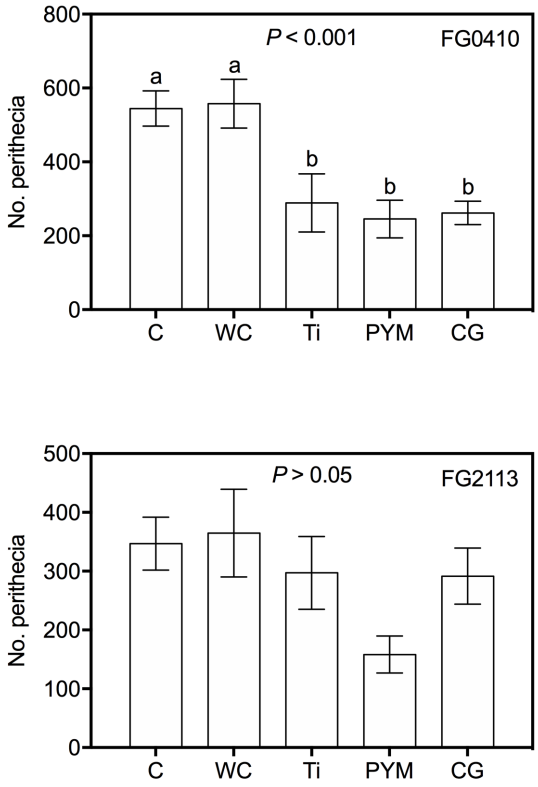

Fig. I.5. Number of perithecia formed on maize stalks by two strains of *Fusarium graminearum* (FG0410 and FG2113) as affected by Tillecur® (Ti), Pure Yellow Mustard (PYM), and Chinese galls (CG) at 2 % concentration (w v^{-1}). The control (C) and water control (WC) refer to untreated stalks and stalks treated with sterile dH_2O, respectively. Average values from two experiments are presented and bars indicate the standard error of the mean (n = 12). The results from a Kruskal-Wallis test were significant for FG0410 (χ^2 = 22.7, df = 4, P < 0.001). All pairwise comparisons were performed using a Student-Newman-Keuls test and different letters indicate significant differences (α = 0.05). The results from a one-way ANOVA were not significant for FG2113 ($F_{4,55}$ = 2.2, P > 0.05).

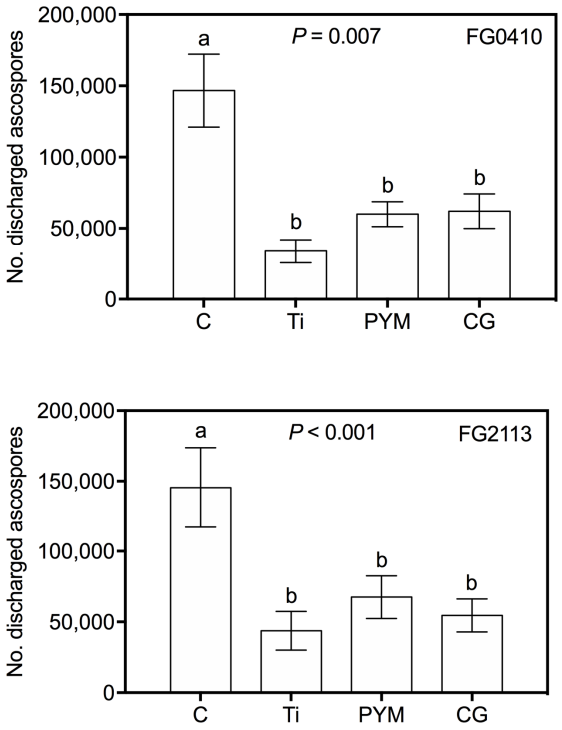

Fig. I.6. Number of ascospores discharged by two strains of *Fusarium graminearum* (FG0410 and FG2113) as affected by Tillecur® (Ti), Pure Yellow Mustard (PYM), and Chinese galls (CG) at 2 % concentration (w v^{-1}). For the control (C), sterile dH_2O was applied. Average values from two experiments are presented and bars indicate the standard error of the mean (n = 12). The results from a Kruskal-Wallis test were significant for FG0410 (χ^2 = 12.3, df = 3, P < 0.01) and FG2113 (χ^2 = 15.7, df = 3, P < 0.001). All pairwise comparisons were performed using a Student-Newman-Keuls test and different letters indicate significant differences (α = 0.05).

Table I.1. Negative ion LC-ESI-qTOF-MS analysis of gallotannins in extracts of Chinese galls.

Compound	Calculated masses (Da)	Observed masses m/z (Da)	Relative abundance (%)	Fragments MS^2 (m/z)
Gallic acid	170.0348	169.0560	19	169.0560, 125.0608
GG	332.1129	331.1254	2	331.1254, 169.0560, 125.0608
2-GG	484.1289	483.1480	1	483.1480, 331.1254, 169.0560, 125.0608
3-GG	636.1294	635.1750	4	635.1750, 483.1480, 331.1254, 169.0560, 125.0608
4-GG	788.1872	787.1890	2	787.1890, 635.1750, 483.1480, 331.1254, 169.0560, 125.0608
5-GG	940.2873	939.2726	1	939.2726, 787.1890, 635.1750, 483.1480, 331.1254, 169.0560, 125.0608
6-GG	1092.2381	1091.2137	1	1091.2137, 939.2726, 787.1890, 635.1750, 483.1480, 331.1254, 169.0560, 125.0608
7-GG	1244.2296	1243.2302	1	1243.2302, 1091.2137, 939.2726, 787.1890, 635.1750, 483.1480, 331.1254, 169.0560, 125.0608
9-GG	1548.2471	1547.2578	3	1547.2578, 1243.2302, 1091.2137, 939.2726, 787.1890, 635.1750, 483.1480, 331.1254, 169.0560, 125.0608
Tannic acid	1700.2751	1699.2727	54	1699.2727, 1547.2578, 1243.2302, 1091.2137, 939.2726, 787.1890, 635.1750, 483.1480, 331.1254, 169.0560, 125.0608
11-GG	1853.2861	1852.2885	5	1852.2885, 1699.2727, 1547.2578, 1243.2302, 1091.2137, 939.2726, 787.1890, 635.1750, 483.1480, 331.1254, 169.0560, 125.0608
12-GG	2005.3059	2004.3014	1	2004.3014, 1852.2885, 1699.2727, 1547.2578, 1243.2302, 1091.2137, 939.2726, 787.1890, 635.1750, 483.1480, 331.1254, 169.0560, 125.0608
14-GG	2309.3371	2308.3265	2	2308.3265, 2156.3150, 2004.3014, 1852.2885, 1699.2727, 1547.2578, 1243.2302, 1091.2137, 939.2726, 787.1890, 635.1750, 483.1480, 331.1254, 169.0560, 125.0608
15-GG	2461.3385	2460.3391	4	2460.3391, 2308.3265, 2156.3150, 2004.3014, 1852.2885, 1699.2727, 1547.2578, 1243.2302, 1091.2137, 939.2726, 787.1890, 635.1750, 483.1480, 331.1254, 169.0560, 125.0608

GG = galloylglucopyranose

Discussion

We believe this is the first report describing the use of botanicals to suppress FG based on assessing the impact on multiple, distinct stages of the pathogen life cycle. The botanicals used for this research included PYM and Ti, which are both based on white mustard seed flour, and CG, which is rich in gallotannins as well as in gallic and tannic acid. All the botanicals tested showed promise suppressing development of structures at different life-cycle stages of the three strains of FG. The only exception was CG, which showed only minor or no effects on suppressing ascospore germination depending on the fungal strain. Overall, the bioassays on mycelium growth, conidia and ascospore germination, and ascospore discharge showed similar patterns among all FG strains examined. However, a differential response was observed in reduction of the number of perithecia on maize stalks; treatment with botanicals reduced the number of perithecia for FG0410 but not for FG2113. The difference in response may indicate that prevention of perithecial development may be inconsistent when applying these botanicals on maize residues, but clearly further research is needed to ascertain these effects at the field scale.

The forcible discharge of ascospores from mature perithecia represents the initial inoculum, and therefore is a key element for FHB infection. Hence, interruption of ascospore discharge in FG might be an effective method to control FHB, as has been previously reported for ascomycetes that rely on ascospores for primary inoculum (Trail, 2007). In the current study, we observed that ascospore discharge from mature perithecia was 2- to 4-fold lower after application of botanicals. After deposition on flowering cereal heads, the germination of ascospores and conidia are the major developmental steps in the pathogenesis of FHB (cited in Dweba et al., 2017). We showed that the germination of both spore types was completely inhibited *in vitro* by applying PYM or Ti. Although application of CG suppressed conidial germination, it did not have a sufficiently suppressive effect against ascospore germination, indicating that reduction in activity of these spores may be more difficult to achieve. Another critical stage is the survival of FG, which overwinters as mycelium in soil and on infected crop residues (Yuen and Schoneweis, 2007; Leplat et al., 2013). Therefore, reducing the mass of hyphae left in the field could be an effective strategy to further minimize the risk of FHB epidemics. All the botanicals tested could be candidates for inclusion in a management strategy to reduce overwintering mycelium in the soil and on infected crop residues. All three botanicals consistently suppressed the mycelium growth of all three strains of FG at concentrations ≥ 1 %.

Sinalbin was the principal glucosinolate present in PYM and Ti (57.4 and 56.4 g kg^{-1},

respectively). Sinalbin is the precursor of p-HBITC, a compound characterized for its antimicrobial activity (Delaquis and Mazza, 1995; Ekanayake et al., 2012; Dufour et al., 2015). Although many studies have investigated the antimicrobial effects of mustards for food preservation and biofumigation purposes, there is limited knowledge on the antimicrobial effects of p-HBITC as a potential biological fungicide against FG. For example, Ekanayake et al. (2006) demonstrated that IsoGard^{TM}, a product based on p-HBITC extracted from moistened ground white mustard seeds, showed antimicrobial activity against pathogenic and spoilage bacteria including *Escherichia coli*, *Staphylococcus aureus*, *Campylobacter jejuni*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Listeria monocytogenes*, *Shigella boydii*, and *Clostridium perfringens*. Furthermore, Quiles et al. (2015) investigated the potential of mustard-based botanicals to reduce aflatoxin contamination of wheat tortillas by *Aspergillus parasiticus* and observed that the gaseous allyl ITC from oriental mustard was more effective than p-HBITC from white mustard, yet substantial reduction in aflatoxin occurred with either treatment. Calmes et al. (2015) studied the mechanisms by which ITCs could cause fungal cell death using *Alternaria brassicicola* as a model organism. The authors demonstrated that exposure to these compounds led to reduced oxygen consumption rate, intracellular accumulation of reactive oxygen species, and mitochondrial-membrane depolarization. Smolinska et al. (2003) studied the effects of different ITCs as inhibitors of *Fusarium oxysporum*. Propenyl-, ethyl-, benzyl-, and in most cases, phenethyl-ITCs, fully inhibited conidia and chlamydospores germination. However, the effects on mycelium growth were predominantly fungistatic and not fungitoxic, indicating that the mycelium may represent a more resistant stage of this fungus. In another study, the mycelium growth of FG was suppressed after the application of four alkenyl aliphatic ITCs (methyl-ITC, propenyl-ITC, butenyl-ITC, pentenyl-ITC) or two aromatic ITCs (benzyl-ITC and 2-phenylethyl-ITC) (Sarwar et al., 1998). In our study, the botanicals PYM and Ti, which contain sinalbin, completely suppressed the mycelium growth of all three strains of FG at a concentration of 2 %.

More alkaline environments reduce the stability of p-HBITC resulting in the formation of a quinone that hydrolyses to ionic thiocyanate, SCN^- (Borek and Morra, 2005). To overcome this challenge in farming practice where mustard-based suspensions in water might be used, the product must be formulated to ensure stability of p-HBITC. Besides mustard-based botanicals, cereal farmers employing minimum tillage and thus encountering higher FHB disease pressure, could include mustard cover crops (e.g. *Brassica juncea* or *Sinapis alba*) in their rotation. After mulching the mustard crops, glucosinolates will be hydrolysed and converted into bioactive ITCs. The latter would suppress various fungal structures in the life cycle of FG present in the field, including mycelium, perithecia, and conidia. In maize-wheat rotations, an additional approach could be to cultivate mustard species as intercrops between maize rows.

Thus, mustard residues after maize harvest might suppress the mycelium of FG growing on the maize stalks and eventually decrease the FG inoculum potential for the subsequent wheat crop.

The gallotannins identified in CG (CGTs) could be responsible for the antifungal activity observed against FG. CGTs can interact with membrane proteins of microorganisms by hydrogen bonding through their hydroxyl groups, which can result in changes in membrane permeability causing cell destruction (Burt, 2004). Huang et al. (2012) studied the chemical composition of CG extract and the comparison between the efficacy of its main components and the whole extract in the inhibition of enamel demineralization. The authors concluded that gallic acid was the main demineralization-inhibiting component of CG and the effect was similar to that of the whole CG aqueous extract. Tian at el. (2009b), evaluated the antibacterial activity of CGTs against *Salmonella typhimurium* and *Bacillus cereus* using a paper disc based diffusion method. Hexa-hepta-galloylglucopyranoses (6-7-GGs), which were also identified in the CG powder used, showed remarkable antioxidant and antibacterial activities suggesting that they could be utilized as food preservatives. *In vitro* studies on FG showed that solutions with 1 % tannic acid or suspensions with 1 % CG inhibited conidia germination by 98-100 % and mycelium growth by 75-80 % (Forrer et al., 2014), which is in agreement with our findings. Moreover, Vogelgsang et al. (2013) conducted *in vitro* and field studies on the effect of aqueous extracts of botanical powders including chamomile, meadowsweet, thyme and CG against *Microdochium majus*. They found that CG had the most suppressive activity and completely inhibited mycelium growth and reduced conidia germination up to 97 %. However, for control of FHB, the incomplete suppression of ascospore germination by CG suggests that it might be less effective in direct control strategies targeting flowering cereal heads.

Filamentous fungi are able to grow in a wide range of pH due to their efficient homeostatic mechanisms (Flaherty et al., 2003). Beyer et al. (2004) found similar conidia germination rates of FG at a pH ranging from 3 to 7. Moreover, disease severity, DON content in grain, yield, and thousand-kernel weight of wheat plants were similar when treated with water (pH = 7.8) or acidified water (pH = 4.0) subsequent to artificial inoculation with conidia of FG and *F. crookwellense* (Forrer et al., 2014). Hence, we can surmise that the suppressive effects of the botanicals against FG in our study are not due to the slight decrease in pH by incorporating the botanicals in dH_2O or in the PDA.

In conclusion, we demonstrated that botanicals based on white mustard seed flour and Chinese galls were able to suppress or fully inhibit growth and development of FG using an array of *in vitro* bioassays. However, it should be noted that results of *in vitro* studies may not be directly transferable to the field. Firstly, the interaction between the pathogen and plant is

not taken into account. Secondly, under field conditions, crops are exposed to a wide range of pathogens and a mixture of several strains simultaneously, which is not the case in *in vitro* bioassays. Therefore, the antifungal effects of the botanicals must be tested *in planta* under both controlled environments and field conditions. Experiments should focus on direct control measures through applications of botanicals to flowering wheat heads. In addition, prevention strategies should be explored in reduced or zero tillage systems through botanical applications to crop residues in order to suppress inoculum of FG for the following crop.

Acknowledgements

The authors warmly thank Anja Marty for technical assistance with the mycelium growth bioassay and G. S. Dunn (Dry Mustard Millers, Canada) for providing the Pure Yellow Mustard flour.

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

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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*) crops 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 mycotoxins 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 % to 42 % and 60 % to 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 botanicals had 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.

Keywords: *Fusarium graminearum*; mycotoxin; wheat; mustard; clover

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 act 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 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).

Thus, 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 cut-and-carry 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.

Material and methods

Experimental design and crop management

In 2016-17 (year 1) and 2017-18 (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 randomized 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. Levis (maturity: medium-late; susceptibility to FHB: high) and 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 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 the cereal leaf beetle occurred at head emergence (BBCH 57-59).

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. II.1 a-c): Silage maize (P8057; DuPont Pioneer, USA) was harvested from a field at Agroscope-Reckenholz (Zurich, Switzerland) leaving stalk pieces of 40 to 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 excessive 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 VP, 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.

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. II.1 d) in autumn and included fresh aboveground biomass of white 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 to 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. II.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 positive control, while autoclaved (non-inoculated) stalks served as negative control.

Fig. II.1. Experimental set-up of the study: (a) maize field after harvest where stalks were collected, (b) inoculated 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.

Measurements

Ascospore deposition and disease incidence

During wheat anthesis, spore traps (Fig. II.2) with *Fusarium* selective medium containing pentachloronitrobenzene were placed in the field in order to catch the ascospores, which were discharged from perithecia. Details about the design of the spore traps and the selective medium can be found in Schöneberg et al. (2018b) 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 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 to 7 pm) and collected in the following morning (9 to 10 am), allowing a period of 14 to 16 h for ascospore deposition. Upon collection, Petri dishes were incubated upside down at 18°C in the dark for 3 to 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 is not able to be quantified. 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 from three times of spore traps placement during wheat anthesis was calculated for each treatment.

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

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.

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.

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 sec^{-1}) in lysis buffer (PL1). Quantitative PCR was performed as described in

Schöneberg et al. (2018b) 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.

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 content 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.

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).

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 test 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 figures 3 and 4 were prepared with Prism 5.0 (GraphPad Software Inc., USA).

Results

Ascospore deposition, disease incidence and *Fusarium graminearum* DNA 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 ($p < 0.001$). 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 were higher in Levis than in Forel ($p < 0.001$). In both years, all mulch layers and Ti applied in spring decreased the ascospore deposition by 35 % to 48 % compared with the plots containing untreated maize stalks inoculated with *F. graminearum*, i.e. positive control (Table II.1, $p < 0.05$). In plots with sterile 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 II.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 II.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 II.1, $p < 0.05$). In the second year, mulch layers and botanicals reduced *F. graminearum* DNA amount in grain by 42 % to 54 % and by 28 % to 47 %, respectively, compared with the positive control (Table II.1, $p < 0.05$).

Table II.1. Ascospore deposition, disease incidence and DNA amount of *Fusarium graminearum* (FG) in grain of wheat crop in year 1 (2016-17) and year 2 (2017-18) 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 crops 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 (Levis and 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 (2016/17)			Year 2 (2017/18)		
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).

Mycotoxins in wheat grain

Overall, the mean DON content in grain was 3.5-fold higher in the second year than in the first (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 Levis than in those from 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. II.3, $p < 0.05$). In the second year, all mulch layers and botanicals decreased DON by 50 % to 58 % and by 22 % to 42 %, respectively, compared with the positive control (Fig. II.3, $p < 0.05$). In the first year, use of Mulch WM, Ti and CG applied in spring decreased ZEN by 73 % to 75 % compared with the positive control (Fig. II.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. II.3, $p < 0.05$).

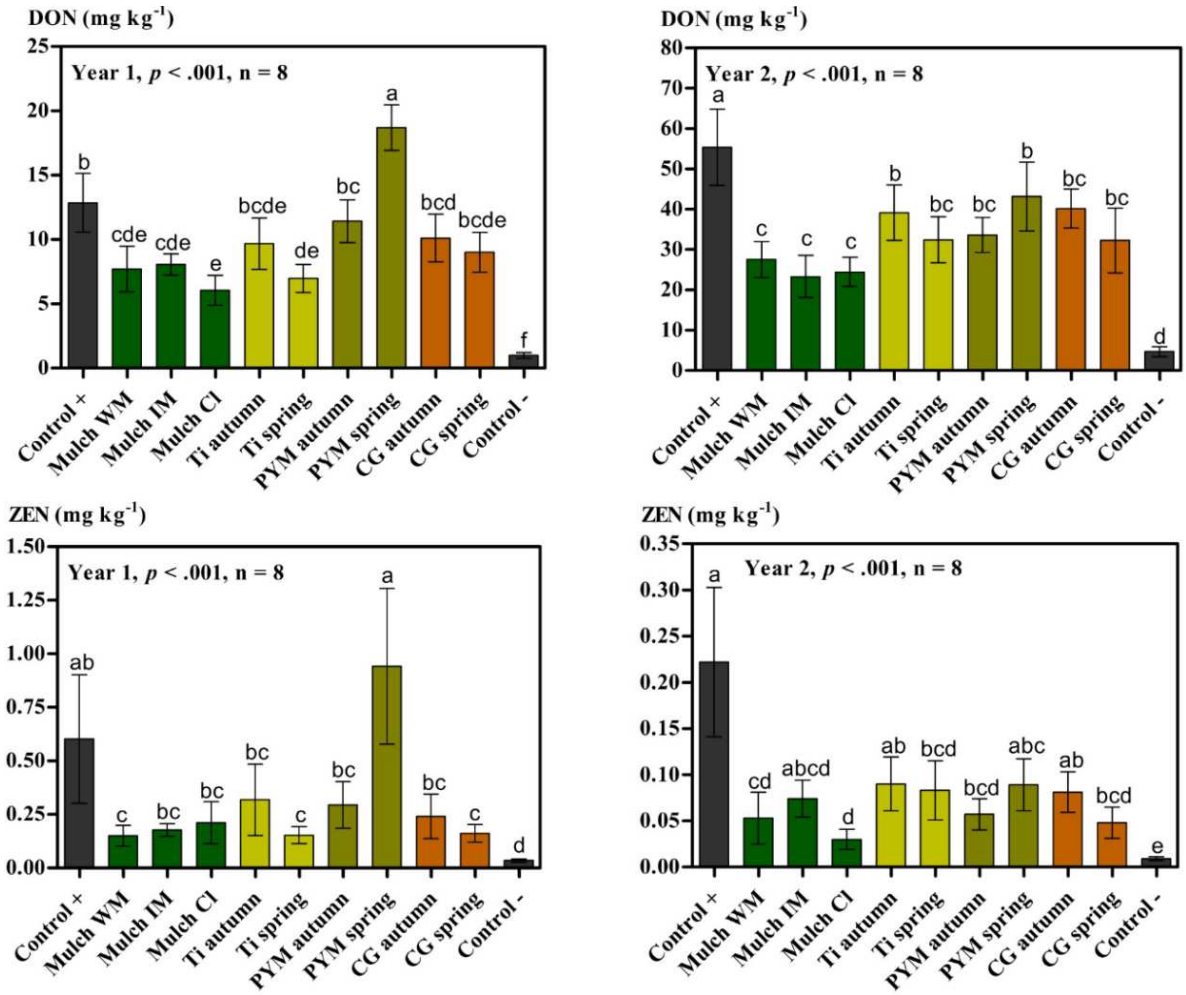

Fig. II.3. Deoxynivalenol (DON, mg kg^{-1}) and zearalenone (ZEN, mg kg^{-1}) contents in wheat grain in year 1 (2016-17) and year 2 (2017-18) as affected by application of mulch layers or botanicals to

inoculated with *Fusarium graminearum* maize stalks. 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 clover crops 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 (Levis and 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).

Grain yield

Overall, the mean grain yield was lower in the second year than in the first (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, Forel resulted in higher yield than Levis (year 1: 8.54 versus 8.19 t ha^{-1}, $p = 0.005$; year 2: 7.80 versus 6.06 t ha^{-1}, $p < .001$). 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. II.4, $p < 0.05$). In the second year, the mulch layer treatments resulted in 8 % to 15 % higher grain yield compared with the positive control, while the highest yield was observed in the negative control (Fig. II.4, $p < 0.05$).

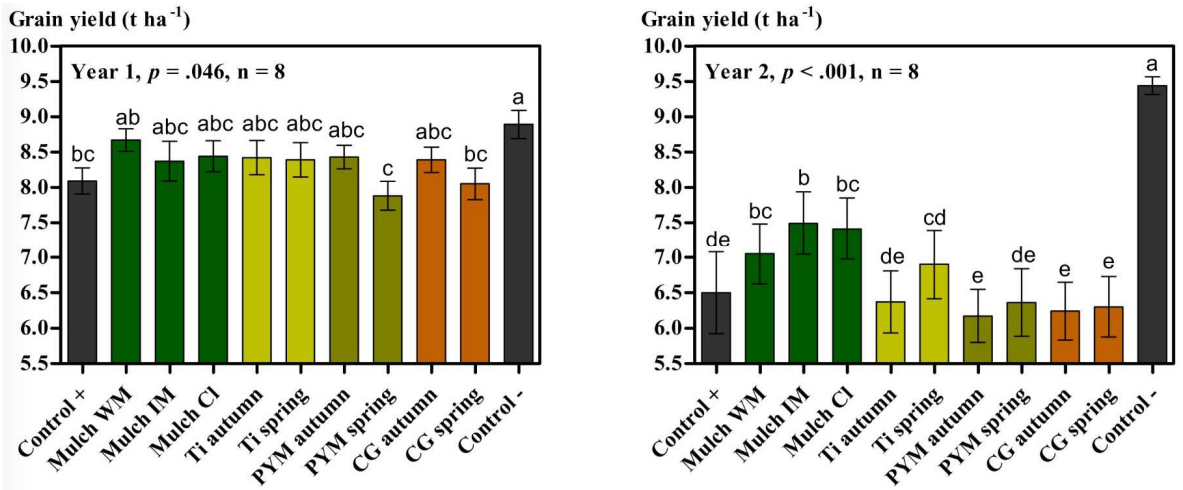

Fig. II.4. Grain yield (t ha^{-1}) of wheat in year 1 (2016-17) and year 2 (2017-18) as affected by application of mulch layers or botanicals to inoculated with *Fusarium graminearum* maize stalks. Control +/- refers to positive/negative control, i.e. untreated inoculated/sterilised maize stalks; Mulch WM/IM/Cl refers to fresh mulch layers harvested from white mustard/Indian mustard/berseem clover crops 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 (Levis and 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).

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 II.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. 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.

Table II.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) content 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	

Table II.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 till early dough (BBCH 71-83) and soft dough till 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

Discussion

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 were 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 in both 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-ITC, while for white mustard are p-hydroxybenzyl- and benzyl-ITC (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 to 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 of 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 evidence for the first time for 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, 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. Our findings are less conclusive regarding the 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 sterile maize stalks (i.e. negative control). The mean grain yield across the whole field was lower in the second year than the first. This could be due to the prevailing climatic conditions (Table II.3) from beginning of anthesis (BBCH 61) to ripening (BBCH 92) of 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 (+3 % and +14 %, respectively) in the second year compared with the first. Moreover, the total precipitation during BBCH 61-83 was

higher by 22 mm in the second year compared with the first. 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. In contrast, ZEN content was 4-fold higher in the first year than in the second (298 versus 76 µg kg^{-1}, $p < 0.001$). ZEN is strongly dependent on post-anthesis rainfall with greater levels 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 (i.e. year 2).

