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Cultural control of Fusarium mycotoxins in cereal rotations through the application of biofumigation

A thesis submitted to Harper Adams University in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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Abstract

Fusarium graminearum is a globally important cereal pathogen that causes yield losses and mycotoxin contamination. Currently, triazole fungicides are used to manage *F. graminearum*, however, their limited effectiveness and concerns over safety have led to the pursuit of safe alternatives such as biofumigation. Biofumigation generally involves growing short term brassica crops, followed by chopping of the plant tissue and rapid incorporation into the soil. Inhibitory substances, particularly isothiocyanates, are released as a result of damage to brassica plant tissue causing suppression of soil-borne pests and diseases. Isothiocyanates were evaluated for their effect against *F. graminearum* under *in vitro* conditions; allyl and methyl isothiocyanates were overall more efficient, showing lower ED_{50} values (35-150 mg l^{-1}) for conidial germination and mycelial growth. Furthermore, *Brassica juncea*, *Raphanus sativus* and *Eruca sativa* were assessed for their biofumigation potential against *F. graminearum* in *in vitro* and microcosm experiments. *Brassica juncea* frozen leaf discs, collected at different growth stages, were effective against mycelial growth showing up to 100% suppression, while the sinigrin content in the leaf tissue corresponded with the level of suppression. In the microcosm experiment, each brassica species significantly suppressed *F. graminearum* inoculum by 41-55%. Furthermore, a field experiment was conducted to investigate the effect of incorporation of brassica cover crops on *F. graminearum* in a wheat-maize rotation. The potential of *B. juncea, E. sativa, R. sativus, B. carinata* and *B. oleracea* var. *caulorapa* L. to suppress *F. graminearum* inoculum in soil, disease incidence in maize and to reduce subsequent mycotoxin contamination in maize was investigated. Incorporation of *R. sativus* significantly decreased the amount of *F. graminearum* DNA by 58% compared with the cultivated fallow treatment. *Fusarium graminearum* DNA and deoxynivalenol in maize was 50-60% lower after incorporation of *B. oleracea* var. *caulorapa* L. but the difference was not significant. The field experiment was not effective in suppressing *F. graminearum* inoculum in soil, and disease and DON contamination in maize, this may have been due to the low glucosinolate content in the cover crops. However the findings, in addition to the promising results from the laboratory studies, provide sound basis for further research.

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Publication

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Presentations

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MycoTWIN-MycoKey International Conference, Bari, Italy, 9-12 November 2021: Poster (online) "Effect of brassica tissues on mycotoxin-producer *Fusarium graminearum*"

Abbreviations

AITC	allyl isothiocyanate
aw	water activity
BITC	benzyl isothiocyanate
cfu	colony forming unit
CZID	Czapek dox iprodione agar
CZPD	Czapek dox propiconazole agar
DMI	demethylation inhibitor
DNA	deoxyribonucleic acid
DON	deoxynivalenol
ED_{50}	effective dose values at 50%
EITC	ethyl isothiocyanate
GSL	glucosinolate
ha	hectare
HPLC	high performance liquid chromatography
ITC	isothiocyanate
MITC	methyl isothiocyanate
PCNB	pentachloronitrobenzene
PDA	potato dextrose agar
PDB	potato dextrose broth
PEITC	2-phenylethyl isothiocyanate
RNA	ribonucleic acid
SDHI	succinate dehydrogenase inhibitor
t	tonne
ZON	zearalenone

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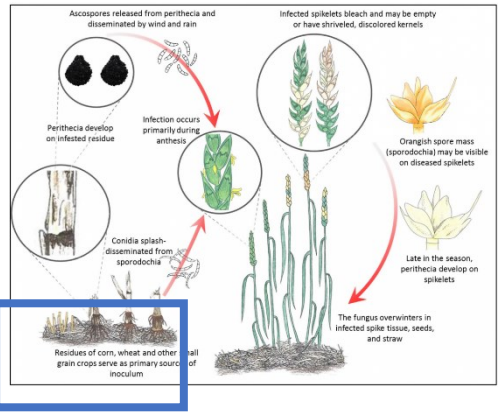

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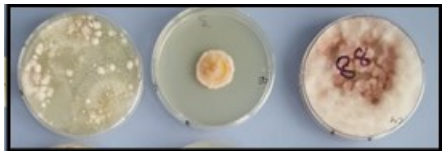

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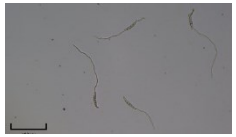

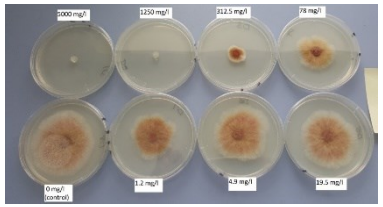

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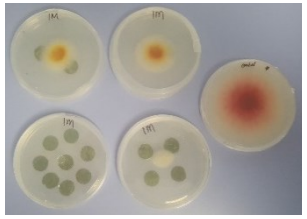


Chapter 1

Chapter 1

1 Literature review

1.1 Importance of cereals

Cereals are the world's most important crops providing the main source of energy in human diets and animal feed (McKevith, 2004). In 2013, cereals were cultivated on 47% of global agricultural land, providing approximately 60% and 50% of calories and protein respectively from human-edible crops (FAOSTAT; Hunter *et al*., 2017). The European Union (EU) is one of the largest producers, supplying 12% of the world market in 2017 and ranking as the fourth-largest global cereal-producer after China, US and India (Eurostat, 2017; World Bank, 2017). With regard to area and quantity, wheat is the most important cereal grown in the EU, and makes up approximately 50% of the total cereals grown. Maize constitutes a further 17% of the cereals grown in the EU. Of the cereals grown in the EU, about 30% are used for human consumption, 65% for animal feed and 3% for biofuels (EC, 2019a). Food demand in 2050 is projected to rise due to increase in world population and increase in per-capita consumption driven by greater wealth. As cereals are the main food source worldwide, the "2050 food demand" mainly reflects cereals demand (Hunter *et al*., 2017). According to a recent analysis, with perspective of sustainability and production goals, 25 to 70% increase in the current production levels will be required to fulfil the crop demand for 2050 (Hunter *et al*., 2017).

1.1.1 Wheat

Wheat is one of the most important agricultural crops globally. In terms of dietary intake, it is the most important staple commodity (OECD/FAO, 2021) and the most widely grown crop with more than 215 million ha grown worldwide (FAOSTAT, 2019). The EU is the largest producer of wheat with approximately 26 million ha cultivated; around 20% of this is exported annually (EC, 2019a). In 2019, the EU produced approximately 155 million t of wheat, accounting for 20% of the total global production (FAOSTAT). In the UK, wheat production ranged from 13 to 16 million t per annum from 1.8 million ha of cultivated land in 2015-2019 period (FAOSTAT). In 2017, approximately 15 million t of wheat, with the value of almost £2 billion was harvested in the UK, making it the third largest wheat producer in the EU, behind France and Germany (Anonymous, 2018; DEFRA, 2018).

Wheat contributes for about 20% of the total calorie intake worldwide (Shiferaw *et al*., 2013). In addition to being a major source of carbohydrates (85%), wheat also contains other important components of diet such as vitamins, fibre, minerals, amino acids and protein (Shewry and Hey, 2015). A unique characteristic that makes it an important global food commodity is attributed to the versatility of its flour which can be processed into a range of products such as bread, biscuits, cakes, noodles, pasta and other processed foods.

Wheat is generally classified into winter and spring which refers to the season in which the crop is sown. Approximately 98% of wheat grown in the UK is winter wheat. Winter wheat is planted mostly in October but it can be drilled from September through to January/February depending on the variety. Spring varieties are sown in February to March. Both spring and winter wheat are harvested in August (AHDB, 2021; Curtis, 2002).

1.1.2 Maize

Maize is one of the most important crops worldwide. It is a major energy source in animal feed. It is the third leading staple after wheat and rice and the most produced crop in the world (OECD/FAO, 2021). An average global production of over 1 billion t of maize has been reported for the seven-year period (2013-2019) (FAOSTAT). In the EU in 2019, the area under maize cultivation was approximately 9 million ha producing 70 million t (FAOSTAT). In the UK, maize is planted over 197,000 ha and is predominantly grown as forage maize for livestock (Anonymous, 2018). Maize is generally grown in rotation with wheat or barley in a two-year cycle. However, a variety of rotations including crops such as wheat, maize, barley, beets, oilseed rape, potato, sunflower, lucerne and temporary grassland is also practised in Europe (Rudelsheim and Smets, 2011).

1.1.3 Fungal diseases of wheat

Fungal diseases of wheat can result in 15 to 20% of yield losses per year (Figueroa *et al*., 2018). The major wheat diseases are take-all, eyespot, septoria leaf blotch, rusts, powdery mildew and head blight.

Wheat rusts are globally distributed and caused by species of *Puccinia*. There are three diseases of rust, leaf or brown rust, stem rust and stripe or yellow rust caused by *P. triticina*, *P. graminis* f. sp. *tritici* and *P. striiformis* var. *striiformis* respectively. These diseases are characterised by the presence of pustules on plant parts. Rusts cause losses due to reduction in grain size and weight. Stem rust can result in additional losses by causing plants to lodge (Salgado *et al*., 2016). According to an estimate, 5.47 million t wheat are lost due to stripe rust annually (Beddow *et al*., 2015). All three wheat rusts together contribute global losses of up to 5 billion US$ per year (P. Pardey, University of Minnesota, unpublished). Although stem rust is rare in the UK (AHDB, 2019a), epidemics of stem rust were observed in 2016 in Europe after more than five decades (EC, 2019b). These epidemics and the stripe rust epidemics in the recent

years raised serious concerns which led to the launch of the EU Horizon 2020-RustWatch project to manage wheat rusts.