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 a good indicator of FHB infection and DON contamination risks. In plots with sterile 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.

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 of white mustard, Indian mustard or berseem clover crops grown in separate fields, i.e. cut-and-carry biofumigation, to infected maize residues. An additional important outcome of this study is that the experimental set-up was appropriate with minimum cross-contamination among 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 counsel 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 the 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 and ensuring grain yield and quality.

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.

4. Chapter III. Innovative cropping systems to improve food safety in wheat: An agronomic and economic assessment

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Abstract

The effective control of Fusarium head blight (FHB), mainly caused by *Fusarium graminearum*, is an important component of agronomic and economic sustainability among cereal farmers worldwide with significant impact on food safety and food security. Within the context of sustainable intensification of agroecosystems, we investigated several innovative cropping systems aiming at decreasing mycotoxins in wheat under reduced tillage intensity. We showed that growing interval cover crops as well as intercrops in maize in a maize-wheat rotation reduced mycotoxins in wheat grain. More specifically, use of white mustard, Indian mustard and winter pea as interval cover crops reduced mycotoxins in spring wheat by up to 72 %, 58 % and 75 %, respectively. Remarkably, with the use of cover crops, the decrease in mycotoxins was comparable with the plough treatment. The use of white mustard and Indian mustard as intercrops decreased deoxynivalenol in winter wheat by 52 % and 32 %, respectively, compared with maize grown as sole crop. Nevertheless, substantial reduction of mycotoxins through maize-mustard intercropping was not observed under very high FHB pressure in 2019. We found similar deoxynivalenol and zearalenone contents in wheat grain under no-tillage and reduced tillage in the maize-intercropping experiment. We observed economic trade-offs of the intercropping and cover cropping systems due to increased operating costs. Hence, policy makers may consider providing incentives to farmers to use cover crops or intercrops in maize-wheat crop rotations. This could contribute to enhanced crop diversification strategies with the overall goal of improving food safety while ensuring the economic viability of these new cropping systems.

Introduction

The world's population continues to increase rapidly and is estimated to reach approximately 9 billion people by the middle of this century. A great challenge of agriculture is to ensure food security while improving food safety through sustainable intensification of agroecosystems (Godfray et al., 2010; Garnett et al., 2013). Grains, such as maize (*Zea mays*) and wheat (*Triticum* spp.), are essential food and fodder crops with historical importance for food security. In 2017, maize and wheat took the second and third position in the global list of production quantity with 1135 and 772 million tonnes, respectively (FAOSTAT, 2019). Moreover, among the worldwide grown cereals, maize and wheat are crops with the highest gross and net production value after rice (FAOSTAT, 2019), thereby representing the utmost contribution to the global economy. The potential yield losses due to phytopathogens have been estimated overall at 9.4 % for maize and 15.6 % for wheat (Oerke, 2006). Hence, the effective control of crop diseases is crucial for ensuring food security and economic sustainability worldwide.

Fusarium head blight (FHB) is one of the most noxious cereal diseases worldwide in terms of food security, food safety and economic viability. The yield losses in wheat due to FHB were estimated between 1.8 % and 8.8 % depending on the geographic region (Savary et al., 2019). Apart from yield reductions, FHB causes grain contamination with hazardous secondary metabolites, named mycotoxins, which put food and feed safety at risk. Mycotoxins are toxic to humans and animals with adverse chronic and acute effects (Escrivá et al., 2015). As a result, maximum levels in food and guidance levels in feed were set for certain mycotoxins, such as deoxynivalenol (DON) and zearalenone (ZEN) (Ferrigo et al., 2016). Severe epidemics in the 1990's affected wheat producers in the United States of America, Canada, Europe, Argentina and China (Singh et al., 2016). In the USA only, FHB caused an economic loss of approximately $ 2.7 billion between 1998 and 2000 (Nganje et al., 2004).

In most parts of the world *Fusarium graminearum* (teleomorph *Gibberella zeae*) is the predominant fungal species that causes FHB (Osborne and Stein, 2007). The fungus overwinters on crop residues where its fruiting bodies, perithecia, are developed. Ascospores are discharged from mature perithecia and infect the wheat heads during anthesis with the aid of wind, but infection may also occur from water-splashed macroconidia.

Crop rotation with non-host species and conventional tillage with mouldboard ploughing are common strategies to control FHB (Parry et al., 1995). In an eight-year survey of wheat, higher levels of DON and amounts of *F. graminearum* DNA were observed in grain samples from fields with maize as previous crop and under reduced tillage (Vogelgsang et al., 2019). However,

reduced tillage has been associated with preservation of soil quality as well as prevention of soil degradation and erosion (Six et al., 2000; Jacobs et al., 2009). In Switzerland, direct payments are provided to farmers who employ reduced tillage practices, representing approximately 28 % of open arable land in 2018 (Anonymous, 2019). Additionally, in several regions of the temperate zone, farmers commonly cultivate wheat after maize since it fits well in the rotation in terms of sowing and harvesting periods. Thus, exploration of alternative cropping systems within a maize-wheat rotation is considered necessary to provide integrated solutions for the control of FHB.

Intercropping is a farming practice that involves two or more crop species, or genotypes, growing simultaneously and could be one route to sustainable intensification (Brooker et al., 2015). Potential benefits of intercropping systems include (i) increased land- and resource-use efficiency; (ii) reduced run-off, nutrient leaching and soil erosion; (iii) enhanced weed suppression; and (iv) improved control of pests and diseases through interspecific facilitation (Brooker et al., 2015; Bybee-Finley and Ryan, 2018). For instance, control of phytopathogens that develop on crop debris could be achieved using intercrop species with antifungal properties, such as the glucosinolate-derived bioactive substances of mustard (Brown and Morra, 1997; Matthiessen and Kirkegaard, 2006). Cover cropping implies the cultivation of a certain plant species before or after a cash crop in a rotation and demonstrates the same benefits as intercropping, but only over time. Under reduced tillage practices, the inclusion of cover crops in the rotation was essential in order to maintain a certain yield level and thus contributes to ecological intensification (Wittwer et al., 2017). Hence, under the umbrella term of sustainable intensification of agroecosystems, intercropping and cover cropping systems should be explored as crop protection strategies against important diseases, such as FHB. Yet, as such cropping systems may have trade-offs in terms of farm profitability, potential economic effects should also be assessed.

The main objective of this study was to investigate the effect of two cropping systems, i.e. ‘maize-intercropping’ and ‘cover cropping’, on the development of FHB and accumulation of mycotoxins in subsequent wheat crop under reduced tillage.

The main research questions were:

(i) Does maize-intercropping reduce grain yield of maize?
(ii) Does maize-intercropping or the use of cover crops after maize decrease the mycotoxin contamination in the subsequent wheat crop?
(iii) Does tillage practice affect the mycotoxin content in wheat grain?
(iv) Are there any economic trade-offs of these cropping systems?

For the 'maize-intercropping' experiment, we used red clover, sudangrass, phacelia, white mustard or Indian mustard as intercrops with grain maize. The intercropping systems were compared with a sole maize crop. After maize harvest, the crop residues of maize and intercrops were either mulched (i.e. no-tillage) or incorporated into the top soil layer after mulching (i.e. reduced tillage). Subsequently, direct sowing of two winter wheat varieties was done. For the 'cover cropping' experiment, we used white mustard, Indian mustard or winter fodder pea as interval cover crops in a silage maize-spring wheat rotation. Cover crops were mulched and then two varieties of spring wheat were established with direct sowing. The cover cropping systems were compared with treatments for which no cover crop was grown, i.e. herbicide or plough applied after silage maize. Besides these agronomic aspects, an economic assessment was conducted by calculating the receipts, operating costs and eventually the gross margin of each cropping system.

Materials and methods

Experimental design, crop management and treatments

Maize-intercropping

The field experiment was conducted twice at Agroscope-Tänikon in Switzerland (2016–2017: 47°28'50"N 8°54'41"E; 2018–2019: 47°29'01"N 8°54'42"E). A split-split-plot design (Table III.1, Fig. SIII.1) in four blocks was used, comprising two tillage practices (no-tillage, reduced tillage) as whole plots, six maize-intercropping systems (sole maize (no intercropping), clover, sudangrass, phacelia, white mustard, Indian mustard; Fig. III.1) as subplots and two winter wheat varieties (Levis, Forel) as sub-subplots. The size of each subplot was 108 m^2 (9 m × 12 m), which was then divided in half for the sub-subplot treatments (4.5 m × 12 m each). To prevent cross-contamination with FHB-causing species, adjacent plots with triticale (×*Triticosecale*) served as buffer zones (6 m × 6 m).

Grain maize (var. Laurinio; KWS, Switzerland) was sown across the entire field (100'000 kernels ha^{-1}, 75 cm distance between maize rows). The intercrops were sown by spreading the seeds at BBCH 13–15 growth stage of maize using a seed broadcaster (APV, Austria). Red clover (*Trifolium pratense* var. Pastor; Feldsaaten Freudenberger, Germany), sudangrass (*Sorghum* × *drummondii* var. HayKing II Hi-Gest®; Alforex Seeds, USA), phacelia (*Phacelia tanacetifolia* var. Angelia; P.H. Petersen Saatzucht Lundsgaard, Germany), white mustard (*Sinapis alba* var. Admiral; Feldsaaten Freudenberger, Germany) and Indian mustard (*Brassica juncea* var. Vittasso; KWS, Italy) were sown at 20, 40, 8, 20 and 8 kg ha^{-1}, respectively. To facilitate the establishment of the intercrops, a field pass with original harrow was done on the same day

in order to improve seed-soil contact and control any emerged weeds. For maize production, mineral fertilisers were applied using targeted rates of 110 N, 95 P and 220 K kg ha^{-1}. For sole maize, a herbicide treatment (Calaris; active ingredients (a.i.): terbuthylazin and mesotrione; Syngenta, Switzerland) was applied between maize rows at BBCH 30–33.

After grain maize harvest with a combine harvester, the following treatments were conducted: for no-tillage, the maize and intercrop residues were mulched on the soil surface; for reduced tillage, the crop residues were mulched and then incorporated into the top soil layer (up to 10 cm depth) by employing one single pass with a rotary tiller, mounted with a fixed packer roller. Approximately two weeks after the soil operations, winter wheat (var. Levis, var. Forel; Saatzucht Düdingen, Switzerland) was sown with direct sowing at 180 kg ha^{-1}. In the adjacent buffer plots, inversion tillage with mouldboard plough was applied to bury all maize residues up to 30 cm soil depth, and then winter triticale (var. Larossa; Saatzucht Düdingen, Switzerland) was sown at 180 kg ha^{-1}. For wheat production, mineral fertilisers were applied using targeted rates of 140 N, 60 P and 80 K kg ha^{-1}. At BBCH 27–29, herbicides (a mixture of Artist (a.i.: flufenacet and metribuzin) and Chekker (a.i.: amidosulfuron and iodosulfuron); Bayer, Switzerland) were applied. At BBCH 55–57, an insecticide (Karate Zeon; a.i.: lambda-cyhalothrin; Syngenta, Switzerland) was applied against cereal leaf beetles.

Table III.1. Maize-intercropping. Split-split-plot design of the field experiments conducted in 2016–2017 and 2018–2019 (see also supplementary Fig. SIII.1).

Whole plot	***Subplot***	***Sub-subplot***
Tillage	*Maize-(inter)cropping*	*Winter wheat variety*
	Sole maize (no intercropping)	
	Clover	
No-tillage	Sudangrass	Levis
Reduced tillage	Phacelia	Forel
	White mustard	
	Indian mustard	

Fig. III.1. Maize-intercropping. Maize as a sole crop (left), maize-white mustard (middle) and maize-clover (right) in July 2016 at Agroscope-Tänikon, 8356 Ettenhausen, Switzerland.

Cover cropping

The field experiment was conducted twice in Switzerland (2016–2017 at Agroscope-Reckenholz: 47°26'15"N 8°31'38"E; 2017–2018 at Agroscope-Tänikon: 47°28'31"N 8°54'14"E). A split-plot design (Table III.2, Fig. SIII.2) in four blocks was used, comprising five cropping systems (herbicide without cover crop, plough without cover crop, white mustard, Indian mustard, winter fodder pea; Fig. III.2) as whole plots and two wheat varieties (Digana, Fiorina) as subplots. The size of each whole plot was 72 m^2 (6 m × 12 m), which was then divided in half for the subplot treatments (3 m × 12 m each). To prevent cross-contamination with FHB-causing species, buffer plots were established as described above in the 'maize-intercropping' experiment.

Silage maize (var. P8057; Pioneer Hybrid International, USA) was the previous crop from the tested cropping systems and was sown across the entire field at 100'000 kernels ha^{-1}. Herbicides (a mixture of Gardo Gold (a.i.: s-metolachlor and terbuthylazin), Callisto (a.i.: mesotrione) and Banvel 4S (a.i.: dicamba); Syngenta, Switzerland) were applied at BBCH 13–15 growth stage. To ensure a sufficient level of FHB infection in the field, maize plants were inoculated with the pin method at BBCH 71–73 growth stage using conidial suspensions of three *F. graminearum* isolates ('0410', CBS 121292, Westerdijk Fungal Biodiversity Institute, The Netherlands; '2113', Research Group Crop Breeding and Genetic Resources, Agroscope, Switzerland; '1145', Fungal Collection of Agroscope, Switzerland; all single-spore isolates from wheat in Switzerland). Equal amounts of each isolate were used and final concentration was adjusted to 10^6 conidia ml^{-1} sterile deionised water containing 0.0125 % Tween® 20. The pin method involved direct penetration (0.5 cm) of the first visible internode above the crown roots with 4-pins previously dipped in the conidial suspension. Each pin had a square pyramid shape. Five maize stalks were inoculated from the middle area of the second, third, sixth and seventh maize row, resulting in ten inoculated stalks for each subsequent wheat subplot.

After the harvest of silage maize, the maize residues were mulched across the entire field. Subsequently, white mustard (var. Salsa; Limagrain, Belgium), Indian mustard (var. Vittasso; KWS, Italy) and winter fodder pea (var. Arkta; Feldsaaten Freudenberger, Germany) were sown at 33, 8.8 and 143 kg ha^{-1}, respectively, using direct sowing. For the 'herbicide without cover crop' treatment, a herbicide (Roundup Profi; a.i.: glyphosate; Leu + Gygax AG, Switzerland) was applied. For the 'plough without cover crop' treatment, maize residues were buried into the soil by inversion tillage with mouldboard ploughing (up to 30 cm depth). White mustard and Indian mustard were mulched before the first frosts, while winter pea was mulched in the beginning of the following spring. Subsequently, spring wheat (var. Digana, var. Fiorina; Saatzucht Düdingen, Switzerland) was sown at 210 kg ha^{-1} using direct sowing,

while in the buffer plots, spring triticale (var. Trado; Saatzucht Düdingen, Switzerland) was sown at 210 kg ha^{-1} after seedbed preparation. The fertilisation inputs for the maize and wheat crops as well as the insecticide application for wheat were the same as described above in the 'maize-intercropping' experiment.

Table III.2. Cover cropping. Split-plot design of the field experiments conducted in 2016–2017 and 2017–2018 (see also supplementary Fig. SIII.2).

	Whole plot	***Subplot***
Previous crop	*Cropping system*	*Spring wheat variety*
Silage maize inoculated with *F. graminaerum*	Herbicide without cover crop Plough without cover crop White mustard Indian mustard Winter pea	Digana Fiorina

Fig. III.2. Cover cropping. Herbicide without cover crop with no-tillage (left), plough without cover crop (middle) and winter fodder pea with no-tillage (right) in November 2017 at Agroscope-Tänikon, 8356 Ettenhausen, Switzerland.

Measurements

Maize-intercropping

Prior to maize harvest, the aboveground biomass of each intercrop species was determined by collecting the plant material from an area of 0.42 m^2 (0.7 m × 0.6 m) from the 2nd and 10th intercrop row and merging it into one composite sample per subplot, which had a total of 12 intercrop rows. The biomass (t ha^{-1}) was determined after drying the samples at 105°C for 48 h. For the grain yield of maize (t ha^{-1}), an area of 12 m^2 (8 m × 1.5 m) was harvested per subplot using a combine harvester and a representative sample of 2 kg was drawn directly from the harvester.

To determine the FHB symptoms (number of symptomatic heads m^{-2}) in wheat, the total number of heads with developed symptoms in an area of 9 m^2 per wheat sub-subplot were counted. To determine yield and seed moisture content of wheat grain, the same area was

harvested using a plot combine harvester (Wintersteiger, Austria). Wheat grain subsamples of 5 g and 150 g were drawn for seed health tests and mycotoxin analysis, respectively, using a riffle divider (Schieritz & Hauenstein AG, Switzerland).

To determine the proportional incidence of FHB causing species in grains (%) for each wheat sub-subplot, seed health tests were conducted (Vogelgsang et al., 2008). This measurement was based on macro- and microscopic observations of the developed fungal colonies (Leslie and Summerell, 2006) by observing 100 grains per sub-subplot.

For mycotoxin analysis, the wheat grain subsamples were finely ground using a mill (Cyclotec™ 1093, Foss Tecator, Sweden; 1-mm screen). Flours were stored at -20°C until mycotoxin extraction. A total of 5 g wheat flour was extracted with 20 ml Milli-Q water:acetonitrile (16:84) solvent solution and shaken for 2 h. Water was purified by a Milli-Q gradient A10 water purification system (MilliporeSigma, USA). Afterwards, each extract was filtered through a folded paper filter (Whatman, 595 ½, 125 mm; GE Healthcare Ltd, UK) and collected in a vial. Then, 2 ml of each extract passed through a 3-ml ISOLUTE® SPE tube (Biotage, Sweden) containing 0.3 g alox:celite (1:1), which was fitted with 20-µm frits at the bottom and the top of each tube, and mounted on a Visiprep SPE Vacuum Manifold (12-port; Supelco, USA). A volume of 200 µl cleaned extract was added into LC-vials with a Hamilton syringe and the eluate was evaporated completely with an airstream using a Visidry Drying Attachment (12-port; Supelco, USA) at 40°C. Finally, a volume of 1 ml Milli-Q water:methanol (90:10) solution was pipetted in each vial and crimped. The following mycotoxin standards were included in the analysis: nivalenol (NIV), deoxynivalenol (DON), 3- and 15-acetyldeoxynivalenol (Ac-DON), fusarenon-x (Fus-X), diacetoxyscirpenol (DAS), HT-2, T-2 (>97 %, premixed standard, Trilogy Analytical Laboratory Inc., USA), monoacetoxyscirpenol (MAS; >98 %, Fermentek, Israel) and zearalenone (ZEN; Sigma-Aldrich, Switzerland). Hereinafter, Ac-DON stands for the sum of 3- and 15-acetyldeoxynivalenol. For each run, reference sample flours (Trilogy Analytical Laboratory Inc., USA) of wheat grain naturally contaminated with either DON or ZEN were included, while a whole-wheat flour (organic product; COOP, Switzerland) without any detected mycotoxins was used for the matrix-matched calibration curve of each standard. Mycotoxins were analysed with liquid chromatography (1260 Series, Agilent, Germany) mass spectrometry (6470 Triple Quad, equipped with JetStream ESI source, Agilent, USA), and the contents in wheat grain are presented in µg kg^{-1} (see Table SIII.1 for instrument parameters). The limit of detection (LOD) and limit of quantification (LOQ) of each analyte are provided in Table SIII.2. When values were below LOQ or LOD, values were replaced by LOQ÷2 or LOD÷2, respectively.

Cover cropping

The FHB symptoms (number of symptomatic heads m^{-2}), grain yield (t ha^{-1}), proportional incidence of FHB causing species in grains (%) and mycotoxins content in grain (µg kg^{-1}) of wheat were determined as described for the 'maize-intercropping' experiment.

Gross margin analysis

For the entire crop rotation of the 'maize-intercropping' and 'cover cropping' experiments, a gross margin analysis was conducted as follows:

$$\mathrm{GM} = \mathrm{R} - \mathrm{OC}$$

where GM is the gross margin, R the receipts and OC the operating costs.

The seed prices of the intercrops, cover crops and wheat were retrieved from UFA-Samen (2019), while the prices of the herbicide and insecticide products were retrieved from the cantonal plant protection office in Switzerland (BBZ-Arenenberg and Strickhof, 2019). The fertilisation costs were obtained from the fertiliser company (Landor, 2019). The costs related to machinery operations were retrieved from Gazzarin (2018) and include the engine performance per h, the operation time per ha in min, the machine cost per ha and the imputed labour costs. The selling prices of grain maize and wheat grain were obtained from Swissgranum (2019), while the selling price of silage maize at 30 % dry matter was obtained from Agridea (2019). In Switzerland, direct payments are granted for the implementation of reduced tillage and no-herbicide use. However, direct payments were not included for the gross margin analysis because agricultural policies frequently change and regulations are usually country-specific. Calculations were performed in Swiss francs (CHF).

Regarding the 'maize-intercropping' experiment, the average values of R and OC were calculated for the sole maize and the different maize-intercropping systems under reduced tillage or no-tillage, pooled across both wheat varieties (Levis, Forel) and experimental years (2016–2017, 2018–2019).

Regarding the 'cover cropping' experiment, the average values of R and OC were calculated for each cropping system, pooled across both wheat varieties (Digana, Fiorina) and experimental years (2016–2017, 2017–2018).

Statistical analysis

Data were analysed with the software Genstat 19th edition (VSN International Ltd., UK) and figures were plotted with Prism 8 (GraphPad Software Inc., USA). Data from both 'maize-intercropping' and 'cover cropping' experiments were analysed separately for each experimental year. The Shapiro-Wilk test was performed to examine whether data follow a normal distribution ($p > 0.05$), and Bartlett's test was used to test whether variances are equal across groups ($p > 0.05$). To approach or reach a normal distribution and homoscedasticity, the response variables were transformed accordingly (e.g. logarithmic, square root transformations). Untransformed data are presented in tables and figures. For post hoc comparisons, Fisher's protected LSD test was used ($\alpha = 0.05$).

Maize-intercropping

A split-split-plot ANOVA was performed to test the effects of 'tillage', 'maize-intercropping' and 'wheat variety' on the response variables in wheat, i.e. 'FHB symptoms', 'grain yield', 'incidence of *Fusarium* species (*F. graminearum*, *F. poae*, *F. avenaceum*, etc.) or *Microdochium* species in grains' and 'DON, Ac-DON or ZEN content in grain'. Hence, the treatment structure (fixed effects) was defined as [tillage × maize-intercropping × wheat variety] and the block structure (random effects) was defined as [block / whole plot / subplot / sub-subplot]. A non-parametric test (Kruskal-Wallis one-way ANOVA) was performed to test the effect of 'maize-intercropping' on 'maize grain yield'. A parametric one-way ANOVA was performed to test the effect of 'year' on the 'dry aboveground biomass of intercrops'.

Cover cropping

A split-plot ANOVA was performed to test the effects of 'cropping system' and 'wheat variety' on the response variables in wheat, i.e. 'FHB symptoms', 'grain yield', 'incidence of *Fusarium* species (*F. graminearum*, *F. poae*, *F. avenaceum*, etc.) in grains' and 'DON, Ac-DON or NIV content in grain'. Hence, the treatment structure (fixed effects) was defined as [cropping system × wheat variety] and the block structure (random effects) as [block / whole plot / subplot].

Results

Maize-intercropping

Maize yield and intercrop biomass

The average grain yield of maize across the entire field was 11.1 and 9.5 t ha^{-1} in 2016 and 2018, respectively. In both years, there was no significant difference between the tested maize-intercropping systems and the sole maize on maize yield (Table SIII.3). In 2016, the highest aboveground dry biomass was observed in sudangrass (1.94 t ha^{-1}), followed by white mustard (1.14 t ha^{-1}), phacelia (0.74 t ha^{-1}), clover (0.56 t ha^{-1}) and Indian mustard (0.48 t ha^{-1}). In 2018, the highest aboveground dry biomass was observed in sudangrass (1.66 t ha^{-1}), followed by clover (0.91 t ha^{-1}), white mustard (0.71 t ha^{-1}), Indian mustard (0.49 t ha^{-1}) and phacelia (0.21 t ha^{-1}). The year effect on the aboveground dry biomass was significant for phacelia (p = 0.001) and white mustard (p = 0.009).

Wheat yield

The average grain yield of winter wheat across the entire field was 6.28 and 6.42 t ha^{-1} in 2017 and 2019, respectively (Table SIII.4).

Experiment in 2016–2017

A three-way interaction was observed among tillage, maize-intercropping and wheat variety on wheat yield (Table SIII.5; p = 0.027). Within the wheat var. Levis, maize-sudangrass resulted in the highest yield compared with the other intercropping systems under no-tillage (p < 0.05), whereas the effect of maize-intercropping was not significant under reduced tillage (Table SIII.6). The effect of maize-intercropping on the yield of var. Forel was not significant under no-tillage or reduced tillage (Table SIII.6). Within Levis, reduced tillage resulted in higher grain yield compared with no-tillage for maize-phacelia (p = 0.026) and maize-white mustard (p = 0.030) intercropping. There was no difference on the yield of Forel between no-tillage and reduced tillage within maize-intercropping system. There was no difference between Levis and Forel on yield within tillage practice and maize-intercropping system.

Experiment in 2018–2019

Significant effects of tillage (p = 0.002), maize-intercropping (p < 0.001) and wheat variety (p < 0.001) were observed on wheat yield (Table SIII.5). Compared with the sole maize (6.75 t ha^{-1}), the maize-intercropping with clover, phacelia and Indian mustard resulted in 7 % to 9 % lower yield of subsequent wheat crop, whereas intercropping with white mustard or sudangrass did not significantly affect wheat yield (Table SIII.7). Reduced tillage had on

average higher yield compared with no-tillage (6.82 versus 6.03 t ha^{-1}). Forel had on average higher yield than Levis (6.68 versus 6.16 t ha^{-1}).

Fusarium head blight and mycotoxins in wheat

On average across the entire field, FHB symptoms, the incidence of *F. graminearum* and *Microdochium* spp. in grains and DON content in grain were 30-, 3-, 4- and 11-fold higher in 2019 compared with 2017 (Table SIII.4). Moreover, Ac-DON was only detected in 2019. Contrarily, the incidence of *F. avenaceum* and *F. poae* was 4- and 9-fold higher, respectively, in 2017 than in 2019 as well as ZEN was only detected in 2017 (Table SIII.4).

Experiment in 2016–2017

A significant interaction was found between tillage and maize-intercropping regarding FHB symptoms (Table SIII.5; $p = 0.026$). Under no-tillage, maize-white mustard intercropping resulted in lower number of FHB symptoms compared with sole maize ($p < 0.05$), while under reduced tillage, no significant differences were observed between maize-intercropping systems and sole maize. For maize-clover and maize-Indian mustard, reduced tillage resulted in higher number of FHB symptoms compared with no-tillage ($p = 0.048$ and $p = 0.012$, respectively). Higher number of FHB symptoms was observed in the wheat var. Levis than in var. Forel ($p < 0.001$).

Overall in 2017, 20 % of wheat grains were infected by a FHB causing species with the most dominant being *F. graminearum* (ratio of 82 %) followed by *F. poae* (7 %), *F. avenaceum* (5 %) and *Microdochium* spp. (4 %) (Fig. III.3 a). Regarding the incidence of *F. graminearum*, significant interactions were found between tillage and maize-intercropping ($p = 0.029$) as well as between tillage and wheat variety ($p = 0.019$) (Table SIII.5). Under no-tillage, maize intercropping with Indian mustard or white mustard resulted in lower *F. graminearum* incidence compared with sole maize ($p < 0.05$), while the effect of intercropping was not significant under reduced tillage. For maize-sudangrass, no-tillage increased *F. graminearum* incidence compared with reduced tillage ($p = 0.042$), while no significant effect of tillage was observed for sole maize or the other intercropping systems. Under both no-tillage ($p = 0.029$) and reduced tillage ($p < 0.001$), grains from Levis showed a higher *F. graminearum* incidence compared with grains from Forel, while no significant effect of tillage was observed within Levis or Forel. The main effects of maize-intercropping ($p = 0.017$) and wheat variety ($p = 0.002$) were significant regarding the incidence of *F. poae* (Table SIII.5). Maize-white mustard resulted in higher *F. poae* incidence (2.4 %) compared with sole maize (0.8 %) and the other intercropping systems (1.1–1.3 %) ($p < 0.05$). The incidence of *F. poae* was higher in grains from Levis compared with grains from Forel ($p = 0.002$), while there was no effect of tillage on

the *F. poae* incidence. The incidence of *F. avenaceum* was not affected by tillage, maize-intercropping or wheat variety.

The average DON and ZEN contents across the entire field were 450 and 180 µg kg^{-1}, respectively (Table SIII.4), while the values of all other measured mycotoxins were below detection or quantification limits (Table SIII.2). Maize-intercropping showed a significant effect on DON ($p < 0.001$) and on ZEN ($p = 0.039$) contents (Table SIII.5). Maize-white mustard and maize-Indian mustard intercropping decreased DON by 58 % and 32 %, respectively, compared with sole maize (590 µg kg^{-1}; $p < 0.05$; Fig. III.4 a). Maize-white mustard and maize-phacelia intercropping decreased ZEN content by 47 % and 34 %, respectively, compared with sole maize (240 µg kg^{-1}; $p < 0.05$). A significant interaction was found between tillage and wheat variety on DON content (Table SIII.5). Grains from Levis contained higher amounts of DON than grains from Forel, both under no-tillage ($p = 0.012$) and reduced tillage ($p < 0.001$), while no significant effect of tillage was observed regardless of the wheat variety (Fig. III.5 a). Use of Levis resulted in 3-fold higher ZEN content compared with Forel ($p < 0.001$). Tillage showed no effect on ZEN (Fig. III.6).

Experiment in 2018–2019

Regarding FHB symptoms in wheat, a significant interaction was observed between tillage and wheat variety ($p = 0.024$), while maize-intercropping had no significant effect (Table SIII.5). There was no difference between no-tillage and reduced tillage on FHB symptoms. Under reduced tillage, Forel had higher number of FHB symptoms than Levis ($p < 0.001$), but the effect was not significant under no-tillage.

Overall in 2019, 49 % of wheat grains were infected by a FHB causing species with the most dominant being *F. graminearum* (ratio of 93 %) followed by *Microdochium* spp. (6 %) (Fig. III.3 b). Neither the maize-intercropping nor tillage showed a significant effect on the incidences of *F. graminearum* and *Microdochium* spp. (Table SIII.5), while use of Levis resulted in higher incidences compared with Forel ($p < 0.001$ and $p < 0.002$; respectively).

The average contents of DON and Ac-DON in grain across the entire field were 5040 and 150 µg kg^{-1}, respectively, while the values of all other measured mycotoxins were below detection or quantification limits (Table SIII.2). Regarding DON, the effects of maize-intercropping ($p = 0.032$) and wheat variety ($p = 0.005$) were significant (Table SIII.5), whereas the effect of tillage was not significant (Fig. III.5 b). The lowest DON contents were observed in maize-Indian mustard and maize-clover intercropping and the highest in maize-phacelia and maize-sudangrass, while maize-white mustard and sole maize led to intermediate values (Fig III.4 b; $p < 0.05$). Grains from Levis contained higher DON contents compared with grains from Forel

(5560 and 4520 µg kg^{-1}, respectively). A three-way interaction was observed among tillage, maize-intercropping and wheat variety on Ac-DON (Table SIII.5; p = 0.019). Within Levis, no significant differences were observed among the tested maize-intercropping systems regardless of the tillage treatment. In contrast, within Forel, use of maize-clover intercropping and sole maize resulted in the highest and lowest Ac-DON contents (170 and 50 µg kg^{-1}, respectively) under no-tillage (p < 0.05), whereas no differences were observed under reduced tillage. No differences were observed between no-tillage and reduced tillage on Ac-DON content with only exception the use of maize-clover intercropping within Forel where no-tillage resulted in higher Ac-DON content (170 and 60 µg kg^{-1} ; p = 0.011). The Ac-DON contents were higher in grains from Levis than Forel, yet results were only significant within sole maize under no-tillage (p = 0.003) as well as within maize-clover (p = 0.016) and maize-Indian mustard (p = 0.047) under reduced tillage.

Cover cropping

Wheat yield

Across the entire field the average grain yield of spring wheat was 4.66 and 4.46 t ha^{-1} in 2017 and 2018, respectively (Table SIII.8). In 2017, yield was similar among cropping systems, whereas in 2018, the use of cover crops (winter pea, Indian mustard or white mustard) improved yield by 7 % to 25 % compared with the herbicide or plough treatments (p < 0.05; Tables SIII.9 and SIII.10). In both years, the wheat var. Fiorina resulted in higher yield than Digana (2017: 4.96 and 4.36 t ha^{-1}, p = 0.027; 2018: 4.59 and 4.33 t ha^{-1}, p = 0.019).

Fusarium head blight and mycotoxins in wheat

On average, FHB symptoms as well as the incidence of *F. graminearum* and *F. avenaceum* in grains were 9-, 7- and 5-fold higher in 2018 compared with 2017 (Table SIII.8). The average contents of DON, Ac-DON and NIV in wheat grain were 35-, 18- and 2-fold higher in 2018 compared with 2017 (Table SIII.8). Contrarily, the average incidence of *F. poae* was 2-fold higher in 2017 compared with 2018 (Table SIII.8).

Experiment in 2016–2017

A significant interaction was observed between cropping system and wheat variety on FHB symptoms (Table SIII.9; p = 0.031). Within Fiorina, winter pea resulted in a ~3-fold higher number of FHB symptoms compared with the other cropping systems (p < 0.05), whereas within Digana, there was no difference among cropping systems. No significant differences between Fiorina and Digana were observed on FHB symptoms within a given cropping system.

Overall in 2017, 8 % of wheat grains were infected by a FHB causing species with the most dominant being *F. poae* (ratio of 41 %) followed by *F. graminearum* (23 %) and *F. avenaceum* (23 %) (Fig. III.3 c). The incidence of *F. poae* and *F. graminearum* was not affected by cropping system. However, the use of winter pea as cover crop resulted in higher incidence of *F. avenaceum* compared with the other cropping systems (7 and range of 0–1 %, respectively; $p < 0.05$). Higher incidence of *F. poae* was observed in grains from Fiorina than Digana ($p = 0.002$), while no differences were found between the two wheat varieties regarding the *F. graminearum* and *F. avenaceum* incidences.

The average contents of DON, Ac-DON and NIV in grain across the entire field were 70, 10 and 40 μg kg^{-1} (Table SIII.8), while the values of the other measured mycotoxins were below the quantification or detection limits (Table SIII.2). The tested cropping systems resulted in similar DON contents in wheat grain ranging from 50 to 100 μg kg^{-1}. Similar NIV and Ac-DON contents were found among cropping systems in Fiorina, while values were below detection limit in Digana. On average, higher DON content was found in Fiorina compared with Digana (120 and 30 μg kg^{-1}; $p < 0.001$).

Experiment in 2017–2018

Both the cropping system ($p = 0.002$) and the wheat variety ($p < 0.001$) showed significant effects on FHB symptoms (Table SIII.9). The highest number of FHB symptoms was found in the herbicide treatment and the lowest in the plough treatment (20 and 4 symptomatic heads m^{-2}, respectively) with cover crops taking intermediate values (7–14 symptomatic heads m^{-2}; $p < 0.05$). A 5-fold higher number of FHB symptoms was found in Digana compared with Fiorina ($p < 0.001$).

Overall in 2018, 28 % of wheat grains were infected by a FHB causing species with the most dominant being *F. graminearum* (ratio of 46 %) followed by *F. avenaceum* (37 %) and *F. poae* (7 %) (Fig. III.3 d). The incidence of *F. graminearum* and *F. poae* was not affected by cropping system, while a significant effect was found for the incidence of *F. avenaceum* ($p = 0.002$). The highest incidence of *F. avenaceum* was observed with the use of winter pea (20 %) and the lowest in the plough (4 %), while moderate incidences were found in the herbicide, white mustard and Indian mustard (7 %, 10 % and 11 %, respectively). The incidence of *F. graminearum* was higher in Digana than Fiorina ($p = 0.005$), whereas the opposite occurred for *F. poae* ($p = 0.006$).

The average contents of DON, Ac-DON and NIV in grain across the entire field were 2580, 180 and 70 μg kg^{-1} (Table SIII.8), while the values of the other measured mycotoxins were below the quantification or detection limits (Table SIII.2). A significant interaction between cropping

system and wheat variety was observed on DON (Table SIII.9; $p = 0.031$). Within Digana and compared with the herbicide treatment, the use of plough, white mustard, Indian mustard or winter pea decreased DON by 69 %, 44 %, 50 % and 75 %, respectively (Fig. III.7 a; $p < 0.05$). Within Fiorina and compared with the herbicide treatment, the use of plough or cover crops (winter pea, white mustard or Indian mustard) decreased DON by 58 % to 82 % (Fig. III.7 b; $p < 0.05$). Within white mustard, Digana resulted in 2-fold higher DON compared with Fiorina ($p = 0.024$), whereas for the other treatments, Digana and Fiorina did not differ significantly. There was a significant effect of cropping system on Ac-DON (Table SIII.9; $p = 0.004$). The plough and cover crop treatments decreased Ac-DON by 69 % to 86 % compared with the herbicide treatment (Fig. III.8; $p < 0.05$). The NIV content did not differ among the cropping systems ranging from 40 to 100 µg kg^{-1}. Digana resulted in 3-fold higher Ac-DON compared with Fiorina ($p < 0.001$).

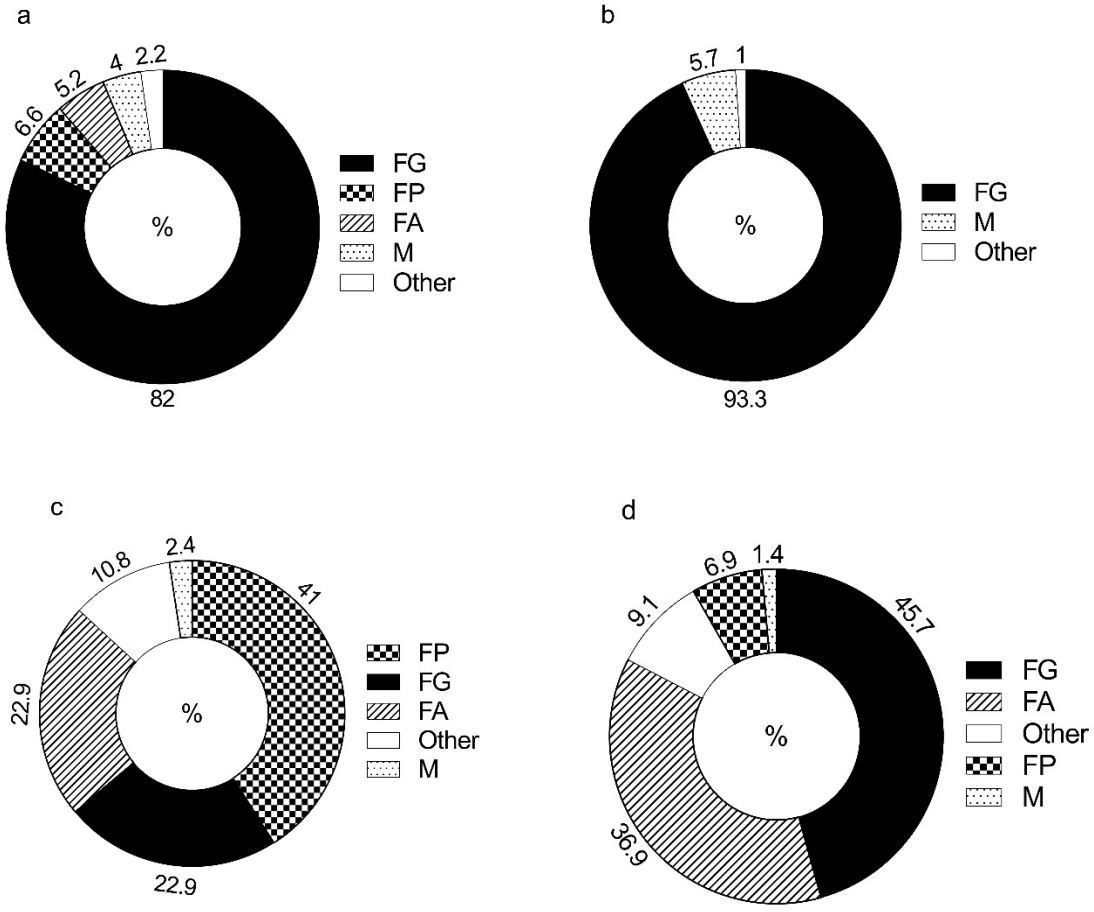

Fig. III.3. Effect of **maize-intercropping** (**a**, 2016–2017; **b**, 2018–2019; n = 96) and **cover cropping** (**c**, 2016–2017; **d**, 2017–2018; n = 40) on the ratio of isolated Fusarium head blight causing species (FG: *Fusarium graminearum*; FP: *F. poae*; FA: *F. avenaceum*; M: *Microdochium* spp.; Other: *F. culmorum*, *F. cerealis*, other *F.* spp.) from wheat grains across the entire field.

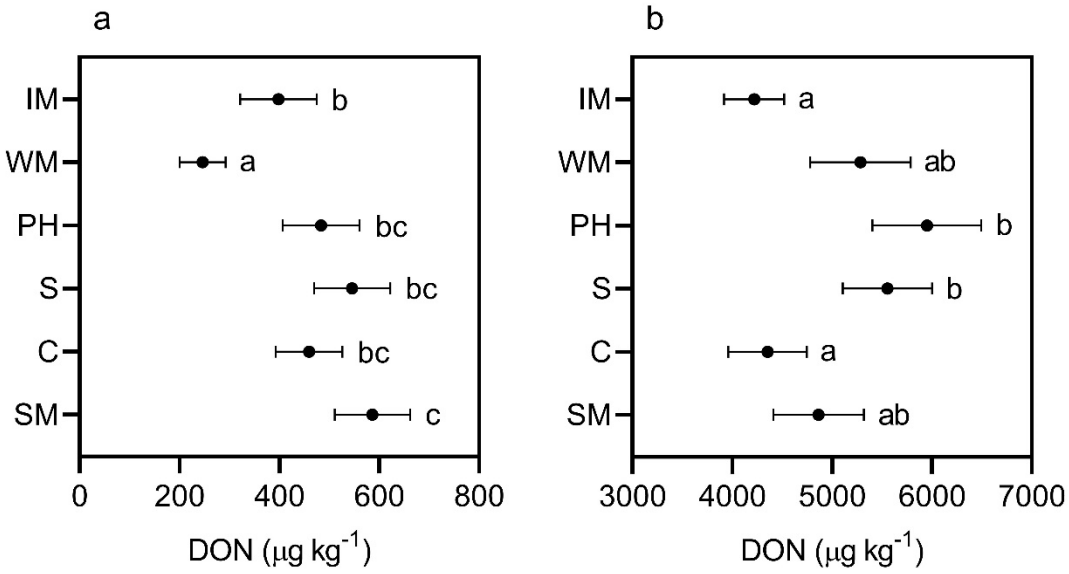

Fig. III.4. Maize-intercropping. Effect of intercropping system (sole maize: SM; clover: C; sudangrass: S; phacelia: PH; white mustard: WM; Indian mustard: IM) on deoxynivalenol (DON) content (µg kg^{-1}) in wheat grain in 2016–2017 (**a**) and 2018–2019 (**b**). Average values of two tillage practices (no-tillage, reduced tillage) and two wheat varieties (Levis, Forel) are presented. Note the different scales of the DON content between the two experimental years. Different letters indicate significant differences according to Fisher's protected LSD test for post hoc comparisons (α = 0.05) and bars represent the standard error of the mean (n = 16).

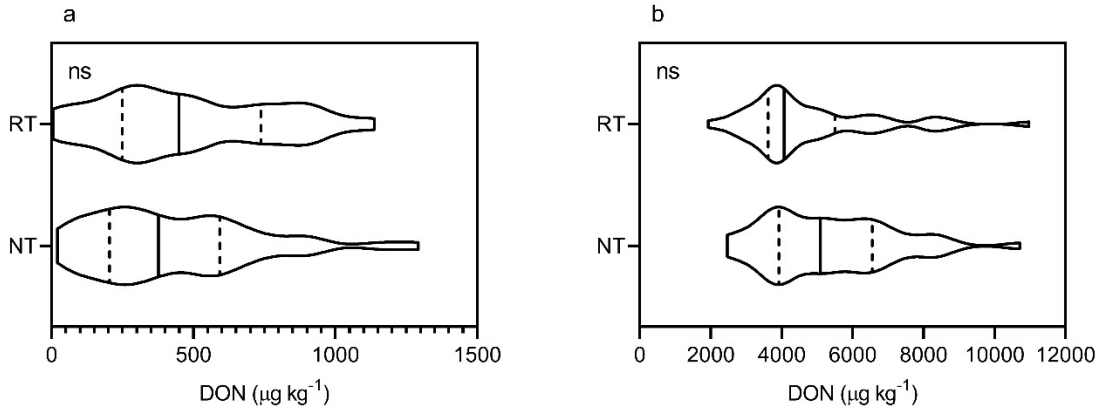

Fig. III.5. Maize-intercropping. Effect of tillage practice (no-tillage, NT; reduced tillage, RT) on deoxynivalenol (DON) content (µg kg^{-1}) in wheat grain in 2016–2017 (**a**) and 2018–2019 (**b**). Average values of six (inter)cropping systems (sole maize: SM; clover: C; sudangrass: S; phacelia: PH; white mustard: WM; Indian mustard: IM) and two wheat varieties (Levis, Forel) are presented (n = 48). Note the different scales of the DON content between the two experimental years. Within each violin plot, continuous and dashed lines indicate the median and quartiles, respectively.
ns: not significant

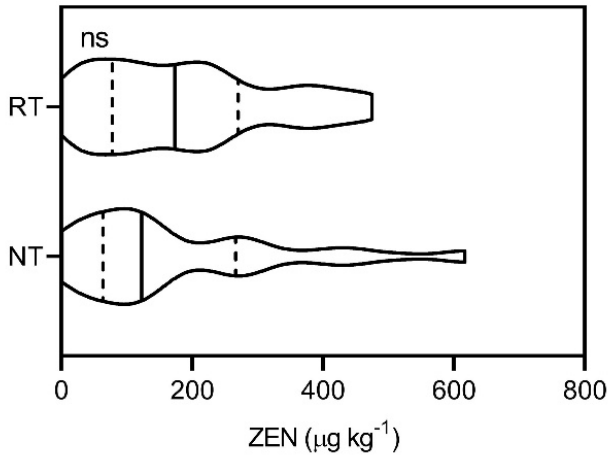

Fig. III.6. Maize-intercropping. Effect of tillage practice (no-tillage, NT; reduced tillage, RT) on zearalenone (ZEN) content (μg kg^{-1}) in wheat grain in 2016–2017. Average values of six (inter)cropping systems (sole maize: SM; clover: C; sudangrass: S; phacelia: PH; white mustard: WM; Indian mustard: IM) and two wheat varieties (Levis, Forel) are presented (n = 48). Within each violin plot, continuous and dashed lines indicate the median and quartiles, respectively.
ns: not significant

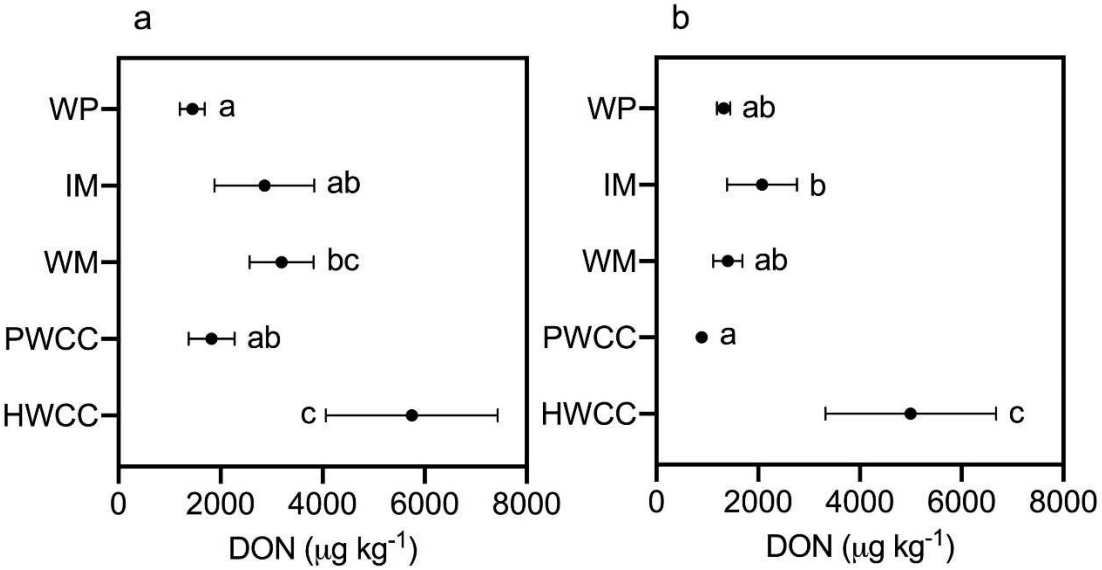

Fig. III.7. Cover cropping. Effect of cropping system (herbicide without cover crop: HWCC; plough without cover crop: PWCC; white mustard: WM; Indian mustard: IM; winter pea: WP) on deoxynivalenol (DON) content (μg kg^{-1}) in wheat grain of var. Digana (**a**) and var. Fiorina (**b**) in 2017–2018. Different letters indicate significant differences according to Fisher's protected LSD test for post hoc comparisons (α = 0.05) and bars represent the standard error of the mean (n = 4).

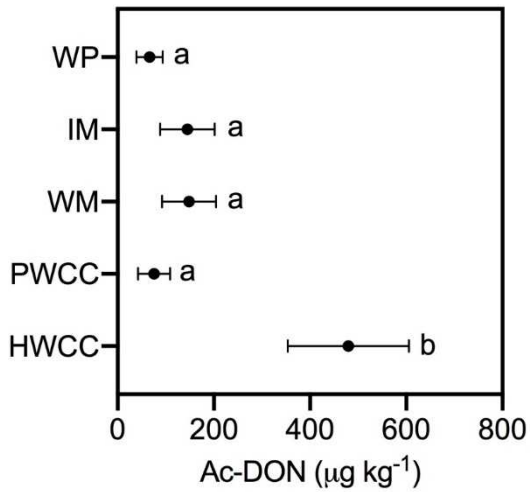

Fig. III.8. Cover cropping. Effect of cropping system (herbicide without cover crop: HWCC; plough without cover crop: PWCC; white mustard: WM; Indian mustard: IM; winter pea: WP) on the sum of 3- and 15-acetyldeoxynivalenol (Ac-DON) content (μg kg^{-1}) in wheat grain in 2017–2018. Average values of two wheat varieties (Digana, Fiorina) are presented. Different letters indicate significant differences according to Fisher's protected LSD test for post hoc comparisons (α = 0.05) and bars represent the standard error of the mean (n = 8).

Gross margin analysis

Maize-intercropping

Regarding the maize crop, the use of sole maize generated the highest receipts (CHF 4011 ha^{-1}) and the lowest total operating costs (CHF 2077 ha^{-1}) resulting in the highest gross margin (CHF 1934 ha^{-1}), which was 19 % to 31 % higher than with the intercropping systems. The differences on the gross margin of maize between sole maize and intercropping systems are explained by treatment-specific operating costs and maize yield variations. The operating costs for the sowing of the intercrops ranged from CHF 167 to 398 ha^{-1} depending on the crop species, whereas the costs for the herbicide treatment of the sole maize was CHF 136 ha^{-1}. Although maize yield was not significantly different between sole maize and maize-intercropping systems, it was slightly higher for the former than the latter yielding higher receipts (Table III.3).

Regarding the wheat crop under no-tillage, maize-sudangrass intercropping yielded the highest receipts (CHF 3293 ha^{-1}) and the highest gross margin (CHF 1651 ha^{-1}). Regarding the wheat crop under reduced tillage, sole maize yielded the highest receipts (CHF 3373 ha^{-1}), resulting in 2 % to 11 % higher gross margin compared with the intercropping systems, which ranged from CHF 1392 to 1550 ha^{-1}. The differences in the gross margin of wheat are explained

by wheat yield variations. Use of reduced tillage resulted in additional operating costs (CHF 160 ha^{-1}) compared with no-tillage. However, due to higher wheat yields, reduced tillage had 2 % to 28 % higher wheat gross margins compared with no-tillage, except for maize-sudangrass where the opposite occurred (Table III.3).

Regarding the entire maize-wheat rotation under no-tillage, the highest gross margin was generated by sole maize (CHF 3471 ha^{-1}) with the maize-intercropping systems ranging from CHF 2609 to 2987 ha^{-1}. Regarding the entire maize-wheat rotation under reduced tillage, the highest gross margin was generated by sole maize (CHF 3505 ha^{-1}) with the maize-intercropping systems ranging from CHF 2886 to 3083 ha^{-1} (Table III.3).