Blotches are another important group of wheat disease. These include Septoria tritici blotch, Septoria nodorum blotch and tan spot of which Septoria tritici blotch is by far the most important disease. These diseases often occur simultaneously in wheat. They cause significant damage due to yield and quality losses. The pathogens of these diseases survive on stubbles and volunteer wheat plants. These diseases are characterised by the formation of necrotic and chlorotic spots on leaves. Septoria nodorum blotch affects leaves and glumes, whereas Septoria tritici blotch and tan spot mainly affect leaves (Figueroa *et al*., 2018). Septoria tritici blotch is caused by *Zymoseptoria tritici*. The estimated losses from Septoria tritici blotch in the EU ranges from 280-1200 million euros per annum (Fones and Gurr, 2015). In the UK, up to 50% yield losses are reported (AHDB, 2019b). Septoria tritici leaf bloch is mainly managed by succinate dehydrogenase inhibitors (SDHI) and demethylation inhibitors (DMI) fungicides (Torriani *et al*., 2015). The causal agent of Septoria nodorum blotch is *Parastagonospora nodorum*. In this disease, leaf blotch and glume blotch may or may not occur simultaneously. In case of glume blotch, brown spots appear on glumes (Salgado and Paul, 2016). Tan spot, caused by *Pyrenophora tritici-repentis*, is rare in the UK but a major disease in France, Germany, Denmark and Sweden (AHDB, 2019b).

Powdery mildew, another foliar disease of wheat, is caused by *Blumeria graminis*. This disease is globally distributed but is particularly important in Europe, China and the Southern Cone of South America (Dubin and Duveiller, 2011). Powdery mildew is characterised by greyish white powdery growth, mainly, on leaves but stems and ears may also be covered with fluffy mycelium growth in severe cases. Although the visual appearance of the disease being more prominent, this does not necessarily reflect the damage potential in the crop. In the UK, reduction in yield is usually less than 10% (AHDB, 2016), however, yield losses as high as 60% and 40% were seen in Brazil and China, respectively (Mehta, 2014). Avoiding excessive nitrogen fertilisation is recommended to manage the diseases. In addition to resistant varieties, chemical control is also found to be useful (AHDB, 2016).

Take-all, caused by *Gaeumannomyces graminis*, is the most important root disease of wheat worldwide. The pathogen causes root rot, stunting of the plant and bleached heads resulting from blockage of water and nutrient uptake. The disease is effectively managed by crop rotation (Cook, 2003). Eyespot caused by *Oculimacula yallundae* and *O. acuformis*, is an important stem base disease of wheat. It causes whiteheads, lodging and reduction in water and nutrient uptake. It can result in 10-30% yield loss. Eyepost infection can be severe if the plants are also infected with take-all (AHDB, 2019b).

1.1.4 Fungal diseases of maize

Maize is attacked by several fungal diseases. Some of the important diseases include, northern leaf blight, ear rot and eyespot.

Ear rots mainly caused by *Fusarium verticillioides* and *F. graminearum* are among the most important diseases of maize. They cause yield losses, quality reduction and mycotoxin contamination of maize grains. Fumonisins, produced primarily by *F. verticillioides* and other *Fusarium* species (Rheeder *et al*., 2002), result not only in significant economic losses but also potential health risks to humans and livestock (Wu, 2004; Wu, 2007). According to a recent evaluation (JECFA, 2017), fumonisin B1 - a possible human carcinogen (IARC, 2002), was found to have the highest occurrence and concentrations in maize among cereals and cereal-based products. Data from the US and Ontario, Canada indicate that ear rot by *F. graminearum* results in yield reduction of more than 1 million t per annum (Mueller *et al*., 2016). In addition to ear rot, *F. verticillioides* and *F. graminearum* also cause stalk rot in maize. Stalk rot typically decreases the maize yield by 10%, however reductions of 30-50% have been recorded in severe cases (Li *et al*., 2010). Losses associated with stalk rot are due to lodging, stalk breakage and disturbance of the grain filling process (Kim *et al*., 2018).

Turcicum leaf blight is one of the most serious foliar disease in the maize-growing regions of the world. The causal agent, *Exserohilum turcicum*, produces cigar-shaped necrotic lesions on leaves (Hooda *et al*., 2017). In Germany, the disease caused 10-30% yield losses in 2002 (Romero, 2016). In 2014 and 2015, turcicum leaf blight was the most damaging of all maize diseases (including non-fungal diseases) in terms of yield loss in the US and Ontario, with approximately 23 million t of loss reported for the two-year period (Mueller *et al*., 2016).

Aureobasidium zeae, the causal agent of maize eyespot, causes spotting of leaves and defoliation in severe cases. Usually, damage by eyespot is minimal but it can reduce yield by 25% to 80% depending on timing and severity of infection (Anonymous, 2014). In Denmark, up to 30% maize yield loss from eyespot was observed in 2011 (Romero, 2016). In the UK, maize eyespot has become an important disease over the last decade (Anonymous, 2014). Eyespot resistance ratings for maize varieties were first included on the Forage Maize descriptive list in 2015. Maize eyespot can be more prevalent in the cool and wet regions in west and south of the UK (Matthews, 2016). Other fungal diseases of maize include common smut, rust, anthracnose, grey leaf spot, and *Aspergillus* ear rot caused by *Ustilago maydis, Puccinia sorghi, Colletotrichum graminicola, Cercospora zeae maydis* and *Aspergillus* species, respectively (Subedi, 2015; Thompson and Raizada, 2018).

1.2 *Fusarium graminearum*

The *Fusarium* genus contains several globally important pathogens with many infecting wheat and maize. Fusarium head blight is mainly caused by species belonging to *Fusarium graminearum* species complex and related species, such as *F. poae*, *F. culmorum* and *F. avenaceum* (Parry *et al*., 1995). Moreover, *F. graminearum* is the most important causal agent of head blight in wheat (McMullen *et al*., 2012; Munkvold, 2003). Symptoms of the disease include bleached spikes or spikelets, sterile florets, discoloured and shrivelled grains. Reduction in wheat yield is caused due to shrivelled and reduced number of kernels (McMullen *et al*., 1997). In addition, *F. graminearum* also produces mycotoxins in these cereals, which is one of the most serious problems

concerning human and animal health (CAST, 2003; Shephard, 2008). Fusarium head blight can result in yield losses as high as 50% in cereals (Gilchrist and Dubin, 2002; Mielniczuk and Skwaryło-Bednarz, 2020), although >70% yield losses were recorded in Argentina in 2012 (Palazzini *et al*., 2015). In the US, Fusarium head blight resulted in yield losses worth $1.176 billion between 2015-2016 (Wilson *et al*., 2018).

There are different factors that are important for the establishment and development of Fusarium head blight such as humid weather conditions during anthesis and previous crop residues (Blandino *et al*., 2010). Density of residues from previous crops directly affects severity of Fusarium head blight and mycotoxin contamination. Blandino *et al*. (2010) investigated the effect of the amount of previous crop debris on Fusarium head blight and DON contamination in wheat after maize as the previous crop. Fusarium head blight severity and DON concentrations were found to be significantly increased with the amount of the residues left by the previous crop. In one of their field experiments, when using maize residues at 0.2, 1.8, 6.1, 10.4 t ha^{-1}, Fusarium head blight severity was recorded at 9%, 13%, 16% and 22% respectively. In the same experiment, a similar trend was also seen for DON contamination; concentrations increasing from 83 µg kg^{-1} at the lowest maize residue quantity to 3536 µg kg^{-1} at the highest residue amount. Maize as a preceding crop is known to be an important risk factor for Fusarium head blight in the subsequent wheat (Champeil *et al*., 2004) because maize leaves large quantities of residues that serve as substrate for *F. graminearum* (Leplat *et al*., 2012). When wheat was grown following maize, Fusarium head blight incidence was 15%, whereas when wheat was grown after oats or alfalfa, 4% incidence was reported (Holbert *et al*., 1919).

1.2.1 Life cycle

Fusarium graminearum survives as mycelium on crop residues and produces ascospores (sexual) and conidia (asexual). These spores and hyphal fragments, air-dispersed and water-splashed, serve as source of inoculum in the subsequent cereal crop (Shah *et al*., 2018; Yuen and Schoneweis, 2007). Bluish-black perithecia are produced from mycelium on crop debris. Later ascospores mature in the perithecia and

are forcibly discharged in the air. Favourable time for perithecia development is during the spring when the conditions are warm and humid (Parry *et al*., 1995). Perithecia and ascospore production also requires light (Tschanz *et al*., 1976). Inch and Gilbert (2003) investigated the survival of *F. graminearum* in damaged kernels on the soil surface or buried in the soil for two years. It was found that perithecia were produced on grains buried at the two depths, 5 and 10 cm, however, perithecia produced ascospores only at soil surface and no ascospores were formed at lower depth. For perithecia production, favourable range of temperature lies between 16 and 31°C, while ascospore production occurs between 13 and 33°C with 25-28°C being the optimal temperature (Keller *et al*., 2014). Conidia are produced within sporodochia and dispersed by water splashes typically at short distances within the canopy (Trail, 2009). Warm and moist conditions are also important for conidia production. Temperature range of 16-36°C was found to be suitable for production of *F. graminearum* conidia and the optimum temperature was 32°C (Sutton, 1982). *Fusarium graminearum* conidia germinate within 6-12 hours after inoculation on glume and hyphae become visible by 12-24 hours post-inoculation (Pritsch *et al.*, 2000). Conidia are said to be more important for causing Fusarium head blight than ascospores (Markell and Francl, 2003). In one study, same concentrations of ascospore and conidia were applied to wheat spikes; fewer colony forming units (cfu) were recovered after ascospore inoculation than conidial inoculation indicating conidia play greater role in the disease (Markell and Francl, 2003). Similarly, in another study conidia were found to be more effective in causing Fusarium head blight than ascospores, however, the difference in the disease severity between these two types of spores was minor (Mitter *et al*., 2006).

Fusarium graminearum could be classified as hemibiotroph, with the caveat that it exhibits a unique relationship with its host, which is different from other hemibiotrophic pathogens (Kazan *et al*., 2012; Shah *et al*., 2018). Hemibiotrophs usually have a short biotrophic phase initially, during which they interact intracellularly with host cells (Münch *et al*., 2008). Whereas *F. graminearum* hyphae initially grows intercellularly and display prolonged interaction with live wheat cells (Brown *et al*., 2017).