Table III.3. Maize-intercropping. Effect of maize-intercropping system (sole maize: SM; clover: C; sudangrass: S; phacelia: PH; white mustard: WM; Indian mustard: IM) under no-tillage or reduced tillage on receipts, operating costs and gross margin of grain maize, wheat grain and entire crop rotation in Swiss Francs (CHF) ha^{-1}. Average values of two experimental years (2016–2017, 2018–2019) and two wheat varieties (Levis, Forel) are presented (n = 16).

	No-tillage					
	SM	C	S	PH	WM	IM
Receipts						
Grain maize	4011	3832	3676	3656	3714	3674
Wheat grain	3179	2716	3293	2891	2894	2890
Operating costs						
Grain maize	2077	2297	2339	2108	2147	2125
Wheat grain	1642	1642	1642	1642	1642	1642
Gross margin						
Grain maize	1934	1535	1336	1548	1567	1549
Wheat grain	1537	1074	1651	1249	1252	1248
Gross margin of entire rotation	3471	2609	2987	2797	2818	2797
	Reduced tillage					
	SM	C	S	PH	WM	IM
Receipts						
Grain maize	4011	3832	3676	3656	3714	3674
Wheat grain	3373	3303	3351	3337	3307	3194
Operating costs						
Grain maize	2077	2297	2339	2108	2147	2125
Wheat grain	1802	1802	1802	1802	1802	1802
Gross margin						
Grain maize	1934	1535	1336	1548	1567	1549
Wheat grain	1571	1501	1550	1535	1505	1392
Gross margin of entire rotation	3505	3036	2886	3083	3072	2942

Cover cropping

Overall, the gross margin of the entire rotation for the cover cropping was 56 % to 70 % lower compared with the maize-intercropping. Cultivation of winter pea after silage maize yielded the highest receipts (CHF 2533 ha^{-1}) for the wheat crop. The lowest receipts (CHF 2293 ha^{-1}) were observed after the herbicide treatment without growing a cover crop (HWCC). However, the highest gross margin over the entire rotation was generated by maize-HWCC-wheat rotation (CHF 1263 ha^{-1}) followed by maize-white mustard-wheat (CHF 1170 ha^{-1}), maize-winter pea-wheat (CHF 1109 ha^{-1}), maize-Indian mustard-wheat (CHF 1082 ha^{-1}) and maize-plough without cover crop-wheat (CHF 1035 ha^{-1}) (Table III.4). The differences on the gross margin are explained by the treatment-specific operating costs which were higher for the cover crops and plough treatments compared with the HWCC treatment.

Table III.4. Cover cropping. Effect of cropping system (herbicide without cover crop: HWCC; plough without cover crop: PWCC; white mustard: WM; Indian mustard: IM; winter pea: WP) on receipts, operating costs and gross margin of silage maize, wheat grain and entire crop rotation in Swiss Francs (CHF) ha^{-1}. The treatment-specific operating costs refer to costs derived from the use of cover crops, herbicide application or plough. Average values of two experimental years (2016–2017, 2018–2019) and two wheat varieties (Digana, Fiorina) are presented (n = 16).

	HWCC	PWCC	WM	IM	WP
Receipts					
Silage maize	2460	2460	2460	2460	2460
Wheat grain	2293	2359	2363	2312	2533
Operating costs					
Silage maize	1659	1659	1659	1659	1659
Treatment	132	425	296	332	526
Wheat grain	1699	1699	1699	1699	1699
Gross margin					
Silage maize	801	801	801	801	801
Wheat grain	594	660	664	613	834
Gross margin of entire rotation	1263	1035	1170	1082	1109

Discussion and conclusions

To the best of our knowledge, this is the first study demonstrating the utilisation of maize-intercropping as a strategy to control FHB in a subsequent cereal crop. Compared with maize grown as sole crop, the use of white mustard and Indian mustard as intercrops of maize decreased DON content in subsequent winter wheat by 52 % and 32 %, respectively. The mechanisms by which intercrops can affect disease dynamics may include changes in the microclimate; alterations of wind, rain and/or vector dispersal; changes of host morphology and physiology; as well as direct pathogen inhibition (Boudreau, 2013). We suggest that the main disease-suppression mechanism of the mustard involves its antifungal properties based on the release of glucosinolate-derived substances (Brown and Morra, 1997; Manici et al., 1997; Drakopoulos et al., 2020). Nevertheless, substantial reduction of FHB and mycotoxins through maize-mustard intercropping was only observed under moderate disease pressure (2017) and not under very high pressure (2019). This might also be explained by the fact that in 2018, the dry biomass of white mustard was 38 % lower compared with 2016. Therefore, the sufficient establishment of the intercrops appears to be crucial to effectively reduce FHB and mycotoxins in subsequent wheat. Another important aspect that should be considered in the design of intercropping systems is any potential interspecific competition which could reduce the yield of the main crop. In our study, none of the tested intercropping systems significantly reduced the yield of grain maize, when intercrops were sown at the BBCH 13–15 growth stage of maize.

One of the most effective cultural practices to manage FHB is the adoption of a suitable crop rotation (Champeil et al., 2004; Shah et al., 2018). Leplat et al. (2013) discussed the importance of choosing the preceding crop in the development of FHB in wheat and suggested assessing the role of less frequently studied cover crops, such as Indian mustard. In fact, we demonstrated that growing white mustard, Indian mustard or winter fodder pea as interval cover crop in a silage maize-spring wheat rotation under no-tillage was an effective strategy to reduce FHB and mycotoxins in wheat. Use of white mustard, Indian mustard and winter pea reduced mycotoxins in wheat grain by up to 72 %, 58 % and 75 %, respectively. Remarkably, with the use of cover crops, the decrease in mycotoxins was comparable to the plough treatment, with which the maize residues were buried into the soil. Nevertheless, it should be noted that winter pea reduced the incidence of *F. graminearum*, but increased the incidence of *F. avenaceum*. This finding is in line with a study from Walder et al. (2017) who observed that another legume cover crop, i.e. hairy vetch (*Vicia villosa*), was a potent alternative host for *Fusarium* spp. (OTU *F. avenaceum* / *F. tricinctum*). The awareness about the pathogenic potential of *F. avenaceum* or other *Fusarium* species on field pea and other legume crops is

relatively recent and merits further research (Šišić et al., 2018). It was previously suggested that agricultural practices targeting individual FHB causing species might create variances on ecological niches that will be filled by other species within the FHB complex (Xu and Nicholson, 2009; Vogelgsang et al., 2019). Therefore, a judicious choice of cover crop species should also account for other potential phytopathological risks for the subsequent crops. In the 'maize-intercropping' experiments, *F. graminearum* was by far the most dominant occurring FHB species in wheat grains, whereas in the 'cover cropping' experiments, a higher species diversity was observed. An increased diversity of mycotoxigenic species could also alter the range of mycotoxins accumulated in the harvested products. Some of these secondary metabolites are considered as emerging mycotoxins and till now, no regulations on allowable limits have been enforced (Gruber-Dorninger et al., 2016). Thus, more research is needed to better understand the interactions between the different FHB causing species and the diversity of mycotoxins in grain.

The effective control of Fusarium head blight (FHB) improves food safety and food security in cereal-based rotation worldwide and thus is an important component of agronomic and economic sustainability. Within the context of sustainable intensification, utilisation of intercrops or cover crops could play a vital role in FHB control resulting in the design of sound cropping systems. A multivariate meta-analysis across the USA including over 100 wheat fields testing the efficacy of triazole-based fungicides showed that, depending on the triazole chemistry, the mean DON content in grain was only reduced by 12 % to 45 % (Paul et al., 2008). This finding indicates a great need for unified efforts to control FHB using integrated management strategies (McMullen et al., 2012). Li et al. (2019) demonstrated that diversifying crop rotations improved the resilience and resistance to biotic stress while ensuring consistent crop yields. Moreover, Dainese et al. (2019) showed that landscape simplification led to species loss and thus negatively impacted ecosystem services and crop productivity. Following our approach, the inclusion of an intercrop or an interval cover crop in a conventional maize-wheat rotation could indeed promote the diversification of agroecosystems.

Certainly, profitability is a crucial factor influencing farmers' decisions to follow alternative cropping systems. Although the inclusion of intercrops or cover crops can bring several benefits to the agroecosystem, such as reduced soil erosion, provision of nitrogen and increased soil organic matter, it also leads to additional costs (Snapp et al., 2005). We detected economic trade-offs associated with maize-intercropping or cover cropping systems. The main reasons for the reduced gross margins of such systems were the increased operating costs, in particular the sowing of the intercrops (CHF 167–398 ha^{-1}) or cover crops (CHF 185–416 ha^{-1}). Nevertheless, the differences on the gross margin between the cropping systems 'silage

maize-herbicide without cover crops-spring wheat' (CHF 1263 ha^{-1}) and 'silage maize-cover crops-spring wheat' (CHF 1082–1170 ha^{-1}) were rather small due to higher wheat yields with the use of cover crops. It is important to be noted that, overall, the gross margin of the entire rotation for the cover cropping was substantially lower (56–70%) compared with the maize-intercropping. The main reasons for the latter are the lower yield of spring wheat than winter wheat as well as the lower revenue from silage maize compared with grain maize.

Interestingly, we found similar DON and ZEN contents in wheat grain under no-tillage and reduced tillage. Hence, our findings suggest that farmers could still choose no-tillage over reduced tillage without increasing the risk for elevated mycotoxin contamination. However, compared with no-tillage, reduced tillage resulted in most cases in higher wheat yields and therefore higher receipts, outweighing the associated increased operating costs. Pittelkow et al. (2015) demonstrated that no-tillage can frequently reduce crop yields as opposed to conventional tillage, but its negative impact can be minimised when combined with residue retention and appropriate crop rotation. This is in line with the findings of the current study, since we observed equal or even higher wheat yields for the cover cropping systems under no-tillage compared with the plough treatment without growing a cover crop.

In summary, our study investigated the effectiveness of innovative cropping systems to reduce mycotoxins in wheat while also considering associated economic trade-offs. We showed that utilisation of mustard as intercrop in maize as well as winter pea or mustard as interval cover crop in a maize-wheat rotation considerably decreased mycotoxins in wheat. However, in years with severe FHB disease pressure ('maize-intercropping' experiment in 2018–2019 and 'cover cropping' experiment in 2017–2018), the use of maize-intercropping systems was not sufficient to decrease mycotoxins in wheat, but significant toxin reductions were still achieved using interval cover crops in a maize-wheat rotation. The findings of this study suggest that higher crop diversification results in more effective management of mycotoxins in cereal-based rotations. Certainly, farmers may face challenges during adoption of such cropping systems and need to carefully weigh potential conflicts between farm profitability and food safety goals. Hence, adjusted agricultural policies could support farmers in the use of intercropping or cover cropping systems in cereal-based rotations with reduced tillage. As a result, these policies could serve as a bridge towards augmented diversification of cropping systems with the overall goal of improving food safety while ensuring farmer's economic viability.

Acknowledgements

The authors warmly thank Pascal Weber, Anja Marty and Sofie Bolt for the excellent technical assistance. 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 (grant agreement No. 678781) and the Swiss State Secretariat for Education, Research and Innovation.

5. Chapter IV. Control of *Fusarium graminearum* in wheat with mustard-based botanicals: from *in vitro* to *in planta*

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Abstract

Fusarium graminearum is a phytopathogenic fungus that causes Fusarium head blight with significant yield reductions in small-grain cereals, such as wheat. Moreover, it contaminates grain with health-threatening mycotoxins, such as deoxynivalenol (DON), jeopardizing food and feed safety. Plant-based biopesticides, i.e. botanicals, have recently gained increased interest in crop protection as alternatives to synthetic chemical products. The main objective of this study was to test the control efficacy of botanicals based on white or Indian/oriental mustard seed flours (Tillecur - Ti, Pure Yellow Mustard - PYM, Pure Oriental Mustard - POM, Oriental Mustard Bran - OMB) on *F. graminearum* infection and mycotoxin accumulation in wheat grain. Botanicals at 2 % concentration showed a higher efficacy in inhibiting mycelium growth *in vitro* compared with a prothioconazole fungicide (F). Under controlled conditions (growth chamber experiment), the spraying agents reduced DON content in grain in the following order: F = Ti = PYM > POM > OMB. The antifungal activity of the mustards may be attributed to their bioactive matrices containing isothiocyanates (ITCs) and phenolic acids. Allyl ITC was detected in POM and OMB at 8.38 and 4.48 mg g^{-1}, while p-hydroxybenzyl ITC was found in Ti and PYM at 2.56 and 2.44 mg g^{-1}. Considerable amounts of various phenolic acids were detected in all botanicals. Under field conditions, only the use of F significantly decreased *F. graminearum* infection and DON content in grain. An additional important finding of this study is that disease control is more difficult when infection occurs with ascospores, which might have several potential implications considering that ascospores are more important in disease epidemics than conidia. Overall, our results suggest that mustard-based botanicals can be considered as effective natural products against Fusarium head blight in small-grain cereals, but for field applications, an appropriate formulation is necessary to stabilize and prolong the antifungal activity, especially against ascospores.

Keywords: Fusarium head blight; antifungal botanical; isothiocyanate; phenolic acid; mycotoxin; conidia; ascospores; wheat

Introduction

Fusarium species are phytopathogenic fungi, which mainly lead to Fusarium head blight (FHB) in small-grain cereals, such as common wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), triticale (× *Triticosecale*), and oats (*Avena sativa*), as well as to Fusarium ear and stalk rot in maize (*Zea mays*). In most parts of the world, the most prevalent FHB causing species in wheat is *Fusarium graminearum* (Osborne and Stein, 2007; Pasquali et al., 2016; Vogelgsang et al., 2019). Epidemics of FHB are frequently resulting in severe economic losses for cereal farmers due to significant reductions in grain yield and quality (Parry et al., 1995). Upon infection of the inflorescences, several *Fusarium* species produce health-threatening secondary metabolites, named mycotoxins, jeopardizing food and feed safety. The most toxicologically important mycotoxins produced by *F. graminearum* are deoxynivalenol (DON) and zearalenone (ZEN). The European Union and many other countries around the globe have established maximum levels in human food and guidance levels in animal feed (Anonymous, 2006; Ferrigo et al., 2016). Thus, the management of FHB in wheat is crucial in order to minimize yield losses and reduce mycotoxin contamination to the lowest possible levels.

The control strategies of FHB in wheat can be categorized in prevention and intervention approaches. The prevention strategies include crop rotation with non-host plant species, management of remaining crop residues with tillage, and breeding for resistance. The intervention strategies mainly focus on protective measures of the cereal heads during anthesis (e.g. fungicides, biological control) and management strategies during or after harvest (e.g. grain processing) (Wegulo et al., 2015). Within the context of minimizing the negative impact of synthetic pesticides on the environment and the arising health-related issues, natural plant protection products have gained increased attention during the last years as an alternative for chemical disease control (Czaja et al., 2015). Botanicals are plant-based products that are frequently degrading faster than synthetic pesticides and therefore having potentially less negative impact on non-target organisms and less environmental risks (Regnault-Roger and Philogène, 2008). Botanicals have also been considered as 'eco-chemical' and 'bio-rational' products for the sustainable management of crop pests and diseases aiming to improve the safety of the producer, consumer, and the environment (Cavoski et al., 2011; Campos et al., 2016).

Mustard plants contain secondary metabolites, named glucosinolates (GSLs), which are hydrolysed with the enzyme myrosinase into bioactive substances, i.e. isothiocyanates (ITCs). The effects of ITCs can be categorized into antimicrobial, herbicidal, and antioxidant. White mustard (*Sinapis alba*) and Indian or Oriental mustard (*Brassica juncea*) are considered as

biofumigation crops, commonly used as green manures in agricultural soils to suppress soil-borne pests and pathogens after biomass incorporation into the soil (Brown and Morra, 1997). Following another approach, botanical suspensions based on mustard seed flours could also be applied to crops for direct control of residue- and air-borne pathogens. In a previous study employing an array of *in vitro* bioassays, botanicals based on white mustard seed flour suppressed or fully inhibited the growth and development of several fungal structures of *F. graminearum* including mycelium, germination of conidia and ascospores, perithecia formation on maize stalks, and discharge of ascospores from mature perithecia produced on carrot agar (Drakopoulos et al., 2019). Apart from the GSLs, mustards also contain phenolic compounds. These compounds are utilized by plants for pigmentation, growth, reproduction as well as for resistance to pests and pathogens (Lattanzio et al., 2006). Phenolic acid derivatives, such as gallic, caffeic, cinnamic, benzoic, protocatechuic, and phenylacetic acids, were recorded to have antifungal activity (Teodoro et al., 2015). Ponts et al. (2011) showed that ferulic, coumaric, caffeic, syringic, and p-hydroxybenzoic acids inhibited 50 % of the radial growth of four *F. graminearum* strains. Schöneberg et al. (2018a) demonstrated that ferulic acid substantially inhibited mycelium growth of *F. graminearum*, *F. langsethiae* and *F. poae*, while p-hydroxybenzoic and vanillic acids had no effect.

The main objective of this study was to test the efficacy of mustard-based botanicals to reduce *F. graminearum* infection and mycotoxin (DON and ZEN) accumulation in wheat. As a first step, the effects of botanicals at different concentrations were tested on a mycelium growth *in vitro* bioassay. Secondly, the efficacy of the botanicals was explored *in planta* under controlled environment using wheat plants that were artificially inoculated, with either conidia or ascospores. Subsequently, the efficacy of the botanicals was investigated under field conditions using a semi-artificial inoculation method with *F. graminearum* in a wheat crop. Finally, the GSLs, ITCs, and phenolic acids present in the botanical powders were identified and quantified.

Material and methods

Fungal strain and spraying agents

The *F. graminearum* strain '2113' was used in the *in vitro* bioassay and growth chamber experiment, while a mixture of three *F. graminearum* strains (i.e. '0410', '2113', '1145'; single-spore isolates from wheat in Switzerland; 15-acetyldeoxynivalenol genotypes) was used for the semi-artificial inoculation method in the field experiment. The botanical powders Tillecur® (Ti; BIOFA, Germany), Pure Yellow Mustard (PYM; product code 106), Pure Oriental Mustard (POM; product code 107), and Oriental Mustard Bran (OMB; product code 403) were used for all experiments. PYM, POM, and OMB were provided by G. S. Dunn (Dry Mustard Millers, Canada). Ti and PYM are based on seed flour from white mustard, while POM is based on seed flour from Indian mustard. OMB is based on the seed husk from Indian mustard. The husk is commonly removed during the milling process and therefore is usually discarded. The synthetic fungicide (F) Proline® (active ingredient: prothioconazole; Bayer AG Crop Science, Switzerland), which is commonly used by cereal farmers to protect against FHB, was included as a comparison in all experiments.

In vitro bioassay - mycelium growth

Botanical suspensions (Ti, PYM, POM, OMB) were tested at 1 % and 2 %, while F was used at the recommended concentration (0.16 %). The methodological procedure for this bioassay is provided in Drakopoulos et al. (2019). In brief, mycelial plugs of 0.5 cm diameter were cut from freshly produced *F. graminearum* colonies and placed in the center of Petri dishes containing potato dextrose agar (PDA; Oxoid Ltd, Basingstoke, UK) with incorporated botanical powders or fungicide. PDA medium without any botanical powder or fungicide served as control. Mycelium was incubated in the dark at 20 °C and 80 % RH, and the colony diameter was measured with a ruler when the fastest growing colony had covered approximately 90 % of the agar surface. Each treatment included four replicates and the bioassay was conducted twice.

Growth chamber experiment - disease severity, yield, fungal DNA, and mycotoxins

Experimental design and procedure

This experiment was conducted in a growth chamber and included three experimental factors ('spraying agent' × 'spore type' × 'wheat variety') in a completely randomized design. The spraying agent included Ti, PYM, POM, OMB, fungicide or the positive control (no antifungal

treatment), the spore type included conidia or ascospores, and the wheat variety included the spring wheat (*Triticum aestivum* L.) varieties Digana or Fiorina (Delley seeds and plants Ltd, Switzerland). Non-inoculated plants (i.e. no infection with conidia or ascospores) served as a negative control. The resulting 26 treatment combinations were replicated four times and the experiment was conducted twice.

Three wheat seeds were sown in 2-liter pots containing potting soil. The pot represented the experimental unit of this study. Prior to sowing, each pot received 6 g of long-term granular fertilizer with micronutrients (N-P-K (MgO) 15-9-12 (2); Standard 5-6 M, Osmocote Exact Standard, Everris, the Netherlands). The plants were kept in a growth chamber with the following climatic conditions: seedling growth under 15 h light at 17 °C, 75 % RH and 9 h dark at 13 °C, 85 % RH; tillering under 15 h light at 19 °C, 70 % RH and 9 h dark at 14 °C, 80 % RH; stem elongation to dough development with 15 h light at 22 °C, 70 % RH and 9 h dark at 16 °C, 80 % RH; ripening with 15 h light at 25 °C, 70 % RH and 9 h dark at 20 °C, 80 % RH. The light intensity (2:3 Cool White, 1:3 Gro-Lux; Sylvania, Germany) was kept in the range of 350 to 400 µmol m^{-2} s^{-1} throughout the experiment.

The conidial suspensions were prepared by flushing off fresh fungal cultures grown for 5 days at 18 °C with a photoperiod 12 h dark / 12 h near-ultraviolet light. Perithecia were produced in carrot agar as described in Drakopoulos et al. (2019) with only a modification of the application of Tween® 60 (Sigma-Aldrich, USA) after the removal of the aerial mycelium. Ascospores were flushed off from the lids of the Petri dishes. Both conidia and ascospores were collected using sterile deionized water (dH_2O) containing 0.0125 % Tween® 20.

When plants reached mid- to full anthesis, one inflorescence (head) per plant (i.e. three heads per pot) was labelled for the respective spraying agent and fungal inoculation. The fungicide was sprayed at 0.16 % v v^{-1}, while botanicals were suspended in sterile dH_2O at 2 % w v^{-1} and stirred for 2 h before application. A blade-type homogenizer (Polytron® PT 3000, Kinematica AG, Switzerland) was used for OMB to reduce the larger particles to smaller fragments. A volume of 20 ml spraying solution was applied onto each wheat head using a spray gun (no. 110 nozzle size, 1.5 bar; Devilbiss PI-7BAR GTi, Carlisle Fluid Technologies, USA). The positive and negative controls were amended with sterile dH_2O. The following day, each head was inoculated with 2 ml *F. graminearum* spore solution containing 1×10^4 conidia or 1×10^4 ascospores ml^{-1} and 0.0125 % Tween® 20 (Sigma-Aldrich, USA) using a glass vial dispenser (0.5 bar; Sarstedt AG and Co. KG, Germany). The negative control was amended with sterile dH_2O containing 0.0125 % Tween® 20. Subsequently, wheat heads were allowed to dry for 1 h before placing the plants into the growth chamber. In order to facilitate infection, plants were incubated for 24 h in a water-saturated environment with fine mist at 20 °C in the dark.

Measurements

For the disease severity, the number of symptomatic spikelets were counted and expressed as percentage of infected spikelets per head. The average value from the three labelled heads per pot was calculated. Grain yield was measured after manual harvesting and combining the seeds from the three labelled heads per pot. Grain samples were finely ground using a ball mill (frequency of 25 sec^{-1} for 60 sec; MM400, Retsch GmbH, Germany) and stored at -20 °C until further analysis. The *F. graminearum* DNA in grain was quantified by CFX96™ Real-Time PCR Detection System for *in vitro* diagnostics (C1000™ Thermal Cycler, Bio-Rad Laboratories, USA) as described for barley in Schöneberg et al. (2018b). In brief, the DNA of 50 mg flour sample was extracted following the protocol of NucleoSpin® 96 Plant II Kit (Macherey-Nagel, Germany) and the amount of total DNA was determined following the Fluorescent DNA Quantitation Kit (BIO-RAD, Switzerland) using a Cary Eclipse Fluorescence Spectrophotometer (Varian, Agilent Technologies, USA) based on the emitted fluorescence of a serially diluted DNA standard. The used qPCR method was originally developed by Brandfass and Karlovsky (2006) and adapted according 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 flour (wheat var. Apogee cultivated in the greenhouse). The limit of quantification (LOQ) was 40 copies per reaction and the limit of detection one tenth of the LOQ. The mycotoxins DON and ZEN in grain were quantified using ELISA kits for enzyme immunoassays (Celer® DON v3 Cod. MD100 and Celer® ZON Cod. MZ670; Tecna, Italy) with the following modifications: A flour sample of 280 mg was weighed into 2-ml Eppendorf tubes and mycotoxins were extracted with 1.4 ml solvent solution of 70 % methanol and 4 % sodium chloride (Sigma-Aldrich, USA). Tubes were then vortexed vigorously and shaken horizontally at 250 rpm for 15 min. Samples were centrifuged at 13000 rpm for 5 min and the supernatant was collected. Reference flours with known DON and ZEN contents were extracted the same way (Trilogy Analytical Laboratory, USA). After extraction, ELISA was conducted following the protocol of the kit. The absorbance microplate reader Sunrise™ (TECAN, Austria) was used. When values were below the respective LOQ (DON: LOQ < 0.04 mg kg^{-1}; ZEN: LOQ < 0.01 mg kg^{-1}), values were replaced by LOQ÷2.

Field experiment - disease incidence, yield, fungal DNA, and mycotoxins

Experimental design and procedure

A field experiment was conducted in 2017 and repeated in 2018 at the research station of Agroscope-Reckenholz in Zurich, Switzerland. A randomized complete block design was followed including two experimental factors ('spraying agent' × 'wheat variety') with four replicates (square plots of 1 m^2 each) in blocks. The spraying agents as well as the spring wheat varieties were the same as in the growth chamber experiment. Border plots of 1 m^2 spring triticale Trado (Saatzucht Düdingen, Switzerland) were grown between the wheat plots. To evaluate the performance of the botanicals in field conditions under FHB pressure, a semi-artificial inoculation method was used as described in Drakopoulos et al. (2020) with small modifications. In brief, maize stalks with 30 cm length were autoclaved and then inoculated with a mixture of three *F. graminearum* strains in equal amounts, containing 2×10^5 conidia ml^{-1} and 0.0125 % Tween® 20. Ten maize stalks were homogeneously distributed in each wheat plot at seedling growth stage. The preparation, concentrations and equipment for the application of the botanicals, fungicide, and the positive control were the same as for the growth chamber experiment. Spraying applications of the botanicals were conducted twice during anthesis of the wheat crop, i.e. at the beginning and at mid-anthesis, while fungicide was applied at the beginning of anthesis. Sterile dH_2O served as positive control. The application rate for all treatments was 600 L ha^{-1}.

Measurements

For the disease incidence, symptomatic wheat heads were counted and expressed as the number of all infected heads per plot. In contrast to the growth chamber experiment, where the disease severity was recorded, only the disease incidence was recorded for the field experiments. The wheat crop was harvested using a plot combine harvester (Wintersteiger, Austria) and grains were dried for 24 h at 28 °C. The seed moisture content (SMC) and hectoliter weight were measured with a moisture tester (GAC 2100, Dickey-John, USA) and grain yield was normalized to 12 % SMC. For each plot, a representative subsample of 150 g was drawn using a riffle divider (Schiertz and Hauenstein AG, Switzerland) and ground with a mill (Cyclotec™ 1093; Foss Tecator, Sweden) using a 1-mm mesh. The amount of *F. graminearum* DNA and mycotoxins DON and ZEN in grain were quantified as described in the growth chamber experiment, except for the extraction method of mycotoxins due to the higher amount of the harvested grain. Flour samples of 5 g were extracted with 25 ml solvent solution 70 % methanol and 4 % sodium chloride, shaken for 10 min in an orbital lab shaker at 250 rpm and then passed through folded filters (Whatman®, Grade 595 ½, Sigma-Aldrich, USA).

Determination of glucosinolates and isothiocyanates in botanicals

Glucosinolate (GSL) extraction from Ti, PYM, POM, and OMB was performed as described in Prestera et al. (1996) with modifications. Twenty g of each botanical powder were weighed in glass tubes and autoclaved at 115 °C for 15 min. Then, 1 g of each autoclaved botanical was mixed with 25 ml dH_2O and homogenized (T18 Ultra-Turrax®, Germany) for 3 min at 11000 rpm. The mixture was centrifuged at 4000 rpm for 10 min at 4 °C. For the extraction of isothiocyanates (ITCs), non-autoclaved botanical powders were processed following the same methodology. The obtained extracts were filtered through a 0.22 mm syringe filter and injected (20 µL) into a Shimadzu LC system (Shimadzu, Japan) equipped with a Gemini C18 column (4.6 x 150 mm i.d. 5 mm; Phenomenex, USA). Quantification of GSLs and ITCs was conducted as described in Herzallah and Holley (2012). For the GSL analysis, the mobile phase consisted of 20 % (v/v) acetonitrile (ACN) and 80 % H_2O with 0.02 M of tetrabutylammonium hydrogen sulphate (TBA) (final pH 5.5). Detection wavelength was established at 227 nm. For the ITCs determination, the same buffer was employed with a different ratio of solvents: ACN 60 % (v/v) and 40 % H_2O with 0.02 M TBA. Detection was established at 244 nm. For both analyses of GSLs and ITCs, elution was carried out isocratically for 20 min at a flow rate of 1 ml min^{-1}.

Determination of phenolic acids in botanicals

Phenolic acids were extracted following the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) methodology as described in Brosnan et al. (2014). The organic residue obtained by the QuEChERS purification was re-suspended in 1 ml H_2O:ACN (90:10), filtered through a syringe filter, and placed into LC amber vials. Chromatographic determination was performed in an Agilent 1200-LC system (Agilent Technologies, USA) equipped with vacuum degasser, autosampler, and binary pump. The column used for the separation was a Phenomenex Gemini NX-C18 (2 mm Å~110 mm, particle size 3 µm). Mobile phases consisted of two solvents; solvent A was 0.1 % formic acid and solvent B was ACN. The gradient elution program was as follows: at time 0 min, 5 % B; then concentration of B increased to 95 % until 30 min and kept constant for 5 min; at time 40 min, concentration of B decreased to 5 %. Flow rate and injection volume were set at 0.3 ml min^{-1} and 20 µL, respectively. Prior to analysis, the column was equilibrated for 3 min. MS analysis was carried out with 6540 Agilent Ultra-High-Definition Accurate-Mass q-TOF-MS, equipped with Agilent Dual Jet Stream electrospray ionization (Dual AJS ESI) interface in negative ionization mode. The following conditions were established: drying gas was nitrogen at 12 L min^{-1}; nebulizer pressure at 50 psi; gas drying temperature at 350 °C; capillary voltage at 3500 V; fragmentor voltage at 200 V; scan range at m/z 50-3000. MS/MS analyses were realized with three energy values: 0, 20, and 40 eV. The

Mass Hunter Workstation software (Agilent Technologies, USA) was employed for integration and data analysis.

Statistical analysis

For the *in vitro* bioassay on mycelium growth, a non-parametric test (Kruskal-Wallis one-way ANOVA) was performed due to largely skewed data and unequal variances among samples. Pooled data from two experiments were used and all pairwise comparisons among treatments were conducted using the Student-Newman-Keuls method ($\alpha = 0.05$).

For the growth chamber experiment, a three-way ANOVA was performed to test significance and interactions among the examined factors ('spraying agent' × 'spore type' × 'wheat variety') using pooled data from two experiments. To check for normality and homogeneity of variances, data were subjected to Shapiro-Wilk and Brown-Forsythe tests, respectively. In order to approach or reach a normal distribution and homogeneity of variances, a logarithmic transformation was used for the response variables 'disease severity', '*F. graminearum* DNA amount in grain', and 'DON content in grain'. The Duncan's method was used for the post hoc comparisons ($\alpha = 0.05$).

For the field experiment, a three-way ANOVA was performed to test significance and interactions among the examined factors ('year' × 'spraying agent' × 'wheat variety'). In order to reach a normal distribution and homogeneity of variances, a square root transformation was used for the response variables 'disease incidence', '*F. graminearum* DNA amount in grain', and 'DON content in grain'. Duncan's method was used for the post hoc comparisons ($\alpha = 0.05$). No analysis was performed for the ZEN content in grain for both growth chamber and field experiments, since the measured values were below the LOQ.

A Spearman's rank-order correlation (r_s) was used to measure the strength and direction of the monotonic relationships among the response variables in the growth chamber and field experiments.

Data were analyzed with the statistical software SigmaPlot 13.0 (Systat Software Inc., USA) and figures were prepared with Prism 5.0 (GraphPad Software Inc., USA). Untransformed data are presented in tables and figures.

Results

In vitro bioassay - mycelium growth

At 2 %, Ti, PYM, and POM completely inhibited mycelium growth of *F. graminearum*, whereas OMB at 2 % reduced the growth by 96 % compared with the control (Fig. IV.1). At 1 %, OMB, PYM, and POM reduced mycelium growth by 50 %, 79 %, and 88 %, respectively, whereas F reduced the growth by 78 % (Fig. IV.1).

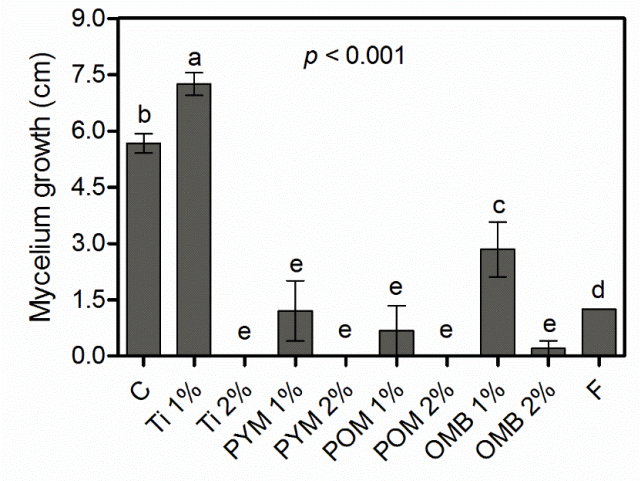


Fig. IV.1. *In vitro* bioassay: mycelium growth (cm) of *F. graminearum* strain '2113' as affected by Tillecur (Ti), Pure Yellow Mustard (PYM), Pure Oriental Mustard (POM) and Oriental Mustard Bran (OMB) at 1 % and 2 % concentrations, and Fungicide (F). C refers to the control treatment. Average values from two experiments are presented and bars indicate the standard error of the mean. Different letters indicate significant differences (α = 0.05).

Growth chamber experiment - disease severity, yield, fungal DNA, and mycotoxins

The results from the negative control showed that no cross contamination occurred during the experimental procedure, since no disease symptoms were observed as well as the amount of *F. graminearum* DNA and mycotoxins in grain were below detection. Hence, this treatment was not included in the statistical analysis, and subsequent reference to control only refers to the positive control.

Regarding disease severity, a significant interaction between spore type and wheat variety (p = 0.016) as well as between spraying agent and spore type (p = 0.027) was observed (Supplementary Table SIV.1). For Digana, inoculation with ascospores resulted in 4-fold higher disease severity compared with conidia inoculation (p < 0.001), while no significant difference was found between conidia and ascospores for Fiorina (p = 0.581) (Fig. IV.2 A). When inoculation was performed with conidia, all spraying agents resulted in lower disease severity compared with the control, except for OMB (Fig. IV.2 B). When inoculation was performed with ascospores, the spraying agents F, Ti, PYM, and POM substantially decreased disease

severity compared with the control and OMB (Fig. IV.2 B). The disease severity was 2- and 5-fold higher for control and OMB, respectively, when inoculation was done with ascospores compared with the conidia treatment (Fig. IV.2 B).

A significant interaction between spore type and wheat variety (p = 0.023) was observed with respect to grain yield (Supplementary Table SIV.1). For Digana, inoculation with ascospores resulted in lower grain yield than with conidia (p < 0.001), while similar yield was obtained between conidia and ascospores for Fiorina (p = 0.693) (Supplementary Fig. SIV.1 A). The main effect of spraying agent on grain yield was significant (p < 0.001) (Supplementary Table SIV.1). Control and OMB resulted in the lowest grain yield, while F and Ti had the highest, with PYM and POM having intermediate yields (Supplementary Fig. SIV.1 B).

Regarding *F. graminearum* DNA amount in grain, there was a significant interaction between spore type and wheat variety (p = 0.002) as well as between spraying agent and spore type (p = 0.016) (Supplementary Table SIV.1). For Digana, inoculation with ascospores resulted in 3-fold higher *F. graminearum* DNA amount in grain compared with conidia, while no significant difference was found between the two spore type inoculations for Fiorina (p = 0.118) (Fig. IV.3 A). When inoculation was done with conidia, the control had the highest *F. graminearum* DNA amount, while the spraying agents PYM, Ti, and F had the lowest, with OMB and POM having intermediate DNA amounts (Fig. IV.3 B). When inoculation was done with ascospores, the control, OMB, and POM had the highest *F. graminearum* DNA amount, while F had the lowest, with PYM and Ti having intermediate DNA amounts (Fig. IV.3 B). The DNA amount of *F. graminearum* was 8-, 6-, 4-, and 3-fold higher for PYM, POM, control, and OMB treatments, respectively, when inoculation was done with ascospores compared with conidia (Fig. IV.3 B).

As for the previous parameters, there was a significant interaction between spore type and wheat variety (p = 0.041) with respect to DON content in grain (Supplementary Table SIV.1). For Digana, inoculation with ascospores resulted in 6-fold higher DON content compared with conidia (p < 0.001), while for Fiorina there was no significant difference between the two spore types (p = 0.151) (Fig. IV.4 A). The main effect of spraying agent on DON content was significant regardless of spore type or wheat variety (p < 0.001) (Supplementary Table SIV.1). DON content was reduced by 100 %, 98 %, 88 %, and 73 % with treatments of F, Ti, PYM, and POM, respectively, compared with control, which had an average DON content of 28.4 mg kg^{-1} (Fig. IV.4 B).

Regarding the associations among the examined dependent variables of the growth chamber experiment, positive relationships were observed among disease severity, *F. graminearum* DNA amount, and DON content (r_s = 0.726 to 0.834, p < 0.001, Table IV.1). Negative

correlations were observed between disease severity and grain yield (r_s = -0.303, p < 0.001) as well as between DON content and grain yield (r_s = -0.307, p < 0.001, Table IV.1).

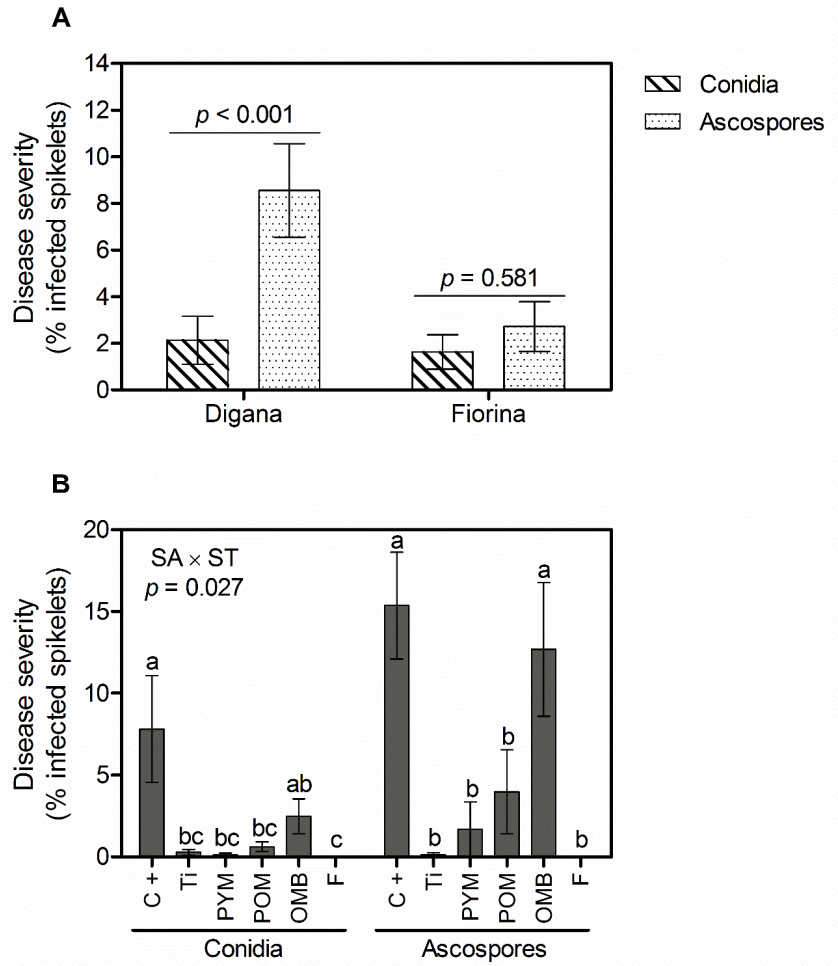


Fig. IV.2. Growth chamber experiment: disease severity (% infected spikelets) as affected by spore type (conidia, ascospores) within wheat variety (Digana, Fiorina) pooled over the spraying agents **(A)** and as affected by spraying agent (SA) within spore type (ST) pooled over the wheat varieties **(B)**. The used spraying agents were Tillecur (Ti), Pure Yellow Mustard (PYM), Pure Oriental Mustard (POM), Oriental Mustard Bran (OMB) and Fungicide (F). Positive control (C +) refers to infected untreated plants. Average values from two experiments are presented and bars indicate the standard error of the mean. Different letters indicate significant differences (α = 0.05).

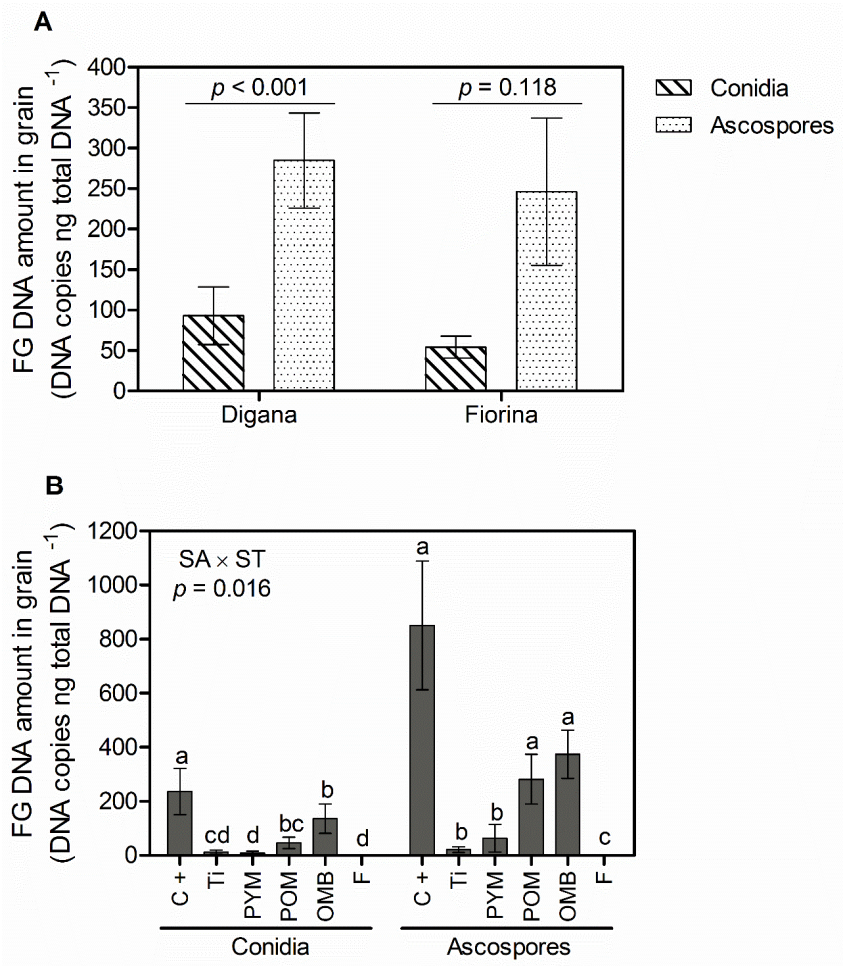


Fig. IV.3. Growth chamber experiment: amount of *F. graminearum* (FG) DNA in grain (DNA copies ng total DNA^{-1}) as affected by spore type (conidia, ascospores) within wheat variety (Digana, Fiorina) pooled over the spraying agents **(A)** and as affected by spraying agent (SA) within spore type (ST) pooled over the wheat varieties **(B)**. The used spraying agents were Tillecur (Ti), Pure Yellow Mustard (PYM), Pure Oriental Mustard (POM), Oriental Mustard Bran (OMB) and Fungicide (F). Positive control (C +) refers to infected untreated plants. Average values from two experiments are presented and bars indicate the standard error of the mean. Different letters indicate significant differences ($\alpha = 0.05$).

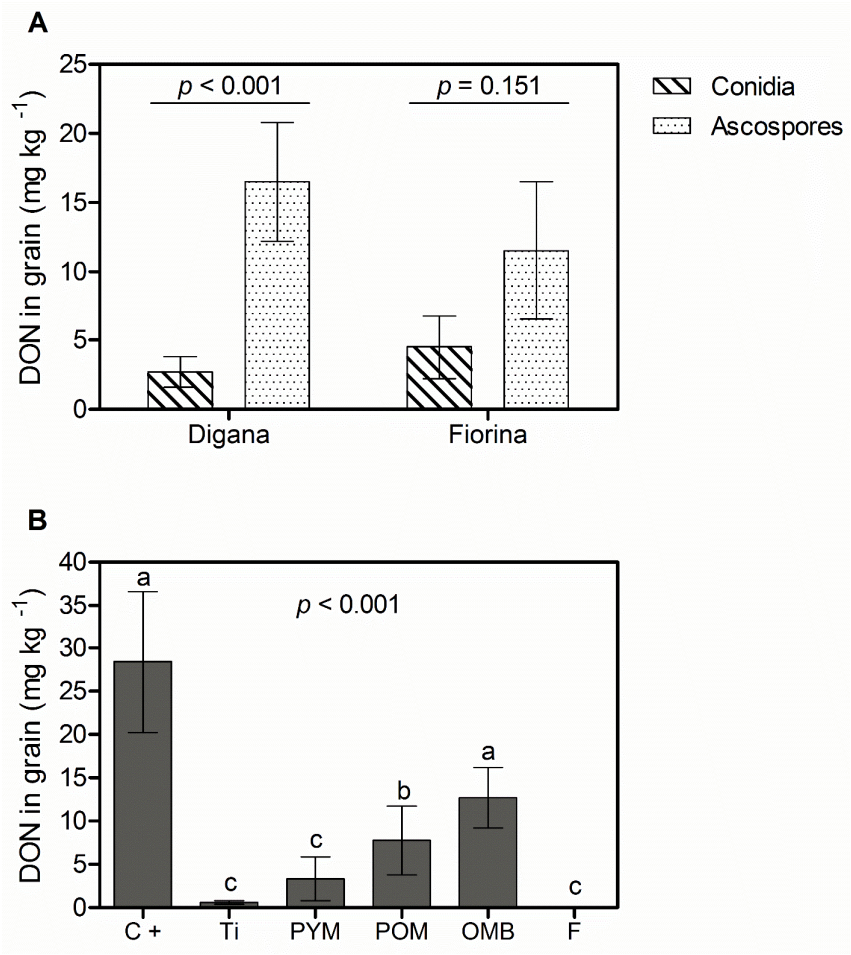


Fig. IV.4. Growth chamber experiment: DON content in grain (mg kg^{-1}) as affected by spore type (conidia, ascospores) within wheat variety (Digana, Fiorina) pooled over the spraying agents **(A)** and as affected by spraying agent pooled over the spore types and wheat varieties **(B)**. The used spraying agents were Tillecur (Ti), Pure Yellow Mustard (PYM), Pure Oriental Mustard (POM), Oriental Mustard Bran (OMB) and Fungicide (F). Positive control (C +) refers to infected untreated plants. Average values from two experiments are presented and bars indicate the standard error of the mean. Different letters indicate significant differences ($\alpha = 0.05$).

Table IV.1. Growth chamber experiment: Spearman's rank correlation coefficient (r_s) examining monotonic associations among the dependent variables disease severity (% infected spikelets), grain yield (g pot^{-1}), *Fusarium graminearum* (FG) DNA amount (DNA copies ng total DNA^{-1}) and DON content (mg kg^{-1}) in grain. Data from two experiments were used for the analysis (n = 192).

	Grain yield	FG DNA amount	DON content
Disease severity	-.303	.726	.817
Grain yield		-.238	-.307
FG DNA amount			.834

Field experiment - disease incidence, yield, fungal DNA, and mycotoxins

Overall and for both wheat varieties, the disease incidence, amount of *F. graminearum* DNA, and DON content in grain were higher in 2018 than in 2017 ($p < 0.001$) (Figs IV.5 A, 6 A, 7 A). In parallel, grain yield and hectoliter weight were lower in 2018 than in 2017 ($p < 0.001$) (Supplementary Figs SIV.2 A, 3 A). The main effects of spraying agent on disease incidence ($p < 0.001$), *F. graminearum* DNA amount ($p = 0.003$), DON content ($p < 0.001$), and grain yield ($p = 0.015$) were significant regardless of the year or the wheat variety (Supplementary Table SIV.2). Compared with the control treatment, use of F reduced disease incidence by 85 %, *F. graminearum* DNA amount in grain by 59 %, and DON content in grain by 64 %, while none of the botanicals showed significant effects (Figs IV.5 B, 6 B, 7 B). The control, PYM, and POM led to the lowest yields (6.72 to 6.93 t ha^{-1}) and F to the highest (7.60 t ha^{-1}), whereas OMB and Ti resulted in intermediate yields (7.11 and 7.16 t ha^{-1}, respectively) (Supplementary Fig. SIV.2 B). Regarding hectoliter weight, there was a significant interaction between year and spraying agent ($p = 0.005$) (Supplementary Table SIV.2). In 2017, there was no difference in hectoliter weight between spraying agents and control, while in 2018, the F treatment resulted in higher values compared with all other treatments (Supplementary Fig. SIV.3 B).

Regarding the associations among the examined dependent variables of the field experiment, positive relationships were observed among disease incidence, *F. graminearum* DNA amount, and DON content ($r_s = 0.869$ to 0.973, $p < 0.001$, Table IV.2). Negative correlations were found between DON content and grain yield ($r_s = -0.732$, $p < 0.001$) as well as between DON content and hectoliter weight ($r_s = -0.815$, $p < 0.001$, Table IV.2).

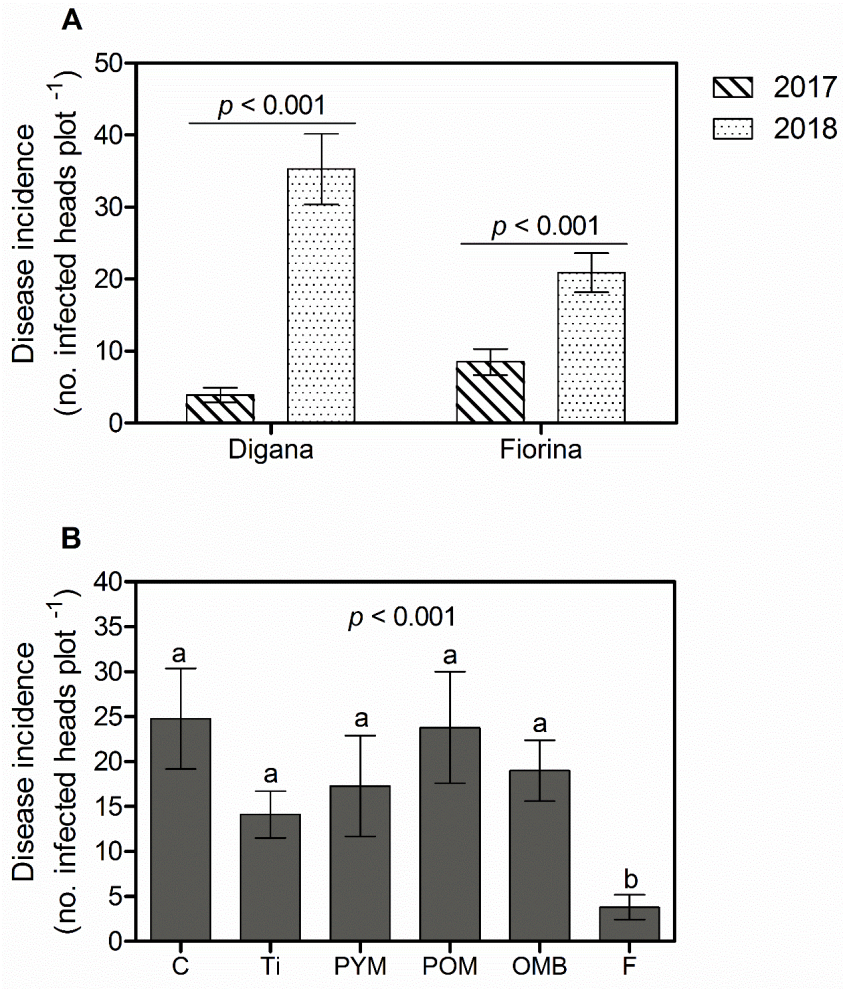


Fig. IV.5. Field experiment: disease incidence (no. infected heads plot^{-1}) as affected by year (2017, 2018) within wheat variety (Digana, Fiorina) pooled over the spraying agents **(A)** and as affected by spraying agent pooled over the wheat varieties and the two years **(B)**. The used spraying agents were Tillecur (Ti), Pure Yellow Mustard (PYM), Pure Oriental Mustard (POM), Oriental Mustard Bran (OMB) and Fungicide (F). Control (C) refers to infected untreated plants. Bars indicate the standard error of the mean and different letters indicate significant differences ($\alpha = 0.05$).