Fusarium graminearum infects wheat from flowering till the soft dough stage. However, wheat heads are most susceptible to infection at anthesis which is usually the time when perithecia are releasing ascospores (Parry *et al*., 1995). The disease is initiated

when the spores germinate on wheat spikelets. Fungal hyphae initially grow in intercellular spaces of floral tissues asymptomatically, characterising the biotrophic phase of *F. graminearum* in wheat. Once the fungus is established within the floret, hyphae penetrate and rapidly proliferate intracellularly and this is accompanied by the development of necrosis, due to cell wall degrading enzymes. This necrotrophic stage is associated with the development of Fusarium head blight symptoms i.e., bleached heads, necrosis, shrivelled kernels. Typically, the first symptoms can be noticed as premature bleaching of spikelets whilst healthy heads remain green. As *F. graminearum* hyphae grow into the rachis, spikelets below or above the point of infection become bleached (Figure 1.1; Trail, 2009). Evidence suggests that *F. graminearum* penetrates host tissues through foot-like structures and compound appressoria which are multicellular types of appressoria formed from irregular hyphae (Boenisch and Schäfer, 2011). The duration between complete *F. graminearum* colonisation of wheat head till bleaching can take up to 10 to 14 days (Figueroa *et al*., 2018). The kernels that are infected at later stages of grain development may look healthy but can be contaminated with mycotoxins (CAST, 2003).

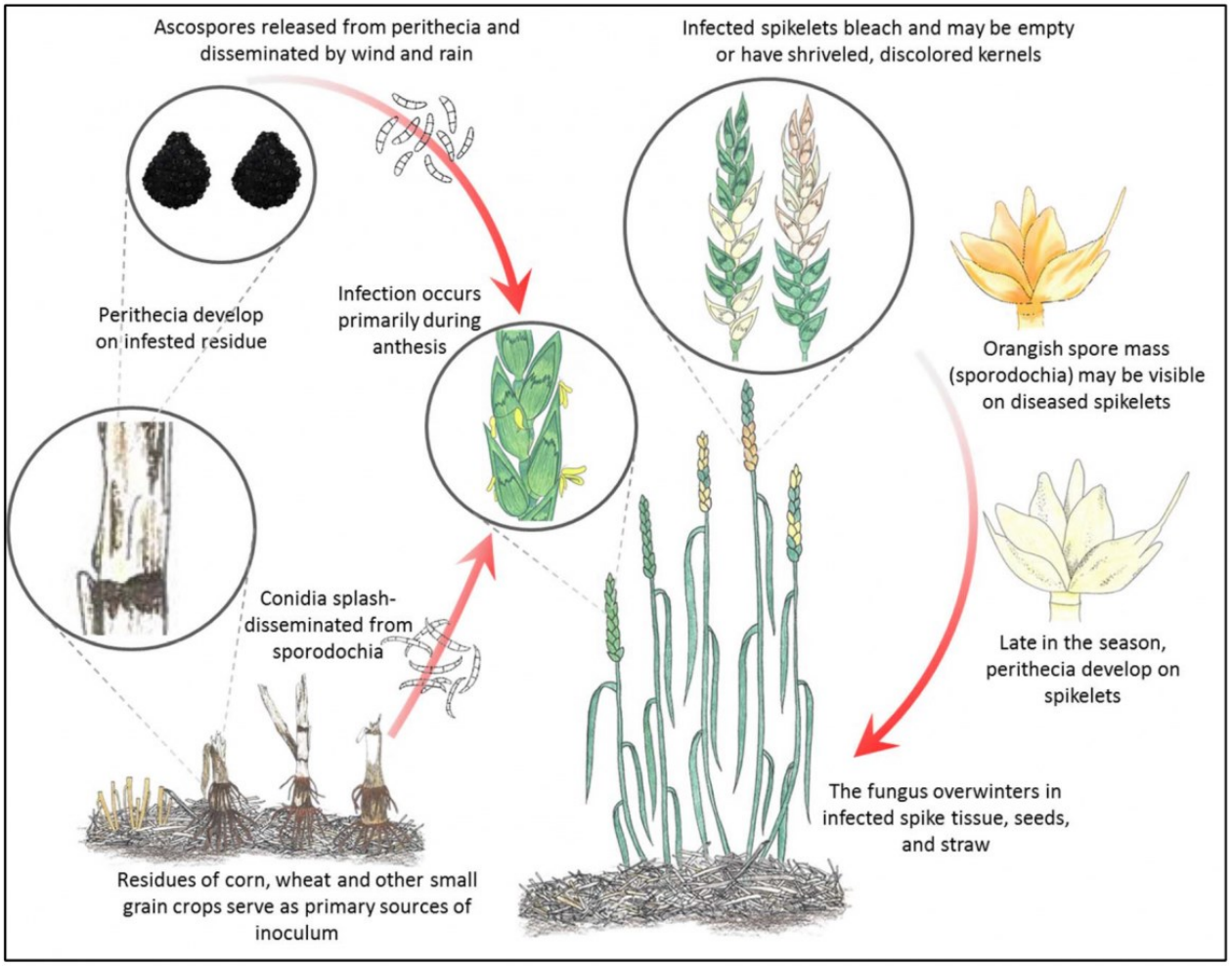


Figure 1.1 Life cycle of *Fusarium graminearum* in wheat (Source: Mills *et al*., 2016)

1.3 Mycotoxins

Mycotoxins are toxic secondary metabolites produced by filamentous fungi. The term "mycotoxin" refers to a poisonous substance produced by fungi. Although all mycotoxins are metabolites of fungi, it should be kept in mind that not every toxic substance synthesised by fungi is termed mycotoxin (Bennett and Klich, 2003). Mycotoxins can be defined as "fungal metabolites which when ingested, inhaled or absorbed through the skin, cause lowered performance, sickness or death in man or animals, including bird" (Pitt, 1996). Mycotoxins are often genotypically specific, but can be formed by one or more fungal species and in some cases one species can produce more than one mycotoxin (Thrane, 1989).

Mycotoxin occurrence in plant-derived foods, feeds and other commodities cause considerable losses related to human health, livestock and poultry productivity (Hussein and Brasel, 2001). Humans are exposed to mycotoxins mainly by consumption of mycotoxin-contaminated products which can lead to health problems. When ingested in high amounts, mycotoxins can cause death whereas long-term, low-level exposure to small quantities of mycotoxins can cause chronic disease symptoms. Mycotoxins can cause a variety of toxic effects; they can be carcinogenic, neurotoxic, teratogenic and immunotoxic. Some mycotoxins are reported to cause autoimmune illnesses, hepatic carcinoma, nephrotoxicity and gastrointestinal disorders (CAST, 2003; Hussein and Brasel, 2001).

Many fungal genera such as *Aspergillus*, *Penicillium* and *Fusarium* are known to synthesise mycotoxins. Aflatoxins are one of the highly toxic group of mycotoxins, mainly produced by *A. parasiticus* and *A. flavus* (Bennett and Klich, 2003).

1.3.1 Fusarium mycotoxins

The *Fusarium* genus is a producer of the largest and most diverse group of mycotoxins which occur abundantly in cereals. Normally, crops are contaminated with Fusarium mycotoxins at the pre-harvest stage but contamination can occur at any point i.e. during processing, storage and transport. It should be noted that different Fusarium

mycotoxins can be synthesised simultaneously by one *Fusarium* species and various *Fusarium* species can produce the same mycotoxin (Kotowicz *et al*., 2014). Fusarium mycotoxins comprise around 140 fungal metabolites out of which fumonisins, zearalenone (ZON) and trichothecenes are the major ones (Sobrova *et al*., 2010; Yazar and Omurtag, 2008).

Four types of trichothecenes viz. A, B, C and D are produced by *Fusarium* species and other fungi, and among all the mycotoxins, they are chemically the most diverse group. *Fusarium* species are known to produce type A and B trichothecenes which are the most important members among the four types. The mycotoxins included in type A trichothecenes are T-2 toxin, HT-2 toxin, diacetoxyscirpenol, monoacetoxyscirpenol and neosolaniol while type B includes deoxynivalenol (DON) and its acetyl derivatives, nivalenol and fusarenon X. Type A trichothecenes are more toxic than the type B members (Krska *et al*., 2007; McCormick *et al*., 2011). Trichothecenes are produced by many species of *Fusarium* including *F. acuminatum* and *F equiseti*, however, *F. sporotrichioides* and *F. poae* are mainly type A producers and *F. graminearum* and *F. culmorum* are known to be type B producers (EFSA 2011; Placinta *et al*., 1999). Synthesis of T-2 and HT-2 toxins principally occurs in *F. langsethiae* but *F. sporotrichioides* and *F. poae* may also be possible producers of these toxins (Nesic *et al*., 2014). Trichothecenes are immunotoxic, neurotoxic and genotoxic. Other toxic effects exhibited by trichothecenes include inhibition of protein synthesis, apoptosis and gastroenteritis (Awad *et al*., 2014; Hussein and Brasel, 2001; Rocha *et al*., 2005).

Fumonisins are commonly produced by *F. proliferatum* and *F. verticillioides* (Rheeder *et al*., 2002). Of the different types of fumonisins identified, the B series is the most common and important one. Fumonisin B_1 is the most toxic and prevalent of the fumonisins and has been categorised in Group 2B as possibly carcinogenic to humans (IARC, 1993). Animal studies have shown that fumonisins exhibit hepatotoxicity and nephrotoxicity (Mathur *et al*., 2001) and may potentially induce immunotoxic effects in humans (Stockmann-Juvala *et al*., 2008). Moniliformin, another Fusarium mycotoxin, is principally produced by *F. proliferatum* but *F. moniliforme* also synthesises moniliformin in addition to fumonisins and fusarin C (Norred *et al*., 1992; Placinta *et al*., 1999). It is immunosuppressive (Li *et al*., 2000), cardiotoxic (Nagaraj *et al*., 1996) and inhibits enzymes including pyruvate dehydrogenase (Gathercole *et al*., 1986), glutathione

peroxidase and glutathione reductase (Chen *et al*., 1990). Other less studied Fusarium mycotoxins include fusaproliferin, gliotoxin and butenolite (Escriva *et al*., 2015; Stanciu *et al*., 2015). *Fusarium equiseti*, in addition to ZON and trichothecenes, produces fusarochromanones (D'Mello *et al*., 1997; Flannigan, 1991).

1.3.2 *Fusarium graminearum* mycotoxins

Fusarium graminearum is known to produce DON, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, nivalenol, fusarenone X and ZON but the most problematic and notable are DON and ZON (D'Mello *et al*., 1999; Kotowicz *et al*., 2014).

1.3.2.1 Deoxynivalenol

Deoxynivalenol is a member of the trichothecene B class of mycotoxins and is important economically due to its abundance in cereals and their products. It is mainly produced by *F. graminearum* but *F. culmorum* is also a known DON producer. The most important environmental factors affecting DON production are temperature and water availability. Optimum conditions for DON production were recorded by Ramirez *et al*. (2006) at 0.995 *a*w and 30°C after 6 weeks. Deoxynivalenol synthesis by *F. graminearum* is also dependent on the pH of the environment as an acidic pH is crucial for DON production (Merhej *et al*., 2010). Gardiner *et al*. (2009) reported that extracellular pH required for maximum DON induction in *F. graminearum* is between 2.4 and 3.1. The chemical structure of DON is presented in Figure 1.2.

Deoxynivalenol is also known as vomitoxin due to its vomit-inducing effect in human and animals after ingestion of DON-contaminated food and feed. Other toxic effects of DON include headache, gastrointestinal inflammation, food refusal, weight loss and reduced immunity. Additionally, DON is an inhibitor of protein, DNA and RNA synthesis and also causes apoptosis (Pestka, 2010; Sobrova *et al*., 2010). Moreover, teratogenic (Zhao *et al*., 2012), genotoxic and cytotoxic (Awad *et al*., 2014) effects of DON were reported in animals. However, the International Agency for Research on Cancer (IARC)

has categorised DON in Group 3 as "not classifiable as to its carcinogenicity to humans" (IARC, 1993). Outbreaks associated with consumption of DON-contaminated cereals have been reported in China (Luo, 1988) and India (Bhat *et al*., 1989).

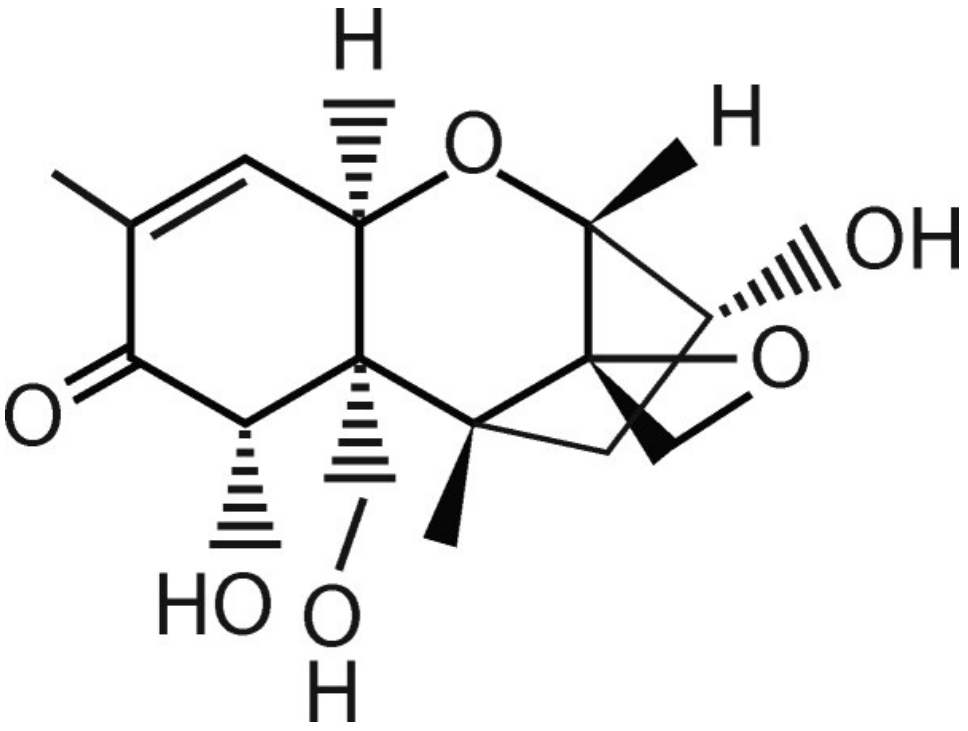


Figure 1.2 Chemical structure of deoxynivalenol (Source: Sobrova *et al*., 2010)

Deoxynivalenol acts a virulence factor. It enables the spread of the fungus in infected plant tissue. The production of DON becomes apparent in the necrotrophic stage when it enables the fungus to spread in the rachis of wheat (Bai *et al*., 2002; Desjardins *et al*., 1996). *In vitro* studies have shown H_2O_2 to be an efficient inducer of DON production

(Audenaert *et al*., 2010; Ponts *et al*., 2007). Hence it seems that reactive oxygen species produced by the host plant as a defense response to infection, are used by *F. graminearum* for its own advantage (Ponts, 2015). Deoxynivalenol has been proved to repress the chitinase activity in the soil-residing *Trichoderma atroviride*, suggesting this mycotoxin might help in competing with antagonists and defending against mycoparasites during the saprophytic phase of *F. graminearum* (Lutz *et al*., 2003). However, DON gives no advantage to *F. graminearum* in saprophytic survival on crop residues (Abid, 2012).

Deoxynivalenol has a high melting point i.e. 151°C to 153°C and due to its heat-stability, DON concentrations are not reduced after cooking. However, some studies have shown that boiling can decrease DON levels in noodles and pasta due to the fact that DON is soluble in water (Kushiro *et al*., 2012; Visconti *et al*., 2004). Despite legislation for safe limits of DON in most parts of the world, the availability of fungicides and development of resistant varieties, DON continues to be a significant concern from economic as well as health perspective (Karlovsky, 2011).

1.3.2.2 Zearalenone

In addition to *F. graminearum*, ZON is also produced by *F. culmorum, F. equiseti, F. semitectum, F. cerealis* and *F. crookwellense* (Bennett and Klich, 2003). Zearalenone, an estrogenic mycotoxin, is one of the most widely distributed Fusarium mycotoxin in agricultural commodities. Zearalenone production mainly occurs pre-harvest, but the toxin may also be produced after harvesting if the crops are not handled properly. It mainly occurs in maize, however, other commodities like wheat, barley, rice, sorghum and oats are also contaminated with ZON worldwide. Zearalenone is also found as a contaminant in cereal products like soybeans, malt, flour and beer (CCFAC, 2000). In addition, ZON derivatives including α-zearalenol, β-zearalenol, α-zearalanol, β-zearalanol and zearalanone can also occur in crops (Bottalico *et al*., 1985; Richardson *et al*., 1985). Low concentrations of α-zearalenol and β-zearalenol were detected in maize by-products, maize silage and soya meal (Schollenberger *et al*., 2006).

Zearalenone is rapidly absorbed from the gastrointestinal tract and metabolised to α and β isomers in mammalian tissues (Zinedine *et al*., 2007). Zearalenone and its

derivatives have binding affinity for estrogen receptors therefore, also called as mycoestrogens and considered as endocrine disruptor chemicals (Frizzell *et al*., 2011).

Zearalenone is responsible for mycotoxicoses in livestock and poultry (Kuiper-Goodman *et al*., 1987). Cases of scabby grain toxicoses linked to both ZON and DON are also reported (Bilgrami and Choudhary, 1998). Being a mycoestrogen, ZON has adverse effects on reproductive system and is responsible for early onset of puberty in young children (Szuets *et al*., 1997; Zhao *et al*., 2013). Uterus enlargement, ovarian dysfunction, infertility and increased production of abnormal spermatozoa may also be caused by ZON (Yang *et al*., 2007; Zatecka *et al*., 2014). In addition, ZON also exhibits immunotoxicity (Hueza *et al*., 2014), hepatotoxicity (Stadnik and Borzecki 2009), hematotoxicity (Maaroufi *et al*., 1996) and genotoxicity (Gao *et al*., 2013). Moreover, studies have shown the potential role of ZON and its derivatives in development of human breast cancer (Belhassen *et al*., 2015; Khosrokhavar *et al*., 2009). The chemical structure of ZON is presented in Figure 1.3.

Despite the worldwide recognition of mycotoxin occurrence and importance, function of many mycotoxins, including ZON, in fungi is not fully understood. Utermark and Karlovsky (2007) investigated the ZON-hydrolysing activity of lactonase produced by *Gliocladium roseum*. It was reported that the growth of filamentous fungi such as *Alternaria alternata* and *Cladosporium herbarum* was inhibited in ZON-amended agar media, suggesting that this mycotoxin helps ZON-producers in fungal competition and may have a role in resisting mycoparasitic attack. The finding was further strengthened by the fact that the inactivation of ZON-specific lactonase gene in *G. roseum* negatively affected the growth of the mutant in media containing ZON whereas the wild type remained unaltered. A previous study suggested that ZON has a role in perithecia development (Mirocha and Swanson, 1983). In contrast, findings from a recent study investigating the role of polyketide synthase genes in perithecial development in *F. graminearum*, suggest that ZON may not have a role in perithecia formation (Kim *et al*., 2021).