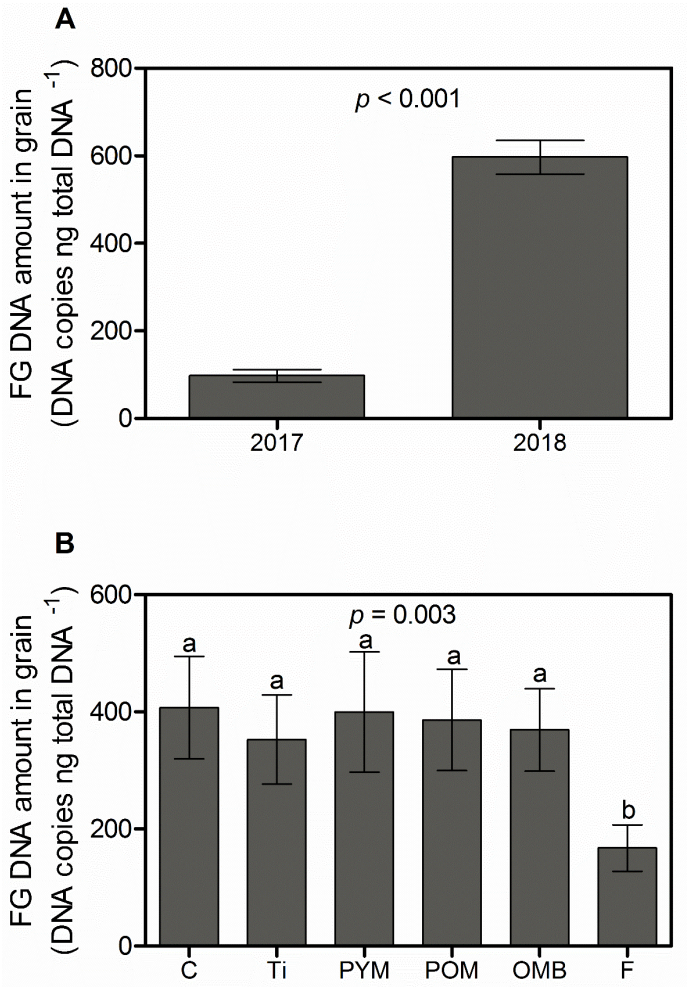


Fig. IV.6. Field experiment: amount of *F. graminearum* (FG) DNA in grain (DNA copies ng total DNA^{-1}) as affected by year (2017, 2018) pooled over the wheat varieties (Digana, Fiorina) and the spraying agents **(A)** and as affected by spraying agent pooled over the wheat varieties and the two years **(B)**. The used spraying agents were Tillecur (Ti), Pure Yellow Mustard (PYM), Pure Oriental Mustard (POM), Oriental Mustard Bran (OMB) and Fungicide (F). Control (C) refers to infected untreated plants. Bars indicate the standard error of the mean and different letters indicate significant differences (α = 0.05).

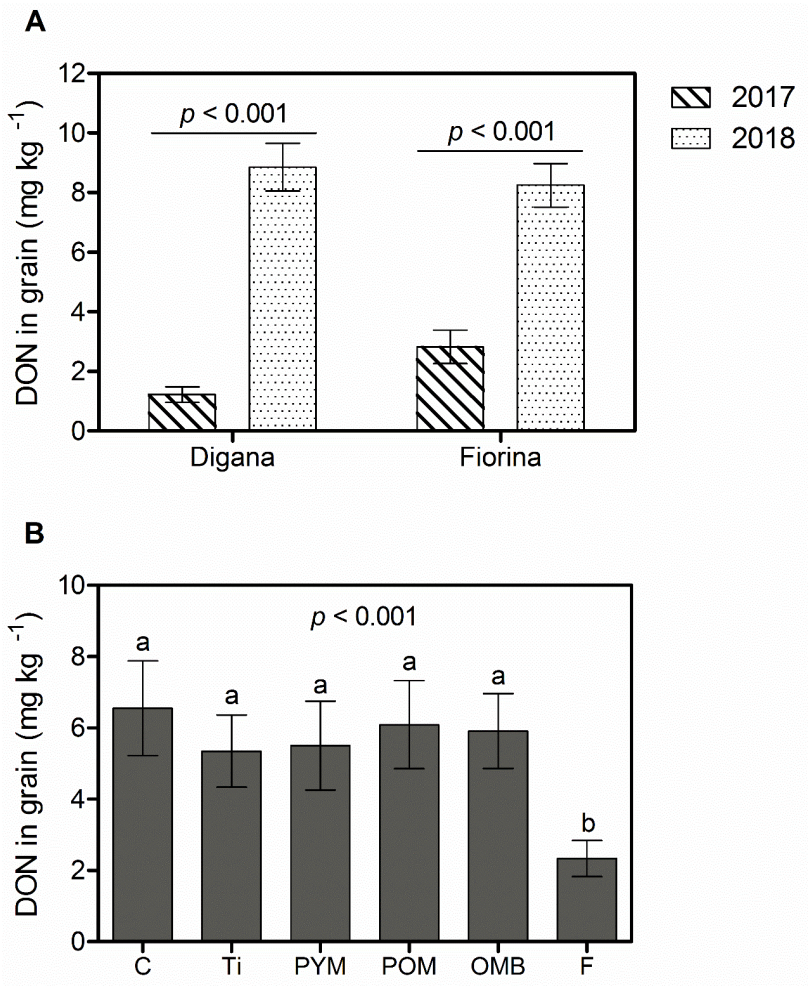


Fig. IV.7. Field experiment: DON content in grains (mg kg^{-1}) as affected by year (2017, 2018) within wheat variety (Digana, Fiorina) pooled over the spraying agents **(A)** and as affected by spraying agent pooled over the wheat varieties and the two years **(B)**. The used spraying agents were Tillecur (Ti), Pure Yellow Mustard (PYM), Pure Oriental Mustard (POM), Oriental Mustard Bran (OMB) and Fungicide (F). Control (C) refers to infected untreated plants. Bars indicate the standard error of the mean and different letters indicate significant differences ($\alpha = 0.05$).

Table IV.2. Field experiment: Spearman's rank correlation coefficient (r_s) examining monotonic associations among the dependent variables disease incidence (no. infected heads plot^{-1}), grain yield (t ha^{-1}), hectoliter weight (kg hl^{-1}), *Fusarium graminearum* (FG) DNA amount (DNA copies ng total DNA^{-1}) and DON content (mg kg^{-1}) in grain. Data from two experiments were used for the analysis (n = 96).

	Grain yield	Hectoliter weight	FG DNA amount	DON content
Disease incidence	-.670	-.823	.869	.904
Grain yield		.788	-.767	-.732
Hectoliter weight			-.793	-.815
FG DNA amount				.973

Determination of glucosinolates, isothiocyanates, and phenolic acids in botanicals

The detailed results of GSLs, ITCs as well as those of the phenolic acids identified in the botanical powders Ti, PYM, POM, and OMB are provided in Tables IV.3 and 4. Ti is characterized by an elevated quantity of the GSL sinalbin (56.4 mg g^{-1}) which is converted into p-hydroxybenzyl isothiocyanate (p-HBITC) through the myrosinase reaction. The concentration of the latter bioactive compound in Ti was 2.56 mg g^{-1}. Sinalbin and p-HBITC were also identified in PYM in similar concentrations, i.e. 57.4 and 2.44 mg g^{-1}, respectively. POM and OMB are characterized by the presence of the GSL sinigrin (19.0 and 11.2 mg g^{-1}, respectively) which is converted into allyl ITC through the myrosinase reaction. Allyl ITC was detected in POM and OMB matrices at 8.38 and 4.48 mg g^{-1}, respectively. Ti contained 14 different phenolic acids in considerable amounts. The compounds with the highest and lowest concentrations were hydroxycinnamic acid (26.1 mg kg^{-1}) and gallic acid (0.12 mg kg^{-1}), respectively. High concentrations of ferulic acid, benzoic acid, dihydroxybenzene, and phenyllactic acid were detected in this matrix (6.46, 6.35, 2.21 and 2.12 mg kg^{-1}, respectively). PYM contained 10 different phenolic acids and the detected compound with the highest concentration in this matrix was benzoic acid (11.8 mg kg^{-1}). The other bioactive compounds were detected in a range of concentrations from 0.11 to 0.52 mg kg^{-1}. POM contained 10 different phenolic acids at a range of 0.18 to 0.63 mg kg^{-1}. Considerable concentrations of p-coumaric, sinapic acid, and vanillic acid were detected in OMB (4.26, 1.26, and 1.01 mg kg^{-1}, respectively).

Table IV.3. Glucosinolates (GSLs, mg g^{-1}) and isothiocyanates (ITCs, mg g^{-1}) identified and quantified in Tillecur (Ti), Pure Yellow Mustard (PYM), Pure Oriental Mustard (POM) and Oriental Mustard Bran (OMB).

	GSLs		ITCs	
Botanicals	**Sinigrin**	**Sinalbin**	**Allyl ITC**	**p-HBITC**
Ti	nd	56.44 ± 0.02	nd	2.56 ± 0.08
PYM	nd	57.41 ± 0.12	nd	2.44 ± 0.05
POM	19.01 ± 1.14	nd	8.38 ± 0.40	nd
OMB	11.24 ± 0.74	nd	4.48 ± 0.96	nd

nd = not detected; ± refers to the standard deviation

Table IV.4. Phenolic acids (mg kg^{-1}) identified and quantified in Tillecur (Ti), Pure Yellow Mustard (PYM), Pure Oriental Mustard (POM) and Oriental Mustard Bran (OMB).

Phenolic acids	Ti	PYM	POM	OMB
1-2-Dihydroxybenzene	2.21 ± 0.01	nd	nd	0.11 ± 0.02
3-4-Dihydroxyhydrocinnamic acid	0.13 ± 0.01	0.20 ± 0.02	nd	nd
Benzoic acid	6.35 ± 0.04	11.83 ± 0.02	0.63 ± 0.02	0.82 ± 0.01
Caffeic acid	0.21 ± 0.01	nd	nd	0.31 ± 0.01
Chlorogenic acid	nd	nd	0.18 ± 0.1	nd
DL-3-Phenyllactic acid	2.12 ± 0.04	0.34 ± 0.01	0.28 ± 0.01	0.30 ± 0.03
Ferulic acid	6.46 ± 0.02	0.33 ± 0.01	0.37 ± 0.02	0.74 ± 0.04
Gallic acid	0.12 ± 0.02	nd	nd	nd
Hydroxycinnamic acid	26.12 ± 0.01	0.52 ± 0.03	0.56 ± 0.01	0.44 ± 0.02
P-Coumaric acid	0.35 ± 0.03	0.21 ± 0.01	0.55 ± 0.02	4.26 ± 0.03
Protocatechuic acid	1.28 ± 0.02	0.25 ± 0.04	nd	nd
Sinapic acid	0.57 ± 0.02	0.27 ± 0.01	0.62 ± 0.04	1.26 ± 0.03
Syringic acid	0.13 ± 0.01	0.11 ± 0.01	0.43 ± 0.04	0.16 ± 0.02
Vanillic acid	0.48 ± 0.02	nd	0.51 ± 0.01	1.01 ± 0.02
Vanillin	0.29 ± 0.01	0.21 ± 0.02	0.30 ± 0.02	0.14 ± 0.01

nd = not detected; ± refers to the standard deviation

Discussion

In this study, the efficacy of mustard-based botanicals against *F. graminearum* infection and mycotoxin accumulation in wheat was evaluated under controlled environments and under field conditions. Furthermore, the effect of inoculum for infection of wheat heads, i.e. with conidia or ascospores, on disease severity and mycotoxin production was investigated. In a previous study, the potential of yellow mustard seed flours (Ti and PYM) to suppress or fully inhibit *in vitro* growth and development of *F. graminearum* was already demonstrated (Drakopoulos et al., 2019). In the current study, further botanicals based on Indian mustard (POM and OMB) and a synthetic prothioconazole fungicide (F) were also included.

Remarkably, at 2 %, all botanicals showed a higher efficacy in inhibiting mycelium growth *in vitro* compared with F. In the growth chamber experiment, the best performing spraying agents to reduce the DON content in grain were F, Ti, and PYM. Mustard-based botanicals have been extensively studied in the past for their potent antimicrobial activity (Saladino et al., 2017). Clemente et al. (2016) found that allyl ITC, which is derived from the main GSL sinigrin of Indian mustard, had stronger bactericidal and bacteriostatic properties than cinnamon essential oil in broth dilution, direct contact, and vapour phase assays. Luciano and Holley (2009) suggested that allyl ITC has a multi-targeted mechanism of action, damaging cellular structures and inhibiting several metabolic pathways. Allyl ITC reduced the growth of the mycotoxigenic fungi *Aspergillus parasiticus* and *Penicillium expansum* on soil medium (Manyes et al., 2015) and completely inhibited the production of aflatoxins, by *A. parasiticus*, as well as beauvericin and enniatin, by *Fusarium poae*, in wheat flour (Meca et al., 2012; Nazareth et al., 2016). In our study, allyl ITC was detected in both matrices of POM and OMB with 8.38 and 4.48 mg g^{-1} concentrations, respectively. Ekanayake et al. (2006) reported the potential use of white mustard essential oil for food preservation due to the presence of p-HBITC, which is produced by hydrolysis of the GSL sinalbin. David et al. (2013) also showed good efficacy of p-HBITC in controlling *Salmonella* sp. in a frozen sauce with vegetable and chicken particulates. In our study, p-HBITC was detected in both matrices of Ti and PYM in the concentrations of 2.56 and 2.44 mg g^{-1}, respectively. Aires et al. (2009), using a disc diffusion *in vitro* assay, showed that ITCs had potent antibacterial activity while the other non-ITC hydrolysis products were much less effective. In addition, the authors indicated that the hydrolysis products from the aromatic group were more effective compared with the aliphatic group. Similarly, the results from the growth chamber experiment showed that Ti and PYM, which contain p-HBITC (aromatic group), had higher efficacy against *F. graminearum* infection and DON accumulation in grain compared with POM and OMB, which contain allyl ITC (aliphatic group).

Besides the bioactive ITCs, several antimicrobial phenolic acids were present in the botanical matrices. The concentrations of the detected acids varied substantially among the studied botanicals and corresponded well with the observed higher efficacies of Ti and PYM against *F. graminearum* in the growth chamber experiment. Phenolic acids, such as ferulic and gallic acids, can cause irreversible changes in membrane properties of pathogenic bacteria (Borges et al., 2013). Studies on the mode of action against fungal pathogens are still scarce. Teodoro et al. (2015) summarized some of the described mechanisms of action against *Candida* species including, among others, cell wall damage, disruption of plasma membrane, and inhibition of isocitrate lyase enzyme activity. Boutigny et al. (2009) found that ferulic acid had a strong inhibitory effect on type B trichothecene biosynthesis in liquid cultures with *F. culmorum*, while Ferrochio et al. (2013) showed that higher doses of ferulic acid reduced the growth rate of *F. verticillioides* and *F. proliferatum* on maize based media. We demonstrated that Ti, which had the highest efficacy in the growth chamber experiment, had 9- to 20-fold higher concentration of ferulic acid compared with the other botanicals. In another study, ferulic acid reduced the production of T-2 toxin by *F. langsethiae* and *F. sporotrichioides*, but in turn, p-coumaric acid stimulated the production of T-2 and HT-2 toxins (Ferruz et al., 2016). This finding might explain our observation that OMB, which contained higher concentrations of p-coumaric acid, showed the lowest efficacy compared with the other spraying agents in the growth experiment. The highest concentrations of benzoic acid were detected in Ti (6.4 mg kg^{-1}) and PYM (11.8 mg kg^{-1}), which are both based on yellow mustard seed flours. Thus, the elevated control of *F. graminearum* DNA and DON might also be due to the higher amounts of benzoic acid, and its mode of action should be further investigated in future studies. Interestingly, Ti contained 50-fold higher concentration of hydroxycinnamic acid than PYM, which could be the reason for its superior performance in reducing DON content in grain under controlled conditions. Consequently, we could speculate that the antifungal activity of the used botanicals in this study is derived from bioactive matrices containing both ITCs and certain phenolic acids.

Under field conditions, only the use of F decreased FHB infection and DON content in grain compared with the control treatment, which stands in contrast to the observed efficacies of the botanicals *in vitro* and *in planta* under controlled conditions. Hence, to improve the efficacy in the field, an effective biopesticide formulation of mustard-based botanicals is needed. In an earlier study, Ti and PYM were applied on maize residues, which had been previously artificially inoculated with *F. graminearum*, to investigate a potential prevention strategy against FHB in a subsequent wheat crop (Drakopoulos et al., 2020). The authors showed that the botanicals were more effective in the second year by decreasing DON and ZEN content in wheat grain up to 41 % and 74 %, respectively. This lack of consistency in terms

of efficacy could be resolved by improving the stability and thus prolonging the activity of ITCs. Allyl ITC in aqueous solution is more unstable under alkaline environments and elevated temperatures (Takatani and Kawakishi, 1995). Liu and Yang (2010) studied the stability and antimicrobial activity of allyl ITC against gram-positive and gram-negative bacteria during long-term storage (180 days) in medium chain triglyceride (MCT) or soybean oil (SBO) dispersed in an oil-in-water (o/w) emulsion system. Higher oil content favoured the stability in the emulsion for both MCT and SBO, while, for the same oil content, allyl ITC in MCT was more stable and more effective than in SBO in inhibiting the studied bacteria. Therefore, an effective oil emulsifier may improve the efficacy of the examined mustard-based botanicals under field conditions by stabilizing and prolonging the activity of ITCs. Tracz et al. (2017) evaluated the potential of allyl ITC to inhibit mycotoxin production by *A. parasiticus*, *Alternaria alternata*, *F. tricinctum*, *F. verticillioides*, and *F. graminearum* in maize kernels placed in hermetically sealed flasks. Treatments with allyl ITC kept mycotoxin production below detectable levels, while residual doses (~16 %) of allyl ITC in kernels were detected even after 30 days, indicating an extended protection period. Overall, the optimal formulation to improve the stability of mustard-based botanicals should be achieved using environmentally friendly additives with minor impact to the agroecosystem.

In general, the use of botanicals has several advantages over the use of synthetic pesticides in integrated pest management (Regnault-Roger and Philogène, 2008). For example, botanicals are enzymatically biodegradable with shorter half-lives, since they were biosynthesized. Faster degradation can be an advantage in terms of environmental impact and effects on non-target organisms, but could also decrease the efficacy of the spraying agent as discussed above. Tembo et al. (2018) assessed the potential trade-offs of using plant extracts on legume crop yields and the regulating ecosystem services of natural enemies. Compared with a synthetic insecticide (lambda-cyhalothrin pyrethroid), plant extracts were as effective in terms of crop yields and better conserved the non-target arthropods, suggesting that plant secondary metabolites could be integrated into agroecological cropping systems. Moreover, botanicals belong to several different chemical families and contribute to the diversification of the biochemical and molecular targets towards pests, therefore limiting or delaying the development of resistance (Regnault-Roger and Philogène, 2008). Recently, there is a movement towards reduced reliance on synthetic pesticides in Europe which would also help to overcome the development of fungicide resistant plant pathogens (Lamichhane et al., 2015). Moreover, new pesticide registration procedures, such as the Food Quality Protection Act in USA, have reduced the availability of synthetic pesticides, leaving more space for the discovery and development of natural product-based pesticides as alternatives in crop protection (Dayan et al., 2009).

An additional remarkable finding of this study is the observed differences between ascosporic and conidial inoculum on *F. graminearum* infection, mycotoxin content, and grain yield under controlled conditions. The disease severity, amount of *F. graminearum* DNA, and DON content were on average higher after inoculating wheat heads with ascospores than conidia, but results were only significant for the wheat variety Digana. Moreover, inoculation with ascospores resulted in lower grain yield of Digana compared with conidial inoculation. In fact, for the botanicals OMB, POM, and PYM, the amount of *F. graminearum* DNA in grain was 3- to 8-fold higher following inoculation with ascospores compared with conidia, pointing out that the efficacy of crop protection products depends on the fungal spore type causing the disease. In contrast, the amount of *F. graminearum* DNA in grain was similar after inoculation with ascospores or conidia when plants were treated with Ti or F, which were the most effective spraying agents in this study. This could indicate that the differences between conidial and ascosporic infections might be eliminated if the efficacy of the product is exceptionally high, which is rare under field conditions. Chinese galls, a botanical rich in gallotannins with antimicrobial activity, inhibited conidia germination, but had minor to no effects on ascospores germination depending on the fungal strain (Drakopoulos et al., 2019). This is additional corroboration for our hypothesis that ascospores of *F. graminearum* are more difficult to control than conidia.

The discovery of the different control efficacies towards the two spore types has several potential implications considering that ascospores are more important in establishing the FHB disease epidemics than conidia. Manstretta et al. (2015) investigated the distribution patterns of ascospores and conidia within a wheat canopy between booting and grain maturity stages using spore traps and found that of the total spores counted, 93 % were ascospores and 7 % conidia. Mitter et al. (2006) indicated that ascospores were less or equally effective in causing FHB compared with conidia, although differences were small and dependent on wheat type and variety. Also, Stack (1989) claimed that experimental results based on conidial inoculations can be expected to be valid for ascospores after comparing four inoculum levels, i.e. 1 to 1000 spores per inoculation site. However, these experiments focused on the disease incidence and severity of stem rot in carnation and head blight in durum wheat. An additional, explanation for the contrary results could be the genetic variation in the populations of *F. graminearum* resulting in different virulence between conidia and ascospores. To our knowledge, the artificial inoculation of inflorescences in studies with FHB causing species is usually performed with conidia. Certainly, conidia are more easily produced than ascospores and some breeders assume that inoculation with conidia might be sufficient to simulate the disease development under field conditions. We suggest that breeding and crop protection programs for resistance and control of *F. graminearum* should be conducted using ascospores

for the artificial inoculation of the plants or with a mixture of both spore types to better mimic disease epidemics. In the current growth chamber experiment, only one *F. graminearum* strain was used. Therefore, this first evidence should be further supported by investigating the effects of spore type from different fungal strains on disease development and mycotoxin accumulation in several wheat varieties as well as in other small-grain cereals, such as barley.

In conclusion, the efficacy of the mustard-based botanicals Ti, PYM, and POM was excellent under controlled conditions indicating that mustards could be highly promising alternatives to synthetic fungicides for FHB control and mycotoxin reduction in wheat. The antifungal activity of the botanicals is possibly due to the detected ITCs and phenolic acids in the bioactive matrices. However, the dramatically decreased efficacy of the botanicals under field conditions underlines the need for an effective biopesticide formulation. Finally, the observed differences between conidial and ascosporic inoculum on disease severity and mycotoxin accumulation suggests that control of *F. graminearum* can be more difficult when infection occurs with ascospores.

Acknowledgments

The authors warmly thank Fabian Zahnd and Jonas Lehner for the excellent technical assistance. Also, we would like to acknowledge G. S. Dunn (Dry Mustard Millers, Canada) for providing us with the botanicals PYM (product code 106), Pure Oriental Mustard (product code 107) and Oriental Mustard Bran (product code 403).

6. Conclusions and outlook

Fusarium head blight (FHB) is a devastating fungal disease of small-grain cereals worldwide. FHB causes yield and economic losses, but most importantly it can result in severe grain contaminations with mycotoxins, jeopardising food and feed safety. As an urgent call for action, in 2015, all United Nations Member States established an agenda to reach 17 Sustainable Development Goals (SDGs) by 2030 including developed and developing countries (UNDP, 2020). Improving food security and food safety, ensuring sustainable food production systems through resilient agricultural practices as well as halting the loss of biodiversity and maintaining soil quality are integral parts of some of the SDGs. It is undoubted that agricultural practices and especially crop protection play a significant role for the realisation of these global goals. More specifically, the effective management of mycotoxins is crucial in order to improve food and feed safety as well as to ensure sufficient levels of food security. Nevertheless, a sustainable intensification of agroecosystems should integrate crop protection measures that pose no risks to the environment and human health.

We investigated the potential of pre-harvest strategies to control FHB and reduce mycotoxins in wheat using innovative cropping systems as well as plant-based biopesticides under reduced tillage practices. Following the experimental approaches of this dissertation, we summarise the range of attained efficacy against fungal structures of *F. graminearum in vitro* (chapter I) as well as the mycotoxin reduction in wheat grain *in planta* (chapters II, III and IV) (Fig. 6.1).

In chapter I, we explored the potential of botanicals to suppress *F. graminearum* by assessing the impact on multiple, distinct stages of the pathogen life cycle *in vitro*. Aqueous extracts of botanicals based on white mustard seed flour and milled Chinese galls showed great promise for the control of FHB. With the use of these botanicals, mycelium growth was reduced by up to 100 %, germination of conidia and ascospores by up to 100 % and 97 %, respectively, perithecia formation on maize stalks by up to 56 % and discharge of ascospores from mature perithecia by up to 77 %. We demonstrated that the bioactive matrix of Chinese galls contains different gallotannins as well as gallic and tannic acids, which are phenolic compounds with antimicrobial activity. While application of Chinese galls suppressed the germination of conidia, it only had minor or no effect on ascospore germination indicating that reduction in activity of these spores may be harder to achieve. This argument is further supported by the findings of chapter IV where disease severity, amount of *F. graminearum* DNA and DON content in grain were on average higher after ascosporic inoculations of wheat heads compared with conidial inoculations. In chapter I, we concluded that the efficacy of the tested

botanicals should be further explored *in planta* by investigating, first, prevention strategies under no-tillage through botanical applications to crop residues in order to suppress the inoculum of *F. graminearum* (chapter II) and, secondly, direct control measures through botanical applications to flowering wheat heads under controlled environments and field conditions (chapter IV).