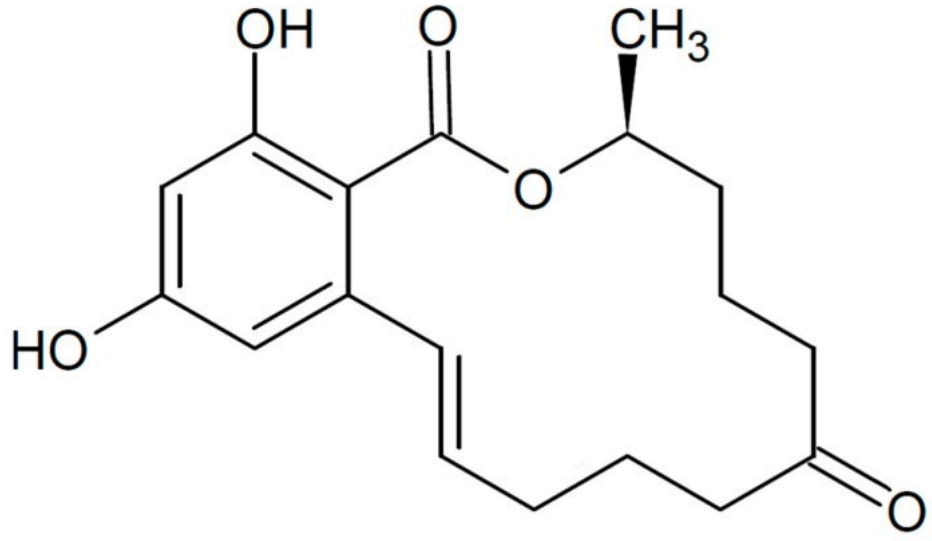


Figure 1.3 Chemical structure of zearalenone (Source: Ropejko and Twaruzek, 2021)

1.3.3 Occurrence of DON and ZON in cereals

For consumer protection, the EU has set legislative limits for ZON at 100 µg kg^{-1} and for DON at 1250 µg kg^{-1} in wheat. In maize the limits are set at 350 µg kg^{-1} and 1750 µg kg^{-1} for ZON and DON respectively (EC, 2006). Since that time there have been several harvests across Europe when high numbers of crops of either wheat or maize have exceeded these legal limits resulting in large costs to the cereal industry. For instance, contamination with concentrations exceeding the EU legal limits of ZON and DON were detected in 29% and 13% wheat samples respectively in England in 2008 (Edwards and Jennings, 2018). Maize samples from Croatia in 2010 were found to be contaminated with these mycotoxins at levels above the legal limits; 50% samples with

DON and 28% samples with ZON (Pleadin *et al*., 2012). Moreover, 83% durum wheat samples from Tunisia in 2007 (Bensassi *et al*., 2010) and 12.5% maize samples from Serbia in 2011 (Jakšić *et al*., 2012) were found contaminated with DON at concentrations higher than the legal limits. Pallez-Barthel *et al*. (2021) reported results from a recent survey conducted in Luxembourg, revealing occurrence of DON at levels above the legal limits in 5% wheat samples collected during a 12-year period (2007-2018). In the same study, 15% of wheat samples from 2018 contained DON levels above legal limits.

An FAO estimate of 25% food contaminated with mycotoxins around the globe is widely cited in the literature. In an analysis, data on occurrence of six types of mycotoxins - total aflatoxins, ochratoxin A, sum of fumonisin B1 and B2, sum of T-2 and HT-2, DON and ZON - in grains and nuts for human consumption and animal feed were reviewed. The data was obtained from European Food Safety Authority and global survey from Biomin Inc., Austria for the period 2010-2015. The results suggest that the “25% estimate” may represent only those samples contaminated with levels exceeding the regulatory limits. However, the prevalence of the detected mycotoxins could be much higher up to 60-80% compared to the widely cited “25%” (Eskola *et al*., 2020).

1.4 Management of *Fusarium graminearum* in cereals

Fusarium head blight can be managed by integrating a variety of cultural, biological and chemical control methods. Management strategies for Fusarium head blight in wheat have recently been reviewed by Shah *et al*. (2018).

1.4.1 Cultural control

Cultural techniques such as crop rotation, weed control and application of appropriate fertilisers, can be used to manage Fusarium head blight. *Fusarium graminearum* survives as mycelium on crop residues and produces sexual spores, ascospores, and

asexual spores, conidia. These spores and hyphal fragments serve as source of inoculum in the subsequent cereal crop (Shah *et al*., 2018; Yuen and Schoneweis, 2007). Therefore, removing or burying cereal crop residues reduces Fusarium head blight inoculum. For instance, in a three year study (Miller *et al*., 1998), 79%, 55% and 46% of kernels were infected with *F. graminearum* in first, second and third year respectively in no till plots, whereas in tilled plots 20%, 40% and 13% kernels were infected. In one survey, when wheat was grown following maize, 15% of the crops were infected with Fusarium head blight. In contrast, only 4% wheat crops were infected when wheat was grown following oats or alfalfa (Holbert *et al*., 1919). Deoxynivalenol concentrations in wheat grains in soybean-wheat rotations were shown to be 25% and 50% lower in wheat-wheat rotations and maize-wheat rotations respectively (Dill-Macky and Jones, 2000).

Weed control may also reduce Fusarium head blight inoculum in field. Field surveys in southern Ontario showed that fields with noticeable amounts of weeds had twice as much Fusarium head blight symptoms compared to the fields without weeds (Teich and Nelson, 1984). The potential significance of weeds in Fusarium head blight disease cycle has also been demonstrated by Suproniene *et al*. (2019), who isolated *F. graminearum* isolates from weeds sampled from fields with cereal crop rotations in Lithuania. *Fusarium graminearum* was isolated from 72% of the 57 weed species (mainly broad-leaved species) that were investigated and these isolates showed Fusarium head blight symptoms when artificially inoculated in wheat in the field. A range of weeds, such as *Fallopia convolvulus*, *Echinochloa crus-galli, Chenopodium album, Amaranthus retroflexus*, *Poa annua, Taraxacum officinale* and *Sonchus arvensis* act as alternative hosts of *F. graminearum* (Postic *et al*., 2012; Suproniene *et al*., 2019).

These cultural control methods play an important role in reducing *F. graminearum* inoculum, however due to the ubiquitous nature of *F. graminearum*, the efficiency of such strategies is limited (Pirgozliev *et al*., 2003).

1.4.2 Biological control

Organisms such as bacteria and fungi are known to have the ability to suppress *Fusarium* spp. and reduce mycotoxin production in cereals. Several bacterial biocontrol agents are reported to have antagonistic effects against *Fusarium* spp. causing head blight, for example, *Lysobacter enzymogenes* (Jochum *et al*., 2006), *Bacillus* spp. (Schisler *et al*., 2002; Zhao *et al*., 2014) and *B. amyloliquefaciens* FLN13 and *Lactobacillus plantarum* SLG17 (Baffoni *et al*., 2015). *Bacillus velezensis* LM2303 when sprayed as bacterial culture broth at anthesis, was shown to reduce disease severity of Fusarium head blight by 72% under field conditions (Chen *et al*., 2018).

Fungal species that are known to be antagonists to *Fusarium* spp. include *Cryptococcus* spp. (Schisler *et al*., 2011), *Aureobasidium pullulans* (Wachowska and Głowacka, 2014), *Clonostachys rosea* (Xue *et al*., 2014) and *Trichoderma* spp. (Matarese *et al*., 2012). Co-culture assays with fungal antagonists showed that *Trichoderma* species including *T. koningiopsis*, *T. gamsii* and *T. viride*, significantly reduced *F. graminearum* mycelial growth by 45-93%; *Clonostachys rosea* and *Cladosporium cladosporioides*, however, did not result in significant suppression (Schoneberg *et al*., 2015). In this study when the antagonists were applied to wheat straw before pathogen inoculation, 88-100% reduction in number of perithecia and ascospore was observed. However, when antagonists were applied after pathogen inoculation of wheat straw, only *Clonostachys rosea* reduced perithecia and ascospore production by 73-100%.

Studies exploring the antagonistic and parasitic potential of microorganisms against *F. graminearum* under controlled environment have shown promising results. However, these biocontrol agents are not always as effective under field conditions. Moreover, the development of commercial biocontrol products from some potential microorganism could face regulatory obstacle. For example, the potential use of *Aureobasidium pullulans* (Wachowska and Głowacka, 2014) and *Cryptococcus flavescens* (Schisler *et al*., 2011) as biocontrol agents may raise safety concerns due to their disease causing ability in humans (Mittal *et al*., 2012) and animals (Kano *et al*., 2012) respectively.

1.4.3 Varietal control

Literature on breeding improvements in wheat for Fusarium head blight resistant cultivars has been previously reviewed (Hollins *et al*., 2003; Shah *et al*., 2017; Zhu *et al*., 2019).

Resistance mechanism of Fusarium head blight has been mainly categorised as resistance to initial infection (Type I), resistance to fungal spread within ear (Type II) (Schroeder and Christensen, 1963) and low DON accumulation (Type III) (Miller *et al*., 1985). Additional two types of resistance were also introduced: resistance to kernel infection, and tolerance (Mesterhazy, 1995). However, only Type I, II and III are widely accepted types of resistance (Bai *et al*., 2018). Type I and Type II resistance are widely exploited and Type III has gained importance due to its effect on grain end use quality. These resistance types are at least to some extent correlated. The most important Fusarium head blight resistance genes are *Fhb1, Fhb2* conferring Type II resistance and *Fhb5* conferring Type I and some Type II resistance (Brar *et al*., 2019).

Currently, a durable and completely resistant cultivar towards Fusarium head blight is not available (Shah *et al*., 2018). Wheat cultivars with partial resistance are however available. Some of the germplasm lines of Fusarium head blight-resistant wheat are Renan (Gervais *et al*., 2003), W14 (Chen *et al*., 2006), Goldfield (Gilsinger *et al*., 2005) and Arina (Paillard *et al*., 2004). Spring cultivars, such as, Arabella, Bombona, Hewilla, Izera, Kandela, Katoda, KWS Torridon, Łagwa, Monsun, Nawra, Ostka Struna, Parabola, SMH 87, Smolicka Radocha, Trappe, Tybalt and Waluta, have shown moderate resistance to Fusarium head blight (Lenc *et al*., 2015). Two Triticum-Secale-Thinopyrum hexaploid lines that have genes to multiple diseases- Fusarium head blight, leaf rust, stem rust race Ug99, have been developed recently (Dai *et al*., 2017). In the UK, wheat varieties such as Skyfall and LG Detroit show high resistance to Fusarium head blight (AHDB, 2021b).

1.4.4 Chemical control

Among chemical fungicides, triazoles are the most effective to manage *F. graminearum* in cereals (Edwards *et al*., 2001). Triazoles are a class of the DMI that inhibits sterol

biosynthesis, ultimately affecting membrane integrity. The most effective fungicides used to suppress Fusarium head blight in wheat are reported to be the triazoles tebuconazole, metconazole and prothioconazole (Paul *et al*., 2008; Pirgozliev *et al*., 2002). Triazole fungicides are also known to be effective against *F. graminearum* infection and mycotoxin contamination (particularly DON) in other cereals (Scarpino *et al*., 2015). D'Angelo *et al*. (2014) reported that tebuconazole and prothioconazole in combination and metconazole are the most effective fungicides when applied within one week after flowering.

Masiello *et al*. (2019) recently compared different fungicide groups at different concentrations, based on recommended doses, under *in vitro* conditions against *F. graminearum*. At the highest tested concentration, the inhibition values for mycelial growth and conidial germination were 77-100% for DMI fungicides (including triazoles) and 100% for the methyl-2-benzimidazole carbamate (MBC) thiophanate-methyl; among SDHI fungicides, boscalid was ineffective whereas isopyrazam caused 93-100% inhibition. Fludioxonil inhibited mycelial growth and conidial germination by 54-94% and 12% respectively whereas folpet completely inhibited conidial germination and reduced mycelial growth by 55% at the highest tested concentrations. In field experiments on maize (also Masiello *et al*., 2019), prothioconazole and thiophanate-methyl reduced *F. graminearum* in kernels by 52% and 48% respectively when applied at the flowering stage. Based on assessment of infection symptoms on maize, prothioconazole also gave the lowest infection severity (13.2%) compared to thiophanate-methyl (33%) and untreated plots (45.1%).

The rising concerns due to the endocrine-disrupting properties of triazoles and their limited effectiveness and high selection pressure for fungicide resistance, managing *F. graminearum* through chemical control is becoming challenging. Although the above discussed management methods can suppress Fusarium head blight, however considering the limitations of individual method, an integrated approach would be more efficient way to manage *F. graminearum* in cereals.

1.5 Biofumigation

1.5.1 Introduction

Conventionally, soil fumigation using synthetic pesticides have been used to combat soil-borne pathogens. In the 1940s, many pesticides like 1,3-dichloropropene, chloropicrin and methyl bromide came to the market (Lembright, 1990). Since then, methyl bromide has been widely used as soil fumigant. However, the initiative taken by the US Environment Protection Agency in the 1980s to protect the ozone layer, led to the phasing out of methyl bromide (class I ozone-depleting substance) under the Montreal Protocol on Substances that Deplete the Ozone Layer (Enebak, 2007). In the EU, these substances are regulated by the Regulation (EC) 1005/2009. Glyphosate, the most widely used herbicide, has recently been targeted due to uncertain concerns over its ecotoxic, neurotoxic and mainly carcinogenic potential (Kissane and Shephard, 2017). The controversial herbicide was on the verge of being banned by the end of 2017, however, the license was renewed for 5 years instead of 10 or 15 years [Commission Regulation (EU) 2017/2324]. Member States such as, Italy and France are already in favour of phasing out glyphosate, thus, the fate of glyphosate seems to be uncertain.

The EU restricted the use of some neonicotinoids in 2013 due to their negative impact on bees. Later on three insecticides (clothianidin, thiamethoxam, imidacloprid) were banned in 2018 (EC, 2021). Two herbicides, Isoproturon and Amitrole, due to their risks to groundwater and aquatic life have been banned since 2016 (Erickson, 2016). Recently, the EU has also constricted the regulations for the use of substances exhibiting endocrine-disrupting properties. Scientific criteria to identify endocrine-disrupting properties of biocidal products [Commission Regulation (EU) 2017/2100] and plant protection products [Commission Regulation (EU) 2018/605] have been issued. Recently, an endocrine disruptor, Mancozeb fungicide has been banned in the EU in 2021 [Commission Regulation (EU) 2020/2087]. Owing to their endocrine-disrupting potential, widely used triazole fungicides are also likely to be withdrawn (Anonymous, 2019). The loss of pesticides puts additional pressure on farmers who are dependent on chemical control options. Consequently, provision of more “greener” non-chemical strategies has become the need of the hour. The European Commission (Directive

2009/128/EC) endorses the use of non-chemical methods to reduce the utilisation of synthetic pesticides and supports research to introduce new low risk plant protection products and biocontrol methods (EC, 2017). In this regard, the EU has financed projects such as, *MyToolBox*-"Safe Food and Feed through an Integrated ToolBox for Mycotoxin Management", IWMPRAISE-"Integrated Weed Management: Practical Implementation and Solutions for Europe" and TROPICSAFE-"Insect-borne prokaryote-associated diseases in tropical and subtropical perennial crops".

The restrictions and public concern over health and environmental impact of synthetic pesticides have led to the pursuit of safe replacement strategies. Alternative strategies to using agrochemicals, such as, solarisation have been used but this method is only effective in regions where solar radiation is intense enough to kill soil-borne pathogens (Lazarovits *et al*., 1991). Other alternate practices include the use of organic amendments such as animal manure, cover crops and compost, which can be effective in suppressing plant pathogens and enhancing soil fertility. Several studies have reported the efficacy of organic amendments in disease suppression (Diab *et al*., 2003; Szczech, 1999; Veeken *et al*., 2005). While exploring such eco-friendly, sustainable and food sovereignty approaches in agriculture, the interest in biofumigation has increased.

Biofumigation is the practical application of allelopathy (Justes *et al*., 2017), a phenomenon defined as "any process involving secondary metabolites produced by plants, microorganisms, viruses and fungi that influence the growth and development of agricultural and biological systems" (Einhellig, 1995). The concept of biofumigation was first introduced in the early 1990s as a biocontrol technique in agriculture exploiting glucosinolate (GSL)-rich brassica crops to suppress agricultural pests through the release of toxic isothiocyanates (ITC) as a result of the hydrolytic breakdown of GSL (Kirkegaard *et al*., 1993). Since then the term has been broadened to include control mechanisms with biopesticidal compounds through the exploitation of biological sources including animal by-products (Ghoname *et al*., 2017; Pokharel, 2012). García-Álvarez *et al*. (2004) defined biofumigation as “the action of volatile substances produced during the biodecomposition of organic matter and by soil microorganisms and the roots of some plants for soil-borne pathogen control”. Taking into account the relevant literature and research experience, biofumigation could also be defined as a control strategy using toxic volatile substances originating from biological sources to

cope with pests, weeds and pathogens, and also enhancing soil health while reducing the environmental impact associated with the conventional application of synthetic pesticides.

1.5.2 General mechanism of biofumigation

Biofumigation is a sustainable agriculture practice which generally utilises volatile biotoxic compounds released from brassica plants. These biofumigant plants, rich in GSL content, are grown between cash crops, shredded thoroughly and then incorporated into the soil (Kirkegaard *et al*., 1993). The production of GSL reaches its peak during the flowering stage and is thus the ideal time to chop the crop (Bellostas *et al*., 2004; Malik *et al*., 2010). Tissue damage results in the release of GSL and the enzyme myrosinase, which are otherwise separately present in the intact tissues. The endogenous enzyme in the presence of water catalyses the breakdown of GSL into glucose and unstable aglycones which are then converted into a variety of products such as, organic thiocyanates, epithionitriles, nitriles, and ITC (Wittstock and Halkier, 2002). Among these compounds, the focus of interest is the ITC owing to their biopesticidal properties. These volatile compounds produce similar effects to synthetic chemical fumigants. In addition to their biocidal effects on pests, biofumigants prove to be efficient also by contributing to the organic content in the soil thereby improving soil health and by allowing the soil microbial antagonists to flourish in the organic matter and suppress plant pathogens (Motisi *et al*., 2010). Moreover, the release of toxic compounds during the decomposition of organic matter contributes to suppression of pathogens and pests (Motisi *et al*., 2010). Biofumigation (including water and plastic film costs) is estimated to cost less than methyl bromide application (García-Álvarez *et al*., 2004). It also offers additional benefit for earth's health by allowing lower greenhouse gas emissions as compared to the use of synthetic pesticides (Lazzeri *et al*., 2012).

Amid scenarios of pest resistance, environmental degradation and ecological issues, biofumigation offers a promising choice in agriculture.

1.5.3 Brassicas as biofumigants

Several members of Brassicaceae family are cultivated and consumed by humans worldwide. These are rich in nutrients, minerals, health-promoting and curative products (Jahangir *et al*., 2009). Apart from this, members of the Brassicaceae family are the main choice as biofumigants due to their GSL content. However, other plant families such as the Moringaceae, Resedaceae and Tropaeolaceae are also known to have potential for biofumigation with Capparaceae exhibiting a wider range of GSL occurrence (Fahey *et al*., 2001). The Brassicaceae family comprises of about 375 genera and 3200 species (LeCoz and Ducombs, 2006). The brassica species containing high concentrations of ITC-producing GSL have potential for biofumigation and these include *Brassica juncea*, *B. rapa*, *B. carinata*, *B. nigra* (Bellostas *et al*., 2004), *B. napus* (Velasco *et al*., 2008), *B. oleracea* (Bellostas *et al*., 2007; Kushad *et al*., 1999) and *Raphanus raphanistrum* (Malik *et al*., 2010) among others (Table 1.1).

Owing to the ability of brassicas to break the life cycle of various disease-producing fungi, they are referred to as break crops. The life cycle of the pathogen is interrupted due to non-availability of a host plant. A wheat-brassica-wheat sequence can expect an average yield benefit ranging from 14 to 33% in subsequent wheat cultivation when compared to wheat-wheat (Kirkegaard *et al*., 2008). Moreover, effective suppression of the diseases take-all caused by *G. graminis* (Kirkegaard *et al*., 1994) and crown rot caused by *F. pseudograminearum* (Kirkegaard *et al*., 2004) in wheat were shown by the application of break crops.