In chapter II, we simulated a maize-wheat rotation under no-tillage by applying maize residues artificially inoculated with *F. graminearum* in field plots after wheat sowing. We investigated prevention strategies to suppress FHB infection and reduce mycotoxins by applications of botanicals or fresh mulch layers to the inoculated maize residues. Botanicals comprised aqueous extracts of white mustard seed flours and milled Chinese galls (same botanicals as in chapter I). Botanicals were more effective in the second experimental year reducing DON and ZEN contents in wheat grain by up to 42 % and 78 %, respectively. For mulch layers, a novel cut-and-carry biofumigation approach was used where 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, Indian mustard and berseem clover consistently decreased mycotoxin contents in both years, i.e. DON and ZEN by up to 58 % and 87 %, respectively. Hence, cut-and-carry biofumigation is a novel approach to supress *Fusarium* inoculum and decrease mycotoxin contamination in grain, especially in cereal-based rotations under minimum tillage. Moreover, cut-and-carry green manures are an excellent source of nitrogen improving inherent soil fertility and soil organic carbon stocks. Sorensen and Grevsen (2016) found that the above-ground biomass of annual legume crops and perennial green manure crops during a growing season yielded a total of 200 and 400-500 kg N per hectare, respectively. Thus, growers who use berseem clover can benefit from the cut-and-carry approach by simultaneously fertilising their crops and controlling residue-borne pathogens through biofumigation. For sufficient coverage of the maize residues in one hectare, we suggest that growers should use the aboveground biomass of white mustard or Indian mustard produced in one hectare (1:1), whereas for berseem clover in half hectare (2:1). Although the cut-and-carry approach would increase the production costs in the short-term, it is anticipated that the long-term agronomic benefits, i.e. reduced *Fusarium* mycotoxins and increased soil fertility, would compensate for the initial economic trade-offs. Hence, a gross margin analysis for the cut-and-carry biofumigation treatments and the application of botanicals to maize residues should be conducted following the same methodology as in chapter III.

In chapter III, we investigated several maize-intercropping and cover cropping systems with the aim to decrease mycotoxins in subsequent wheat under reduced tillage practices. An

important aspect to consider in the design of intercropping systems is the interspecific competition which could potentially lower the yield of the main crop. In our case, none of the tested intercropping systems significantly reduced the yield of grain maize. Use of white mustard and Indian mustard as intercrops of maize decreased the DON content in grain of winter wheat by 58 % and 32 %, respectively, compared with sole maize and effects were significant under both no-tillage and reduced tillage practices. However, maize-intercropping did not significantly reduce FHB and mycotoxins under very high disease pressure, which was the case in the second experimental year. In the cover cropping study, we examined the effect of interval cover crops in a silage maize-spring wheat rotation under no-tillage. The cover cropping systems were compared with treatments where no cover crops were grown, i.e. plots where herbicide or plough was applied after the harvest of silage maize. We showed that the interval cover crops white mustard, Indian mustard and winter pea substantially reduced mycotoxins in wheat, i.e. DON and Ac-DON by up to 75 and 86 %, respectively. Remarkably, the decrease in mycotoxins with cover crops was comparable with that from the plough treatment. Nevertheless, it should be noted that winter pea reduced the incidence of *F. graminearum* in wheat grains, but increased the incidence of *F. avenaceum*. Therefore, a careful choice of cover crop species should also account for potential pathogen and mycotoxin risks caused by other important fungal species. Furthermore, we conducted a gross margin analysis which showed economic trade-offs of both maize-intercropping and cover cropping systems due to increased operating costs. We suggest that policy makers may consider supporting farmers to grow cover crops or intercrops in cereal-based rotations, e.g. maize-wheat. Eventually, this could serve as a bridge towards enhanced crop diversification strategies with the overall goal to improve food safety while ensuring farmers' economic viability.

In chapter IV, we investigated direct control strategies to combat *F. graminearum* and reduce mycotoxins in wheat using mustard-based botanicals. We followed an experimental approach from *in vitro* to *in planta* evaluating the efficacy of aqueous extracts from white mustard seed flours (same as in chapter I) and Indian mustard seed flours (newly added products). The botanicals showed equal or higher efficacies in inhibiting mycelium growth *in vitro* than the fungicide treatment. Under controlled environments in the growth chamber, botanicals based on white mustard were as effective as the fungicide in reducing DON in grain. The antifungal activity of the mustards is attributed to their bioactive matrices containing isothiocyanates (ITCs) and phenolic acids. However, under field conditions, only the use of fungicide decreased significantly FHB infection and DON in wheat. Dougoud et al. (2019) summarised the factors affecting the efficacy of botanicals as follows: (i) there is a variation in active ingredients in botanical plant material due to genotypic and environmental factors as well as storage effects;

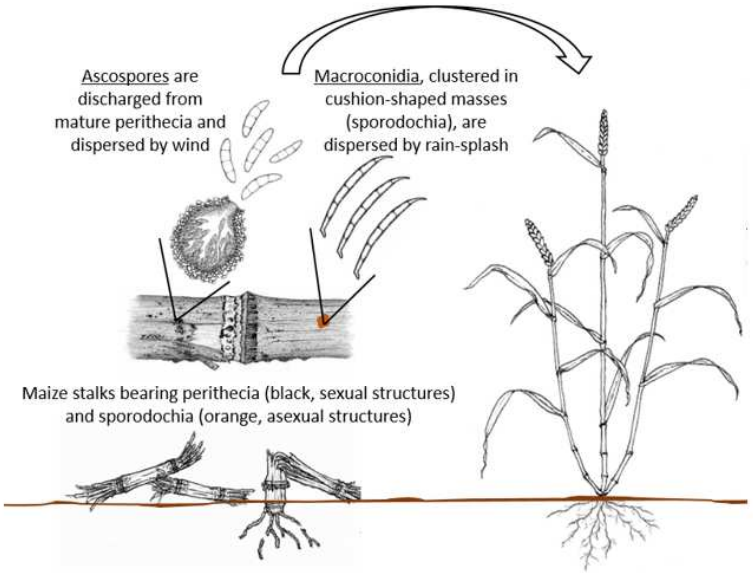
(ii) processing, such as extraction time and solvent; (iii) adjuvants, such as addition of appropriate surfactants and stickers, could improve the efficacy; (iv) complex interactions and implications, such as effects on non-target organisms. We recommend that future research should focus on developing effective formulation strategies to improve the stability and prolong the bioactivity of the tested botanicals. Moreover, the genotypic and environmental factors resulting in variable concentrations of the active ingredients in mustard seeds, e.g. isothiocyanates and phenolic acids, should be further explored.

Recently, there is a movement towards reduced reliance on synthetic pesticides to minimise any potential negative impact on the environment and human health. In 2020, a joint declaration of intent between the partners of the European research alliance "Towards a chemical pesticide-free agriculture" was signed aiming to create a scientific roadmap to find alternatives to the use of chemical pesticides in Europe (Anonymous, 2020). Furthermore, in 2017, the Federal Council of the Swiss Confederation adopted an action plan aiming to define goals and measures for pesticide risk reduction and sustainable use of crop protection products (BLW, 2017). Within this context, in 2019, the Swiss 'PestiRed' project was initiated with the overall goal to reduce pesticide use by 75 % without reducing average crop yields by more than 10 % through the development of innovative production systems (BLW, 2019). The PestiRed project involves a broad farmers' network across three regions that extends over a crop rotation period of 6 years including both agronomic and economic aspects. The principal concepts of conservation agriculture and agroecology are the main focus within PestiRed, including some crop protection measures which were already investigated during the course of this dissertation. The effects of various cover cropping, intercropping and tillage systems on the regulation of weeds, pests and diseases, such as FHB, will be investigated. Some challenges of these systems include the right decision of cover crop or intercrop species, the sowing date, as well as the time and type of destruction. Our findings provide several inputs on these aspects: (i) none of the tested maize-intercropping systems reduced maize yield when sown at BBCH 13–15 growth stage of maize; (ii) white mustard, Indian mustard and winter fodder pea can be successfully sown as cover crops with direct sowing immediately after the harvest of silage maize in the beginning of September; (iii) mustard and winter pea can be mulched before the winter frost and in early spring, respectively; (iv) in case that soil conservation is of primary focus, no tillage should be preferred over reduced tillage (~10 cm depth) without increasing the risk of mycotoxin contamination for the subsequent wheat crop.

With this dissertation, we shed light on alternative crop protection measures using innovative cropping systems and plant-based biopesticides to reduce the risk of *Fusarium* mycotoxins in wheat. We improved the knowledge and provided recommendations on sustainable

mycotoxin management at national level, e.g. see PestiRed, but also at global level, e.g. see SDGs of the United Nations. In summary, white mustard and Indian mustard could be cultivated as cut-and-carry biofumigant crops, cover crops and intercrops of maize to reduce mycotoxins in wheat, with the former two agronomic practices being more effective than the latter. Moreover, berseem clover as a cut-and-carry biofumigant crop and winter pea as a cover crop are not only effective strategies to combat FHB, but could also improve soil fertility and soil organic carbon stocks. For the direct control of FHB, botanicals based on white mustard seed flour were highly effective, though significant mycotoxin reduction was only achieved under controlled conditions in the growth chamber and not in the field. Within the context of sustainable crop protection, cereal growers and consumers could benefit from the recommended pre-harvest strategies by decreasing the risk of mycotoxin contamination in harvest products and thus improving grain yield and quality. Yet innovation in cropping systems, such as intercropping and cover cropping, may result in increased production costs for the grower. Hence, cereal growers should carefully weigh potential conflicts between farm profitability and food safety goals. This challenge could be addressed by appropriate agricultural policies at both producer and consumer levels, fostering sustainable cultivation systems that contribute to safe food and feed as well as to ensure food security.

Simplified life cycle of *Fusarium graminearum* (teleomorph *Gibberella zeae*) in a maize-wheat rotation:



Use of botanicals to suppress different stages of the life cycle of *Fusarium graminearum* [**chapter I**]:

Mycelium growth
+48 to -100 %

Perithecia formation on maize stalks
-20 to -56 %

Ascospore discharge
-54 to -77 %

Germination rates
Ascospores **+2 to -97 %**
Conidia **+6 to -100 %**

Prevention of Fusarium head blight and mycotoxins in wheat using (i) cut-and-carry biofumigation and (ii) botanicals [**chapter II**]; (iii) Intercropping and (iv) cover crops [**chapter III**]:

(i) Applying fresh mulch layers to infected maize stalks
mycotoxins in winter wheat
DON: -33 to -58 %
ZEN: -65 to -87 %

(ii) Applying botanicals to infected maize stalks
mycotoxins in winter wheat
DON: +25 to -42 %
ZEN: +56 to -78 %

(iii) Maize-intercropping
mycotoxins in winter wheat
DON: +9 to -58 %
ZEN: -12 to -47 %

(iv) Cover cropping
mycotoxins in spring wheat
DON: -44 to -75 %
Ac-DON: -69 to -86 %

Control of *Fusarium graminearum* in wheat with mustard-based botanicals: From *in vitro* to *in planta* [**chapter IV**]

Mycelium growth *in vitro*
+28 to -100 %

Growth chamber *in planta* experiment
DON: -55 to -98 %

Field experiment
No significant DON reduction in spring wheat: -7 to -18 %

Fig. 6.1. Summary of the experimental approaches, followed in the doctoral study, demonstrating the range of efficacy against fungal structures of *Fusarium graminearum* (chapter I) and mycotoxin production (chapters II, III and IV). The plus and minus signs refer to increased or decreased, respectively, fungal growth and mycotoxin production. The values of other measured mycotoxins, which are not mentioned here, were below or close to the quantification limit. DON: deoxynivalenol; ZEN: zearalenone; Ac-DON: sum of 3- and 15-acetyldeoxynivalenol.

The outcomes of the current dissertation point towards new research directions:

- To further improve the efficacy of botanicals against FHB in prevention strategies (chapter II) and direct control measures (chapter IV) in the field, future research should target formulation development strategies to prolong the bioactivity and persistency of the products. For instance, it would be interesting to test a biopesticide consisting of mustard seed flour and milled Chinese galls, hypothesising that the tannins of the latter would protect against UV degradation of the former botanical. In addition, the variation of the active ingredients of the botanicals as affected by genotypic and environmental factors as well as the effect of application timing and any potential induced plant resistance should be examined.
- Another interesting approach would be to extract and isolate the bioactive ingredients present in the powders of mustard and Chinese galls, assess the efficacy of each individual substance against *F. graminearum* and prepare a liquid biopesticide formulation. Moreover, a liquid suspension would facilitate the preparation and application processes by farmers compared with powder suspension in water.
- The effect of the tested intercrops and cover crops as well as the formulated botanicals on non-target organisms (e.g. predatory mites, honeybees and earthworms) should be assessed comparing with synthetic fungicides.
- The observed differences between ascosporic and conidial inoculums of *F. graminearum* on FHB severity and *Fusarium* mycotoxins in soft wheat (chapter IV) should be further investigated. For this reason, a new study has been initiated to explore the effects of the two spore types of two *F. graminearum* strains on disease severity and mycotoxin accumulation in barley and durum wheat.
- Winter pea as cover crop and berseem clover as cut-and-carry biofumigant crop significantly reduced FHB and mycotoxins in wheat. It would be important to investigate the mode of actions of these legumes against FHB causing species.
- The effect of living mulches on hampering the discharge of ascospores from maize residues in wheat should be explored. For this reason, we have initiated field experiments to investigate the effect of wheat-clover intercropping on FHB infection, mycotoxin contamination in wheat grain and wheat yield in a maize-wheat rotation under no-tillage.
- Although the effects of cropping factors on FHB infection and *Fusarium* mycotoxins have been well-investigated in wheat, a few analogue studies are available for other important small-grain cereals, such as barley. Hence, we have initiated a *Fusarium* mycotoxin survey collecting grain and straw samples of commercially grown barley across Switzerland. A farmer's questionnaire was included to collect information

about the cropping history of each barley field to investigate the main influencing factors of disease development and mycotoxin production.

- To promote alternative agronomic systems, such as intercropping and cover cropping, future research should explore a holistic approach of the agroecosystem. Besides the effects on *Fusarium* mycotoxins and the economic parameters, other agronomic factors need to be included, such as pests and other diseases, weed suppression, soil fertility and attraction or repellence of beneficial organisms. Also, it is recommended to evaluate the cropping systems with the highest efficacy against FHB of this dissertation under on-farm experiments in collaboration with a broad network of farmers (e.g. in PestiRed project). Moreover, future investigations should include farmers' acceptance to implement such agronomic measures at global level. This could be a particularly important aspect in developing countries where pesticides are scarce or not available, and therefore alternative cropping systems and homemade botanicals could reduce the risks of mycotoxin accumulation in food products. For example, smallholder farmers in subsistence agriculture could use non-edible parts from certain crop or tree species with antimicrobial properties to control pests and diseases.
- It is recommended to promote public participation in scientific research with overall goal to increase the awareness of non-expert individuals regarding the impact of mycotoxin contamination on food safety and food security. Interdisciplinary projects with Citizen Science could involve participatory monitoring of disease symptoms in the field, excursions in cereal mills and open workshops organised by farmers and extension agents including participation of the public. Also, it would be important to involve students from early age, e.g. elementary school, in these activities. Consequently, the knowledge transfer from producers to consumers on mycotoxin contamination will be enhanced, facilitating agricultural policies to implement certain measures that promote the sustainable management of mycotoxins throughout the entire supply chain.

Appendix

Chapter I

Supplementary Tables

Table SI.1. The pH of aqueous suspensions of, and in potato dextrose agar (PDA) containing, Tillecur® (Ti), Pure Yellow Mustard (PYM) or Chinese galls (CG) botanical powders.

	Suspensions in dH_2O (w v^{-1})		Incorporated in PDA[a]		
	1 %	4 %	1 %	2 %	4 %
Ti	5.4	5.3	5.4	5.4	5.3
PYM	5.3	5.0	5.5	5.4	5.3
CG	4.0	3.9	4.5	4.3	4.2

[a]Incorporation of botanicals at 1 %, 2 %, and 4 % w v^{-1} in PDA (39 g $liter^{-1}$ dH_2O).

Table SI.2. Results of the pairwise comparisons (Student-Newman-Keuls test) among treatments (Control - C; Tillecur® - Ti; Pure Yellow Mustard - PYM; Chinese galls - CG) for bioassays assessing the effects on (a) mycelium growth, (b) conidia germination, and (c) ascospore germination of *Fusarium graminearum* strains FG0410, FG2113, and FG1145. The strain FG1145 was not included in the ascospore germination bioassay due to its inability to produce sufficient perithecia and, therefore, ascospores. Only significant comparisons are presented followed by the corresponding *P* values.

(a) Mycelium growth

FG0410		FG2113		FG1145	
Pairwise comparison	*P* value	Pairwise comparison	*P* value	Pairwise comparison	*P* value
Ti 0.5% vs PYM 1%	<0.001	Ti 0.5% vs PYM 1%	<0.001	Ti 0.5% vs PYM 2%	<0.001
Ti 0.5% vs PYM 2%	<0.001	Ti 0.5% vs PYM 2%	<0.001	Ti 0.5% vs Ti 2%	<0.001
Ti 0.5% vs Ti 2%	<0.001	Ti 0.5% vs Ti 2%	<0.001	Ti 0.5% vs PYM 1%	<0.001
Ti 0.5% vs CG 1%	<0.001	Ti 0.5% vs CG 1%	<0.001	Ti 0.5% vs Ti 1%	<0.001
Ti 0.5% vs CG 0.5%	<0.001	Ti 0.5% vs Ti 1%	<0.001	Ti 0.5% vs CG 1%	<0.001
Ti 0.5% vs Ti 1%	<0.001	Ti 0.5% vs CG 0.5%	<0.001	Ti 0.5% vs CG 0.5%	<0.001
Ti 0.5% vs PYM 0.5%	<0.001	Ti 0.5% vs PYM 0.5%	<0.001	Ti 0.5% vs C	<0.001
Ti 0.5% vs C	<0.001	Ti 0.5% vs C	<0.001	Ti 0.5% vs PYM 0.5%	0.001
C vs PYM 1%	<0.001	C vs PYM 1%	<0.001	PYM 0.5% vs PYM 2%	<0.001
C vs PYM 2%	<0.001	C vs PYM 2%	<0.001	PYM 0.5% vs Ti 2%	<0.001
C vs Ti 2%	<0.001	C vs Ti 2%	<0.001	PYM 0.5% vs PYM 1%	<0.001
C vs CG 1%	<0.001	C vs CG 1%	0.009	PYM 0.5% vs Ti 1%	<0.001
C vs CG 0.5%	<0.001	C vs Ti 1%	0.01	PYM 0.5% vs CG 1%	<0.001
C vs Ti 1%	0.006	C vs CG 0.5%	0.01	PYM 0.5% vs CG 0.5%	<0.001
C vs PYM 0.5%	0.005	C vs PYM 0.5%	0.002	PYM 0.5% vs C	0.005
PYM 0.5% vs PYM 1%	<0.001	PYM 0.5% vs PYM 1%	0.002	C vs PYM 2%	<0.001
PYM 0.5% vs PYM 2%	<0.001	PYM 0.5% vs PYM 2%	<0.001	C vs Ti 2%	<0.001
PYM 0.5% vs Ti 2%	<0.001	PYM 0.5% vs Ti 2%	<0.001	C vs PYM 1%	<0.001
PYM 0.5% vs CG 1%	<0.001	CG 0.5% vs PYM 1%	<0.001	C vs Ti 1%	<0.001
PYM 0.5% vs CG 0.5%	<0.001	CG 0.5% vs PYM 2%	<0.001	C vs CG 1%	<0.001

Ti 1% vs PYM 1%	<0.001	CG 0.5% vs Ti 2%	<0.001	C vs CG 0.5%	0.005
Ti 1% vs PYM 2%	<0.001	Ti 1% vs PYM 1%	<0.001	CG 0.5% vs PYM 2%	<0.001
Ti 1% vs Ti 2%	<0.001	Ti 1% vs PYM 2%	<0.001	CG 0.5% vs Ti 2%	<0.001
Ti 1% vs CG 1%	<0.001	Ti 1% vs Ti 2%	<0.001	CG 0.5% vs PYM 1%	<0.001
Ti 1% vs CG 0.5%	<0.001	CG 1% vs PYM 1%	<0.001	CG 0.5% vs Ti 1%	<0.001
CG 0.5% vs PYM 1%	<0.001	CG 1% vs PYM 2%	<0.001	CG 0.5% vs CG 1%	<0.001
CG 0.5% vs PYM 2%	<0.001	CG 1% vs Ti 2%	<0.001	CG 1% vs PYM 2%	0.02
CG 0.5% vs Ti 2%	<0.001			CG 1% vs Ti 2%	0.001
CG 0.5% vs CG 1%	<0.001			CG 1% vs PYM 1%	0.001
CG 1% vs PYM 1%	0.002			Ti 1% vs PYM 2%	0.002
CG 1% vs PYM 2%	<0.001			Ti 1% vs Ti 2%	<0.001
CG 1% vs Ti 2%	<0.001			Ti 1% vs PYM 1%	<0.001

(b) Conidia germination

FG0410		FG2113		FG1145	
Pairwise comparison	*P* value	Pairwise comparison	*P* value	Pairwise comparison	*P* value
C vs PYM 2%	<0.001	PYM 0.5% vs CG 2%	<0.001	Ti 0.5% vs Ti 2%	<0.001
C vs CG 2%	<0.001	PYM 0.5% vs PYM 2%	<0.001	Ti 0.5% vs CG 2%	<0.001
C vs CG 1%	<0.001	PYM 0.5% vs Ti 2%	<0.001	Ti 0.5% vs PYM 2%	<0.001
C vs Ti 2%	<0.001	PYM 0.5% vs PYM 1%	<0.001	Ti 0.5% vs CG 1%	<0.001
C vs Ti 1%	<0.001	PYM 0.5% vs CG 1%	<0.001	Ti 0.5% vs CG 0.5%	<0.001
C vs CG 0.5%	<0.001	PYM 0.5% vs Ti 1%	<0.001	Ti 0.5% vs PYM 1%	<0.001
C vs PYM 1%	<0.001	PYM 0.5% vs CG 0.5%	<0.001	Ti 0.5% vs Ti 1%	<0.001
C vs Ti 0.5%	0.003	C vs CG 2%	<0.001	PYM 0.5% vs Ti 2%	<0.001
C vs PYM 0.5%	<0.001	C vs PYM 2%	<0.001	PYM 0.5% vs CG 2%	<0.001
PYM 0.5% vs PYM 2%	<0.001	C vs Ti 2%	<0.001	PYM 0.5% vs PYM 2%	<0.001
PYM 0.5% vs CG 2%	<0.001	C vs PYM 1%	<0.001	PYM 0.5% vs CG 1%	<0.001
PYM 0.5% vs CG 1%	<0.001	C vs CG 1%	<0.001	PYM 0.5% vs CG 0.5%	<0.001
PYM 0.5% vs Ti 2%	<0.001	C vs Ti 1%	<0.001	PYM 0.5% vs PYM 1%	<0.001
PYM 0.5% vs Ti 1%	<0.001	C vs CG 0.5%	<0.001	PYM 0.5% vs Ti 1%	<0.001
PYM 0.5% vs CG 0.5%	<0.001	Ti 0.5% vs CG 2%	<0.001	C vs Ti 2%	<0.001
PYM 0.5% vs PYM 1%	<0.001	Ti 0.5% vs PYM 2%	<0.001	C vs CG 2%	<0.001
Ti 0.5% vs PYM 2%	<0.001	Ti 0.5% vs Ti 2%	<0.001	C vs PYM 2%	<0.001
Ti 0.5% vs CG 2%	<0.001	Ti 0.5% vs PYM 1%	<0.001	C vs CG 1%	<0.001
Ti 0.5% vs CG 1%	<0.001	Ti 0.5% vs CG 1%	<0.001	C vs CG 0.5%	<0.001
Ti 0.5% vs Ti 2%	<0.001	Ti 0.5% vs Ti 1%	<0.001	C vs PYM 1%	<0.001
Ti 0.5% vs Ti 1%	<0.001	Ti 0.5% vs CG 0.5%	<0.001	C vs Ti 1%	<0.001
Ti 0.5% vs CG 0.5%	<0.001	CG 0.5% vs CG 2%	<0.001	Ti 1% vs Ti 2%	<0.001
Ti 0.5% vs PYM 1%	<0.001	CG 0.5% vs PYM 2%	0.002	Ti 1% vs CG 2%	<0.001
PYM 1% vs PYM 2%	0.02	CG 0.5% vs Ti 2%	0.003	Ti 1% vs PYM 2%	<0.001
PYM 1% vs CG 2%	0.003	CG 0.5% vs PYM 1%	0.004	Ti 1% vs CG 1%	<0.001
PYM 1% vs CG 1%	0.009	CG 0.5% vs CG 1%	0.005	Ti 1% vs CG 0.5%	0.007
PYM 1% vs Ti 2%	<0.001			PYM 1% vs Ti 2%	<0.001
PYM 1% vs Ti 1%	0.003			PYM 1% vs CG 2%	<0.001
CG 0.5% vs PYM 2%	0.009			PYM 1% vs PYM 2%	<0.001
CG 0.5% vs CG 2%	<0.001			PYM 1% vs CG 1%	<0.001
CG 0.5% vs CG 1%	0.002			CG 0.5% vs Ti 2%	<0.001

CG 0.5% vs Ti 2%	<0.001		CG 0.5% vs CG 2%	<0.001
CG 0.5% vs Ti 1%	<0.001		CG 0.5% vs PYM 2%	<0.001
			CG 0.5% vs CG 1%	<0.001

(c) Ascospore germination

FG0410		FG2113	
Pairwise comparison	*P* value	Pairwise comparison	*P* value
PYM 0.5% vs Ti 2%	<0.001	Ti 0.5% vs Ti 2%	<0.001
PYM 0.5% vs PYM 2%	<0.001	Ti 0.5% vs PYM 2%	<0.001
PYM 0.5% vs PYM 1%	<0.001	Ti 0.5% vs Ti 1%	<0.001
PYM 0.5% vs Ti 1%	<0.001	Ti 0.5% vs PYM 1%	<0.001
PYM 0.5% vs CG 0.5%	0.006	C vs Ti 2%	<0.001
PYM 0.5% vs CG 1%	0.002	C vs PYM 2%	<0.001
PYM 0.5% vs CG 2%	0.002	C vs Ti 1%	<0.001
PYM 0.5% vs C	0.02	C vs PYM 1%	<0.001
Ti 0.5% vs Ti 2%	<0.001	PYM 0.5% vs Ti 2%	<0.001
Ti 0.5% vs PYM 2%	<0.001	PYM 0.5% vs PYM 2%	<0.001
Ti 0.5% vs PYM 1%	<0.001	PYM 0.5% vs Ti 1%	<0.001
Ti 0.5% vs Ti 1%	<0.001	PYM 0.5% vs PYM 1%	<0.001
Ti 0.5% vs CG 0.5%	<0.001	CG 0.5% vs Ti 2%	<0.001
Ti 0.5% vs CG 1%	<0.001	CG 0.5% vs PYM 2%	<0.001
Ti 0.5% vs CG 2%	<0.001	CG 0.5% vs Ti 1%	<0.001
Ti 0.5% vs C	0.001	CG 0.5% vs PYM 1%	<0.001
C vs Ti 2%	<0.001	CG 1% vs Ti 2%	<0.001
C vs PYM 2%	<0.001	CG 1% vs PYM 2%	<0.001
C vs PYM 1%	<0.001	CG 1% vs Ti 1%	<0.001
C vs Ti 1%	<0.001	CG 1% vs PYM 1%	<0.001
C vs CG 0.5%	0.007	CG 2% vs Ti 2%	<0.001
C vs CG 1%	0.002	CG 2% vs PYM 2%	<0.001
C vs CG 2%	0.002	CG 2% vs Ti 1%	<0.001
CG 2% vs Ti 2%	<0.001	CG 2% vs PYM 1%	<0.001
CG 2% vs PYM 2%	<0.001		
CG 2% vs PYM 1%	<0.001		
CG 2% vs Ti 1%	<0.001		
CG 1% vs Ti 2%	<0.001		
CG 1% vs PYM 2%	<0.001		
CG 1% vs PYM 1%	<0.001		
CG 1% vs Ti 1%	<0.001		
CG 0.5% vs Ti 2%	<0.001		
CG 0.5% vs PYM 2%	<0.001		
CG 0.5% vs PYM 1%	<0.001		
CG 0.5% vs Ti 1%	<0.001		
Ti 1% vs Ti 2%	0.009		
Ti 1% vs PYM 2%	0.05		
Ti 1% vs PYM 1%	0.03		
PYM 1% vs Ti 2%	0.02		
PYM 2% vs Ti 2%	0.007		

Chapter II

No supplementary material.