Table 1.1 Common brassica biofumigants and their predominant glucosinolates

Brassica plant	Common name	Predominant glucosinolates	Reference
Brassica oleracea	Brussels sprouts, cabbage, kale, cauliflower	Sinigrin, glucobrassicin	Kushad *et al*., 1999
	Broccoli	Gluconapin, glucoraphanin, glucobrassicin, neoglucobrassicin	Kushad *et al*., 1999; Vallejo *et al*., 2003
B. juncea	Indian or brown mustard	Sinigrin, gluconasturtiin	Ngala *et al*., 2015a; Njoroge *et al*., 2008
B. napus	Rapeseed	Progoitrin, glucobrassicanapin, gluconapin	Velasco *et al*., 2008
B. carinata	Ethiopian mustard	Sinigrin	Ngwene *et al*., 2017
B. nigra	Black mustard	Sinigrin	Amiri-Jami *et al*., 2016
B. chinensis	Pak choi	Gluconapin	Bhandari *et al*., 2015
Raphanus sativus	Radish	Glucoraphasatin, gluconapin, gluconasturtiin, glucoraphenin	Wang *et al*., 2017; Yi *et al*., 2016
R. raphanistrum	Wild radish	Glucotropaeolin, glucoerucin, gluocoraphenin	Malik *et al*., 2010
Eruca sativa	Rocket	Glucoraphanin, glucoerucin, glucotropaeolin	Cataldi *et al*., 2007; Kim and Ishii, 2006; Rossetto *et al*., 2013
Sinapis alba	White mustard	Sinalbin	Hopkins *et al*., 1998; Popova and Morra, 2014
Nasturtium officinale	Watercress	Gluconasturtiin	Jeon *et al*., 2017; Voutsina *et al*., 2016

1.5.4 Glucosinolates

Glucosinolates are secondary metabolites occurring in Brassicaceae members including *B. juncea, E. sativa, R. sativus* and *B. carinata*. Glucosinolates are sulphur- and nitrogen-containing molecules with variable side chains derived from amino acids. About 200 different GSL have been identified (Clarke, 2010) which are categorised as aliphatic, aromatic or indole groups on the basis of the amino acid precursors (Figure 1.4). Aliphatic GSL are derived from alanine, methionine, valine, leucine or isoleucine; aromatic GSL derived from phenylalanine or tyrosine; indole GSL derived from tryptophan (Ishida *et al*., 2014; Wittstock and Halkier, 2002). With regard to biofumigation, only the aromatic and aliphatic GSL are more important because of their enzymatic degradation into ITC (Bones and Rossiter, 1996; Rask *et al*., 2000). The biosynthesis of GSL occurs as a result of chain elongation phase, core structure formation and subsequent modification of side chains by processes including hydroxylation, oxygenation and alkenylation (Ishida *et al*., 2014).

The type and quantitative levels of GSL differ between species (Bhandari *et al*., 2015) and between development stage of the plant and plant organ (Brown *et al*., 2003; Malik *et al*., 2010). Glucosinolates are defense molecules that combat against herbivores (Giamoustaris and Mithen, 1995) and microbial pathogens (Buxdorf *et al*., 2013; Schlaeppi *et al*., 2010). The difference in GSL profile could also be explained by the fact that GSL being the defense arsenal accumulate in those parts that are important for the survival of the plant at that particular stage in the plant development. For instance, GSL concentrations are highest in seeds and fruits maximising the protection of the reproductive phase (Brown *et al*., 2003). Variance in the composition and concentration of GSL also occur within varieties of same species (Bellostas *et al*., 2007). Aromatic GSL commonly occur in roots whereas the aliphatic types are found in shoots (Bhandari *et al*., 2015; Zhu *et al*., 2013). The variation also occurs depending on environmental and climatic conditions such as temperature, soil pH and moisture content (Bellostas *et al*., 2004; Rosa *et al*., 1996). Consequently the hydrolysis products and type of ITC produced also vary in their toxicity with different pathogens responding differently to these products (Hu *et al*., 2015; Nazareth *et al*., 2016; Smith and Kirkegaard, 2002).

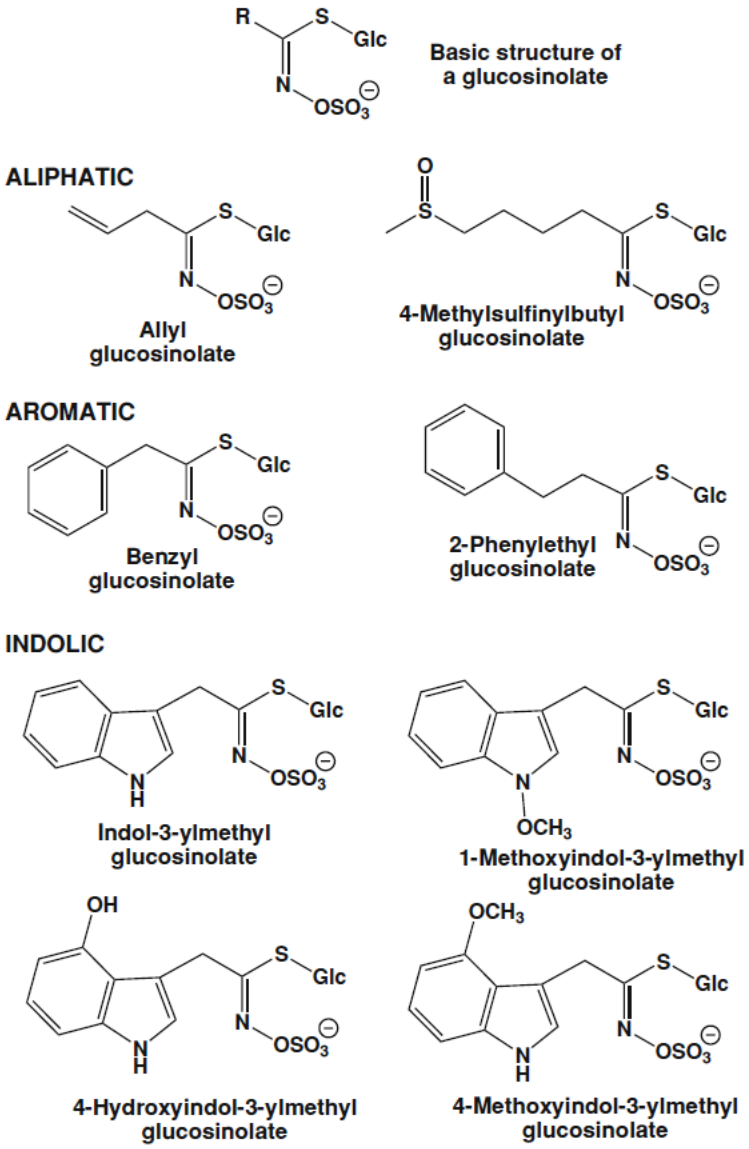


Figure 1.4 Structures of the three main types of glucosinolates with examples (Courtesy of Jonathan Gershenzon, Department of Biochemistry, Max Planck Institute for Chemical Ecology, Jena, Germany)

Glucosinolates and their breakdown products are also found to be present in the soils following biofumigation. In a study, up to 13% of the total GSL present in the incorporated brassica plant parts were detected in the soil for up to 8 days (Gimsing and Kirkegaard, 2006) indicating that intact GSL can remain in soil for a significant time following biofumigant incorporation. These authors found that the highest total ITC concentration of 90.6 nmol g^{-1} (for *B. juncea*) was detectable after 30 minutes following incorporation, however, trace amounts of ITC could be detected up to 12 days later.

With regard to GSL content, as mentioned earlier, roots of brassica plants typically contain largely aromatic GSL whereas the shoots mainly produce aliphatic GSL (Kirkegaard and Sarwar, 1998). Considering this aspect and the significance of fine shredding of plant tissues for maximum ITC liberation, Matthiessen and Shackleton (2005) suggested the use of brassica species with greater above-surface plant biomass and high potential for aliphatic GSL production. Thorough crushing of the above-surface plant parts is feasible as compared to the chopping the below-surface parts which would negatively affect the soil structure and which is not convenient practically.

1.5.5 Myrosinase - the catalyst of biofumigation

Myrosinase enzymes are located primarily in specialised cells known as myrosin cells, that are dispersed throughout the brassica tissues (Andréasson *et al*., 2001). Within the myrosin cells, myrosinase enzymes are present in protein containing vacuoles; the myrosin grains (Thangstad *et al*., 1990) (Figure 1.5). On the other hand, GSL are distributed throughout the plant organs and located in translucent vacuoles or moved for long-term storage in sulphur-rich cells called the S-cells (Jørgensen *et al*., 2015; Koroleva *et al*., 2010).

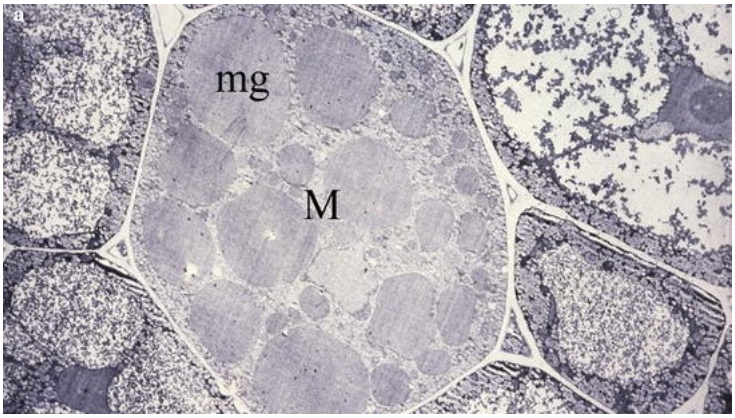


Figure 1.5 Transmission electron microscopy image of a myrosin cell in *Raphanus sativus* indicated by M, surrounded by ground tissue cells. One of the myrosin grains is indicated as mg (Courtesy of Atle Bones, Department of Biology, Norwegian University of Science and Technology, Trondheim, Norway)

Glucosinolates are non-toxic unless they are hydrolysed by tissue disruption. For instance, tissue damage by herbivory allows physical contact of GSL and myrosinases. The interaction then leads to the catalysis of GSL into a range of biologically active substances including toxic ITC (Bones and Rossiter, 1996; Wittstock and Burow, 2010). However, not all GSL undergo chemical breakdown by myrosinase released from plants. Intact GSL can also be released through root exudates in the soil and be converted to ITC, contributing to biofumigation. Al-Turki and Dick (2003) observed myrosinase activity in rhizosphere soil of *Sinapis alba* grown under glasshouse conditions. Myrosinase activity was also reported in soil extracts collected around field grown *B. napus* as compared to pasture soil extracts in which myrosinase activity was not detected (Borek *et al*., 1996). In both studies, the source of the myrosinase enzyme was not clear. However, there is evidence that myrosinase activity occurs in soil without GSL-containing plants or where biofumigant plants have not been cultivated for a minimum of five years (Gimsing *et al*., 2006). Gimsing and Kirkegaard (2009) suggests that in such cases, myrosinase could be produced by soil microorganisms and may also be released from the roots of GSL-containing plants. Previously, myrosinase activity by *Aspergillus* species has been reported (Rakariyatham *et al*., 2005; Sakorn *et al*., 1999). The fungal species were able to break down sinigrin when incubated in media supplemented with

sinigrin. Bacterial species such as a strain of *Lactobacillus* sp. (Palop *et al*., 1995) and three strains of *Bifidobacterium* sp. (Cheng *et al*., 2004) were also found to have the capability to degrade GSL, indicating that myrosinase or myrosinase-like activity is involved.