Chapter III

Supplementary Tables

Table SIII.1. LC-MS/MS parameters used for the mycotoxin analysis.

Eluent A: water + 5 mM ammoniumacetate + 0.2 % acetic acid
Eluent B: methanol + 5 mM ammoniumacetate + 0.2 % acetic acid
Column: Agilent Zorbax Eclipse plus C18, rapid resolution HD, 2.1 mm × 50 mm, 1.8 µm
Pre-column: Phenomenex Security Guard C18, 4 mm × 2 mm
Flow: 0.300 ml min^{-1}
Injection volume: 10 µl
Temperature: 40°C
Gradient: 0.0 min 5 % B, 0.1 min 5 % B, 0.2 min 10 % B, 6.5 min 95 % B, 8.5 min 95 % B, 9.0 min 5 % B, 12.0 min 5 % B
Drying gas temperature: 250°C
Drying gas flow: 12 L min^{-1}
Nebulizer: 55 psi
Sheath gas temperature: 350°C
Sheath gas flow: 12 L min^{-1}
Capillary: 3500 V pos / 2500 V neg
Nozzle voltage: 0 V pos / 0 V neg
Dwell time: 7 msec
Cell accelarator: 7 V

Analyte	Precursor Ion	Product (m/z)	Fragmentor (V)	Collision energy (V)	Polarity
NIV	371	281	108	8	neg
NIV	371	311	108	4	neg
DON	355	295	95	6	neg
DON	355	59	95	20	neg
FUS-X	413	353	95	8	pos
FUS-X	413	263	65	8	pos
Ac-DON	356	137	95	8	pos
Ac-DON	339	137	105	12	pos
MAS	342	307	80	6	pos
MAS	342	265	80	6	pos
DAS	384	307	75	5	pos
DAS	384	247	105	6	pos
HT-2	447	345	135	14	pos
HT-2	447	285	135	16	pos
T-2	484	185	120	14	pos
T-2	484	215	125	14	pos
ZEN	317	175	190	16	neg
ZEN	317	131	190	24	neg

nivalenol (NIV), deoxynivalenol (DON), fusarenon-x (Fus-X), acetyldeoxynivalenol (Ac-DON), monoacetoxyscirpenol (MAS), diacetoxyscirpenol (DAS), zearalenone (ZEN)

Table SIII.2. Limit of detection (LOD) and limit of quantification (LOQ) in µg kg^{-1} for the examined analytes (nivalenol (NIV), deoxynivalenol (DON), sum of 3- and 15-acetyldeoxynivalenol (Ac-DON), fusarenon-x (Fus-X), monoacetoxyscirpenol (MAS), diacetoxyscirpenol (DAS), HT-2, T-2 and zearalenone (ZEN) in wheat.

	NIV	DON	Ac-DON	Fus-X	MAS	DAS	HT-2	T-2	ZEN
LOD	2.3	12	3	32	1.4	0.3	1.4	0.4	0.1
LOQ	7.7	40	10	107	4.8	1.0	4.5	1.4	0.2

Table SIII.3. Maize-intercropping. Effect of maize-(inter)cropping system (sole maize: SM; clover: C; sudangrass: S; phacelia: PH; white mustard: WM; Indian mustard: IM) on grain yield (t ha^{-1}) of maize. No significant differences were observed between intercropping systems and sole maize within each experimental year (2016, 2018); ± represent the standard error of the mean (n = 8).

2016	SM	C	S	PH	WM	IM
	12.0 ± 0.3	11.7 ± 0.4	10.5 ± 0.7	10.9 ± 0.5	10.8 ± 0.8	11.0 ± 0.5
2018	SM	C	S	PH	WM	IM
	10.0 ± 0.2	9.3 ± 0.8	9.7 ± 0.3	9.2 ± 0.4	9.6 ± 0.2	9.2 ± 0.3

Table SIII.4. Maize-intercropping. Average values of grain yield (t ha^{-1}); Fusarium head blight (FHB) symptoms (number of symptomatic heads m^{-2}); incidence of *F. graminearum* (FG), *F. avenaceum* (FA), *F. poae* (FP) and *Microdochium* spp. (M spp.) in grains (%); deoxynivalenol (DON), sum of 3- and 15-acetyldeoxynivalenol (Ac-DON) and zearalenone (ZEN) content in grain (µg kg^{-1}) across the entire wheat field in the experimental year 2016–2017 and 2018–2019 (n = 96).

	Grain yield	FHB symptoms	FG	FA	FP	M spp.	DON	Ac-DON	ZEN
2016–2017	6.28	2	16	1.0	1.3	0.8	450	2	180
2018–2019	6.42	58	46	0.3	0.1	2.8	5040	150	0.1

Table SIII.5. Maize-intercropping. Significance levels (*p*-values) for the main effects of tillage (Ti), maize-(inter)cropping (MI) and wheat variety (WV) and their interactions within each experimental year (2016–2017, 2018–2019) on the response variables in wheat, i.e. grain yield; Fusarium head blight (FHB) symptoms; incidence of *F. graminearum* (FG), *F. avenaceum* (FA), *F. poae* (FP) and *Microdochium* spp. (M spp.) in grains; deoxynivalenol (DON), sum of 3- and 15-acetyldeoxynivalenol (Ac-DON) and zearalenone (ZEN) content in grain.

	Grain yield	FHB symptoms	FG	FA	FP	M spp.	DON	Ac-DON	ZEN
2016–2017									
Ti	0.175	0.166	0.743	0.153	0.553		0.497		0.446
MI	0.455	0.002	0.095	0.471	0.017		0.001		0.039
WV	0.071	<0.001	<0.001	0.091	0.002		<0.001		<0.001
Ti × MI	0.065	0.026	0.029	0.470	0.173	na	0.073	na	0.309
Ti × WV	0.010	0.168	0.019	0.603	0.847		0.016		0.706
MI × WV	0.615	0.544	0.483	0.300	0.475		0.325		0.574
Ti × MI × WV	0.027	0.095	0.674	0.307	0.071		0.368		0.681
2018–2019									
Ti	0.002	0.799	0.819			0.165	0.289	0.426	
MI	<0.001	0.706	0.358			0.432	0.032	0.344	
WV	<0.001	<0.001	<0.001			0.002	0.005	<0.001	
Ti × MI	0.461	0.844	0.914	na	na	0.942	0.557	0.313	na
Ti × WV	0.342	0.024	0.175			0.638	0.478	0.282	
MI × WV	0.477	0.325	0.406			0.438	0.860	0.591	
Ti × MI × WV	0.265	0.515	0.748			0.224	0.860	0.019	

na: not applicable

Table SIII.6. Maize-intercropping. Effect of maize-(inter)cropping system (sole maize: SM; clover: C; sudangrass: S; phacelia: PH; white mustard: WM; Indian mustard: IM) on grain yield (t ha^{-1}) of wheat within wheat variety (Levis, Forel) and tillage practice (no-tillage, NT; reduced tillage, RT) in 2016–2017. Different letters indicate significant differences according to Fisher's protected LSD test for post hoc comparisons (α = 0.05) and ± represent the standard error of the mean (n = 4).

		Maize-(inter)cropping					
Wheat variety	Tillage	SM	C	S	PH	WM	IM
Levis	NT	6.42 ± 0.3 ab	5.52 ± 0.6 a	6.92 ± 0.2 b	5.92 ± 0.2 ab	5.49 ± 0.2 a	5.43 ± 0.6 a
	RT	7.10 ± 0.2	6.56 ± 0.5	6.31 ± 0.4	7.22 ± 0.2	6.94 ± 0.5	6.81 ± 0.3
Forel	NT	6.47 ± 0.4	5.25 ± 0.3	6.51 ± 0.3	6.26 ± 0.3	5.60 ± 0.6	6.19 ± 0.5
	RT	5.97 ± 0.4	7.05 ± 0.4	6.28 ± 0.4	6.21 ± 0.4	6.20 ± 0.5	6.06 ± 0.4

Table SIII.7. Maize-intercropping. Effect of maize-(inter)cropping system (sole maize: SM; clover: C; sudangrass: S; phacelia: PH; white mustard: WM; Indian mustard: IM) on grain yield (t ha^{-1}) of wheat in 2018–2019. Average values of two tillage practices (no-tillage, reduced tillage) and two wheat varieties (Levis, Forel) are presented. Different letters indicate significant differences according to Fisher's protected LSD test for post hoc comparisons (α = 0.05) and ± represent the standard error of the mean (n = 16).

SM	C	S	PH	WM	IM
6.75 ± 0.1 cd	6.06 ± 0.2 a	6.92 ± 0.2 d	6.18 ± 0.2 ab	6.47 ± 0.2 bc	6.17 ± 0.1 ab

Table SIII.8. Cover cropping. Average values of grain yield (t ha^{-1}); Fusarium head blight (FHB) symptoms (number of symptomatic heads m^{-2}); incidence of *F. graminearum* (FG), *F. avenaceum* (FA) and *F. poae* (FP) in grains (%); deoxynivalenol (DON), nivalenol (NIV) and sum of 3- and 15-acetyldeoxynivalenol (Ac-DON) content in grain (µg kg^{-1}) across the entire wheat field in the experimental year 2016–2017 and 2017–2018 (n = 40).

	Grain yield	FHB symptoms	FG	FA	FP	DON	NIV	Ac-DON
2016–2017	4.66	1	1.9	1.9	3.4	70	40	10
2017–2018	4.46	11	12.7	10.3	1.9	2580	70	180

Table SIII.9. Cover cropping. Significance levels (*p*-values) for the main effects of cropping system (CS) and wheat variety (WV) and their interactions within experimental year (2016–2017, 2017–2018) on the response variables in wheat, i.e. grain yield; Fusarium head blight (FHB) symptoms; incidence of *F. graminearum* (FG), *F. avenaceum* (FA) and *F. poae* (FP) in grains; deoxynivalenol (DON), nivalenol (NIV) and sum of 3- and 15-acetyldeoxynivalenol (Ac-DON) content in grain.

	Grain yield	FHB symptoms	FG	FA	FP	DON	NIV	Ac-DON
2016–2017								
CS	0.395	0.155	0.173	<0.001	0.987	0.727		
WV	0.027	0.507	0.074	0.316	0.002	<0.001	na	na
CS × WV	0.386	0.031	0.644	0.230	0.775	0.487		
2017–2018								
CS	0.020	0.002	0.136	0.002	0.278	0.007	0.082	0.004
WV	0.019	<0.001	0.005	0.702	0.006	<0.001	0.849	<0.001
CS × WV	0.757	0.151	0.280	0.822	0.958	0.031	0.757	0.248

na: not applicable

Table SIII.10. Cover cropping. Effect of cropping system (herbicide without cover crop: HWCC; plough without cover crop: PWCC; white mustard: WM; Indian mustard: IM; winter pea: WP) on grain yield (t ha^{-1}) of wheat in 2018. Average values of two wheat varieties (Digana, Fiorina) are presented. Different letters indicate significant differences according to Fisher's protected LSD test for post hoc comparisons ($\alpha = 0.05$) and ± represent the standard error of the mean (n = 8).

HWCC	PWCC	WM	IM	WP
3.99 ± 0.1 a	4.18 ± 0.1 ab	4.46 ± 0.2 abc	4.69 ± 0.1 bc	4.98 ± 0.2 c

Supplementary Figures

Fig. SIII.1. Maize-intercropping. Drone image displaying one experimental block during wheat cultivation. The whole plots (tillage practices) are indicated by the continuous line rectangles, whereas the dashed lines indicate the split of the 12 subplots (maize-(inter)cropping systems) into sub-subplots (two wheat varieties). The drone flight took place in June 2017 at Agroscope-Tänikon, 8356 Ettenhausen, Switzerland.

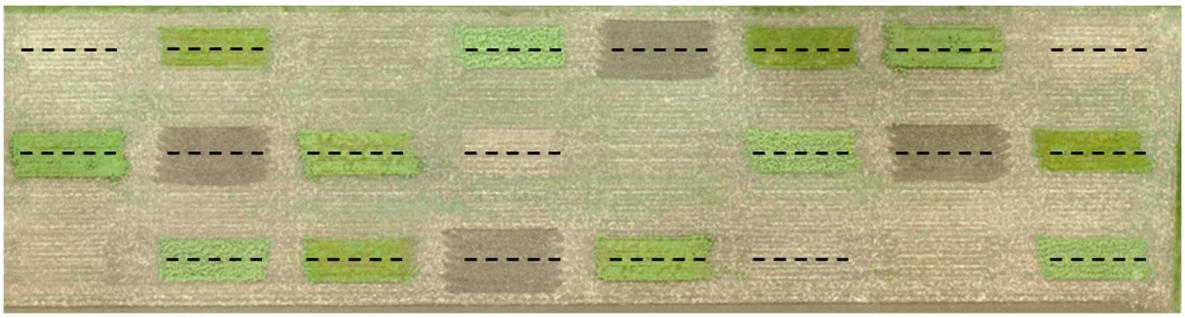


Fig. SIII.2. Cover cropping. Drone image displaying the entire experimental field. The whole plots (cropping systems) were split in half, as indicated by the dashed lines, subsequently creating subplots (two wheat varieties). The drone flight took place in November 2017 at Agroscope-Tänikon, 8356 Ettenhausen, Switzerland.

Chapter IV

Supplementary Tables

Table SIV.1. Growth chamber experiment: summary of significance levels (*p*-values) from a three-way ANOVA presenting main effects and interactions among spraying agent (SA), spore type (ST) and wheat variety (WV) on disease severity, grain yield, *Fusarium graminearum* (FG) DNA amount and DON content in grain. Data from two experiments were used for the analysis (n = 192).

Source of variation	Disease severity	Grain yield	FG DNA amount	DON content
SA	< 0.001	< 0.001	< 0.001	< 0.001
ST	0.002	0.005	< 0.001	< 0.001
WV	0.032	< 0.001	0.899	0.612
SA × ST	0.027	0.288	0.016	0.054
SA × WV	0.196	1	0.736	0.634
ST × WV	0.016	0.023	0.002	0.041
SA × ST × WV	0.480	0.576	0.275	0.640

Table SIV.2. Field experiment: summary of significance levels (*p*-values) from a three-way ANOVA presenting main effects and interactions among year (Y), spraying agent (SA) and wheat variety (WV) on disease incidence, grain yield, hectoliter weight, *Fusarium graminearum* (FG) DNA amount and DON content in grain. Data from two experiments were used for the analysis (n = 96).

Source of variation	Disease incidence	Grain yield	Hectoliter weight	FG DNA amount	DON content
Y	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
SA	< 0.001	0.015	< 0.001	0.003	< 0.001
WV	0.230	0.027	0.014	0.076	0.157
Y × SA	0.150	0.340	0.005	0.548	0.640
Y × WV	< 0.001	< 0.001	< 0.001	0.424	0.022
SA × WV	0.474	0.567	0.292	0.336	0.261
Y × SA × WV	0.613	0.827	0.632	0.944	0.937

Supplementary Figures

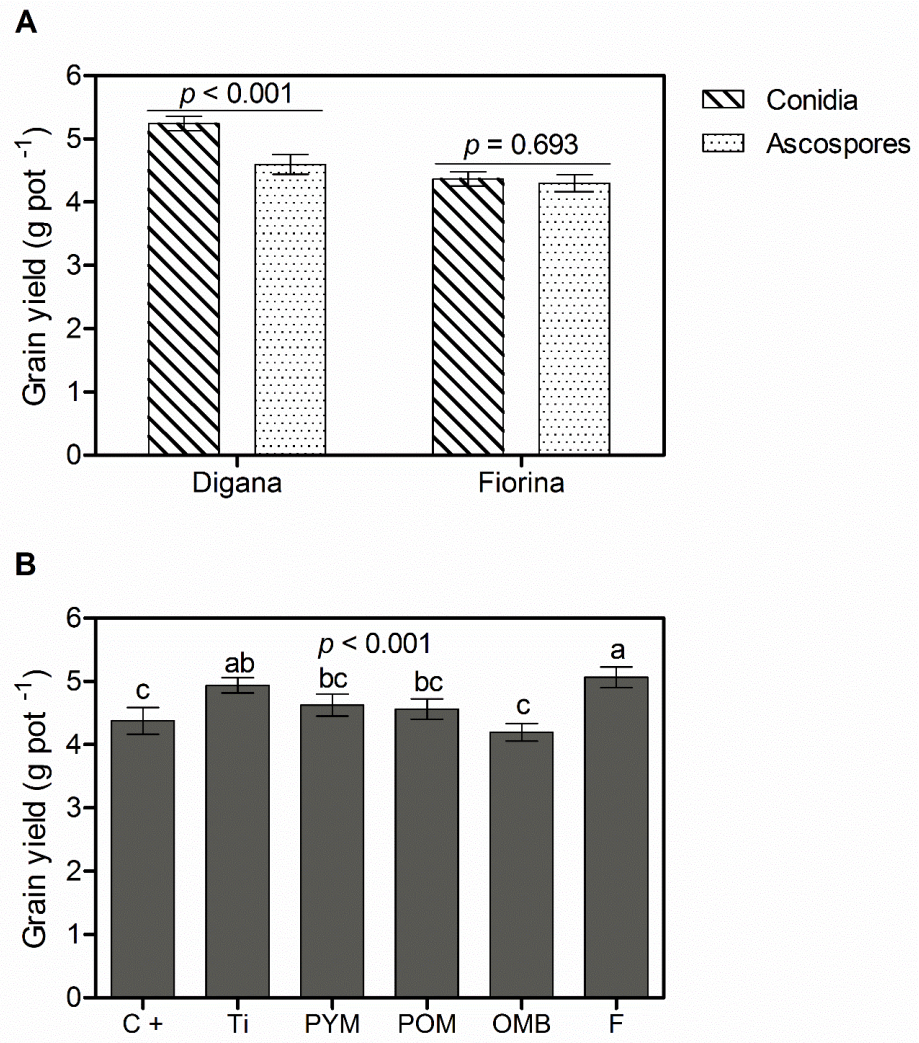


Fig. SIV.1. Growth chamber experiment: grain yield (g pot^{-1}) as affected by spore type (conidia, ascospores) within wheat variety (Digana, Fiorina) pooled over the spraying agents **(A)** and as affected by spraying agent pooled over the wheat varieties and the spore types **(B)**. The used spraying agents were Tillecur (Ti), Pure Yellow Mustard (PYM), Pure Oriental Mustard (POM), Oriental Mustard Bran (OMB) and Fungicide (F). Positive control (C +) refers to infected untreated plants. Average values from two experiments are presented and bars indicate the standard error of the mean. Different letters indicate significant differences (α = 0.05).

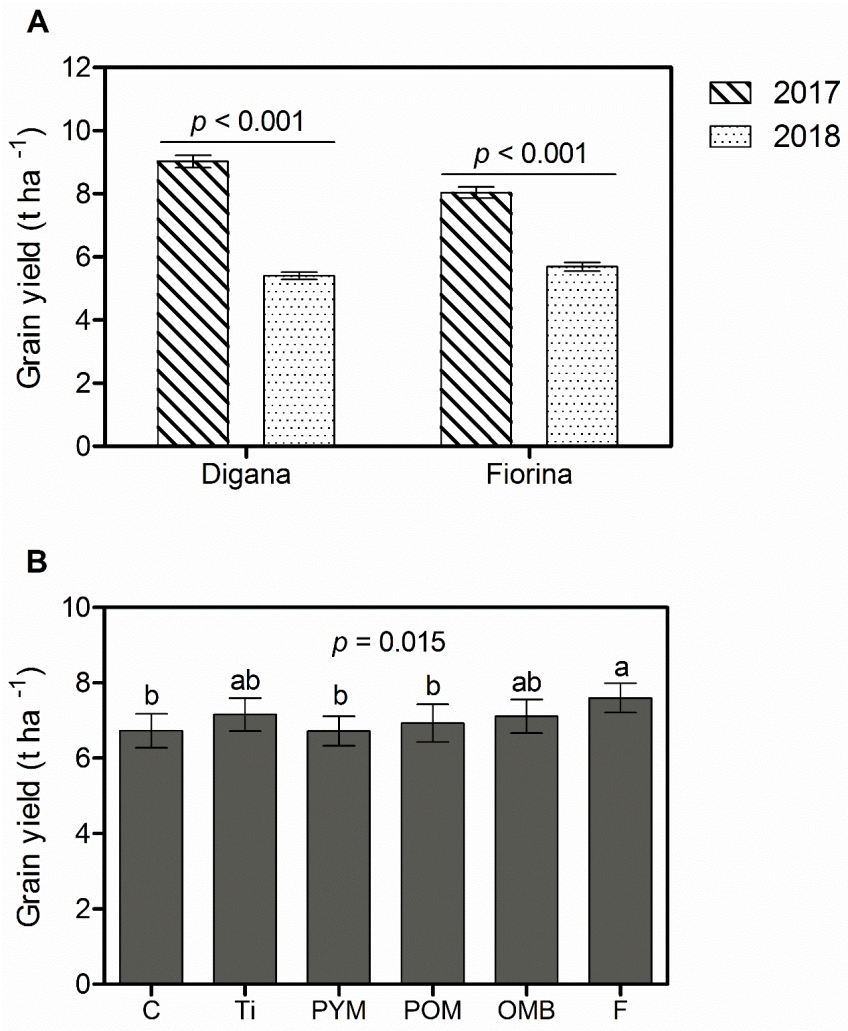


Fig. SIV.2. Field experiment: grain yield (t ha^{-1}) as affected by year (2017, 2018) within wheat variety (Digana, Fiorina) pooled over the spraying agents **(A)** and as affected by spraying agent pooled over the wheat varieties and the two years **(B)**. The used spraying agents were Tillecur (Ti), Pure Yellow Mustard (PYM), Pure Oriental Mustard (POM), Oriental Mustard Bran (OMB) and Fungicide (F). Control (C) refers to infected untreated plants. Bars indicate the standard error of the mean and different letters indicate significant differences ($\alpha = 0.05$).

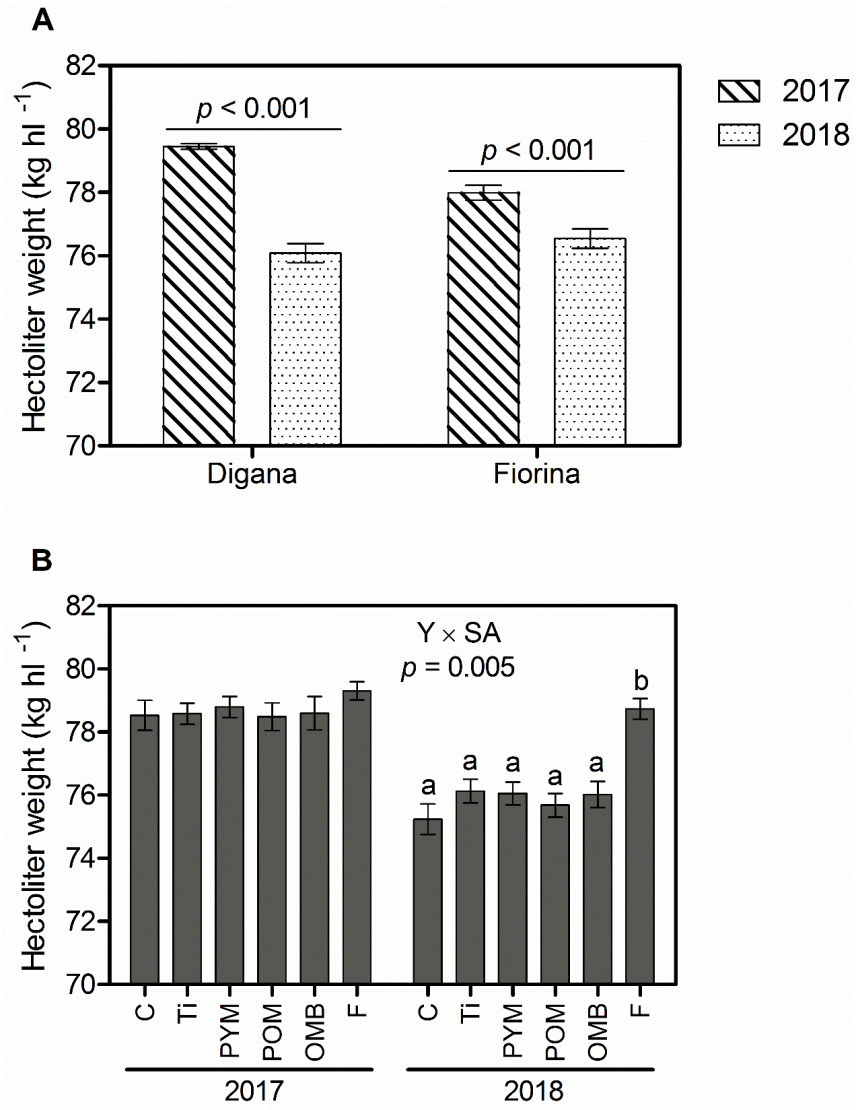


Fig. SIV.3. Field experiment: hectoliter weight (kg hl^{-1}) as affected by year (2017, 2018) within wheat variety (Digana, Fiorina) pooled over the spraying agents **(A)** and as affected by spraying agent (SA) within year (Y) pooled over the wheat varieties **(B)**. The used spraying agents were Tillecur (Ti), Pure Yellow Mustard (PYM), Pure Oriental Mustard (POM), Oriental Mustard Bran (OMB) and Fungicide (F). Control (C) refers to infected untreated plants. Bars indicate the standard error of the mean and different letters indicate significant differences (α = 0.05).

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