1.5.6 Isothiocyanates

Isothiocyanates are the most common degradation products of GSL in brassicas. About 100 ITC have been identified, 20 of which are known to be produced by brassica species (de los Santos *et al*., 2016). Isothiocyanates are released as a result of enzymatic breakdown of GSL by myrosinase at neutral pH (Figure 1.6). Major GSL and their respective ITC are shown in Table 1.2. These volatile substances have toxic effects against broad range of noxious organisms including nematodes, weed, fungi and insects. Depending on the chemical structure, different GSL produce different ITC with varied toxicity. Plant pathogens belonging to the same species or different isolates of the same species show varying sensitivity to different ITC. The pathogen's sensitivity to these ITC also varies depending on which stage they are in their life cycle (Mari *et al*., 1993; Smith and Kirkegaard, 2002). The various GSL with different side chain groups give different toxic potential to the subsequent ITC produced (Brown and Morra, 1997; Warton *et al*., 2001). The varying levels of toxicity are dependent on the rate of penetration, resistance to degradation and accumulation of ITC in the cells (Ye and Zhang, 2001; Zhang and Talalay, 1998). Aliphatic ITC are known to be more toxic than aromatic ones (Matthiessen and Shackleton, 2005; Smolinska *et al*., 2003) suggesting the variation could be due to different side chains or different mode of action (Calmes *et al*., 2015).

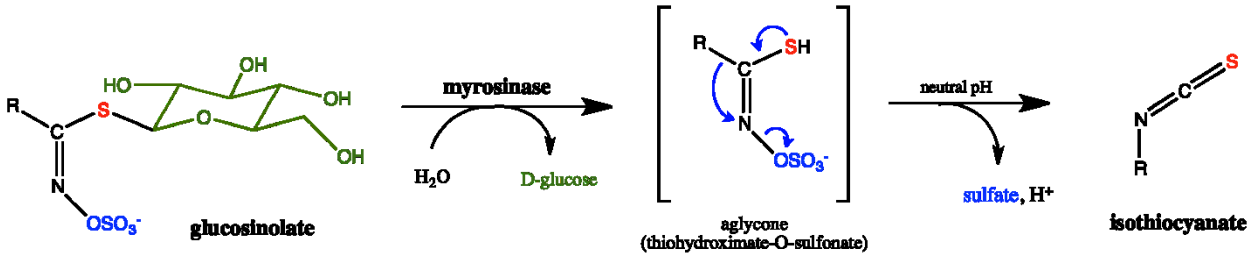


Figure 1.6 Isothiocyanate formation from glucosinolate by hydrolytic reaction of myrosinase (Source: Halkier and Gershenzon, 2006)

Reports on the mechanism of action by which ITC exert inhibitory effect on fungal pathogens is scarce. The antibacterial effect of ITC is associated with inhibition of RNA synthesis (Nowicki *et al*., 2016) and disruption of biofilm formation (Kaiser *et al*., 2017). Isothiocyanates are also known to disrupt mitochondrial function in mammalian cancer cells (Bo *et al*., 2016; Xiao *et al*., 2008). In an attempt to scrutinise if a similar effect occurs in ITC-exposed fungal cells, Calmes *et al*. (2015) found that ITC altered mitochondrial function and triggered production of reactive oxygen species in *Alternaria brassicicola*. Moreover, reduction in oxygen consumption was also observed in the fungal cells suggesting a possible disruptive effect on metabolic pathways. Further research is needed to better understand the mechanism by which ITC exhibit inhibitory effect on fungi.

Isothiocyanate levels in soil may decrease because of volatilisation, chemical degradation and sorption (Hanschen *et al*., 2015). Additionally, ITC are also reported to be subject to microbial degradation. Findings from studies examining sterilised soils suggest that ITC are biodegraded by soil microorganisms as autoclaving increased the stability of ITC (Price *et al*., 2005; Rumberger and Marschner, 2003; Warton *et al*., 2003) and in most cases the degradation took place in a few hours. With regard to environment protection, the degradation seems

advantageous but from a biofumigation perspective, rapid degradation is undesirable because persistence of ITC in the soil for sufficient period of time to satisfy the objective of biofumigation is vital (Gimsing *et al*., 2009).

Table 1.2 Major glucosinolates occurring in Brassicaceae (Hanschen and Schreiner, 2017; Ishida *et al*., 2014; Malik *et al*., 2010)

Glucosinolate group (molecular structure)	**Glucosinolate - trivial name**	**Glucosinolate - side chain**	**Respective Isothiocyanate (ITC)**
Aliphatic	Sinigrin	2-Propenyl	Allyl ITC
	Glucoraphanin	4-Methylsulfinylbutyl	Sulphoraphane
	Gluconapin	3-butenyl	3-butenyl ITC
	Glucoiberverin	3-Methylthiopropyl	3-Methylthiopropyl ITC
	Glucoiberin	3-Methylsulfinylpropyl	3-Methylsulfinylpropyl ITC
	Glucoerucin	4-Methylthiobutyl	4-Methylthiobutyl ITC
	Glucobrassicanapin	4-Pentenyl	4-Pentenyl ITC
	Glucoraphenin	4-Methylsulfinyl-3-butenyl	4-Methylsulfinyl-3-butenyl ITC
Aromatic	Gluconasturtiin	2-Phenylethyl	2-Phenylethyl ITC
	Glucotropaeolin	Benzyl	Benzyl ITC

1.5.7 Other breakdown products of GSL

In addition to ITC, other less toxic compounds are also produced as a result of GSL hydrolysis. Depending on factors including pH, soil conditions and availability or non-availability of specific proteins and metal ions, hydrolysis products such as thiocyanates, oxazolidine-2-thiones, epithionitriles and nitriles are also released (Redovnikovic *et al*., 2008) (Figure 1.7).

Specifier proteins are supplementary proteins, present in GSL-containing plants, that determine the profile of hydrolytic products of GSL. These proteins have no catalytic activity on GSL but transform the unstable aglycones into non-ITC products. In the absence of specifier proteins, the aglycones produced from the myrosinase-catalysed GSL, usually form ITC. However, in the presence of epithiospecifier proteins, epithionitriles and nitriles are produced from alkenyl and non-alkenyl GSL respectively. On the other hand, nitrile specifier proteins favour the formation of simple nitriles from all structural types of GSL (Wittstock *et al*., 2016). Nitrile formation is also favoured by acidic pH (pH 2-5) and presence of ferrous ions in soil (Hanschen *et al*., 2015).

Isothiocyanates as hydrolysis products of GSL are intensively studied and their role in biofumigation is well established. However, the biological activity of non-ITC breakdown products and their role in biofumigation is poorly understood. In one study (Yim *et al*., 2016), *B. juncea* and *R. sativus* were cultivated and incorporated in soil and the breakdown products were analysed. Biofumigation with *B. juncea* produced nitriles (3-butenenitrile, 3-phenylpropanenitrile) and ITC (allyl ITC, 2-phenylethyl ITC) ranging from 0.012 to 0.314 nmol g^{-1} and 0.227 to 15.035 nmol g^{-1} respectively. On the other hand, *R. sativus* did not produce nitriles. In another study (Kupke *et al*., 2016), GC-MS analysis of degradation products of GSL in broccoli and cauliflower revealed that around half of the total breakdown products were nitriles. However, in a study investigating the degradation of benzyl GSL (glucotropaeolin) in soil (Gimsing *et al*., 2007), only trace amounts of nitriles were recovered in the subsoil. Given that the pH of the soil in this study was reported to be neutral, it can be suggested that the conditions did not favour the formation of nitriles.

Organic thiocyanates are formed in the presence of thiocyanate specifier proteins from sinigrin, glucotropaeolin and 4-methylthiobutyl GSL. A common structural characteristic of these GSL is the ability of their side chains to form stable carbon

cation which might be a prerequisite for thiocyanate formation (Kuchernig *et al*., 2012). Thiocyanates are known to have biocidal properties. Toxicity of thiocyanates and nitriles towards insects, such as, the house fly (*Musca domestica*) and the lesser grain borer (*Rhizopertha dominica*) have been shown in *in vitro* studies (Peterson *et al*., 1998; Peterson *et al*., 2000). Crambe (*Crambe abyssinica*) was found to contain allyl, 4-pentenyl, 3-butenyl, 2-hydroxy-3-butenyl and 4-hydroxybenzyl GSL in a previous study (Tsao *et al*., 1996). The researchers further investigated insecticidal activity of crambe-related GSL breakdown products (Tsao *et al*., 2002). The LC_{50} of allyl thiocyanate for house fly was 0.1 µg cm^{-3} which was almost similar to the LC_{50} of the commercial pesticide, chloropicrin (0.08 µg cm^{-3}). Whereas, the LC_{50} of allyl thiocyanate for lesser grain borer (0.55 µg cm^{-3}) was half that of chloropicrin (1.3 µg cm^{-3}). The effect of biofumigation on a variety of weed species was investigated by Lefebvre *et al*. (2018) in laboratory studies. More than 90% mortality was observed in *Daucus carota* (wild carrot), *Chenopodium album* (common lambsquarters) and *Galinsoga quadriradiata* (hairy galinsoga) when the weed seeds were exposed to mustard powder. Analysis of the mustard plant material showed that the main hydrolytic compounds produced were allyl ITC (2455 µg g^{-1}) and allyl thiocyanate (1431 µg g^{-1}). Complete mortality of weeds by the application of thiocyanate at a rate of 1793 kg ha^{-1} has also been documented (Harvey, 1931).

In addition to GSL, brassicas also contain other sulphur-rich compounds such as sulphoxides and sulphur-containing amino acids (cysteines and methionine). During decomposition of plant tissues, these compounds are broken down to toxic products such as dimethyl sulphide, carbon disulphide and methanethiol (Bending and Lincoln, 1999; Chin and Lindsay, 1993) which may also contribute to biofumigation effect (Gimsing and Kirkegaard, 2009).

Bending and Lincoln (1999) measured volatile compounds produced over two weeks following incorporation of *B. juncea* leaf material into soil. In this microcosm study, the non-GSL compounds dimethyl sulphide, dimethyl disulphide, carbon disulphide and methanethiol were produced up to 406, 39, 152 and 992 nmol g^{-1} leaf dry weight respectively. Overall, higher amounts of the compounds were produced in sandy-loam soil when compared to formation in clay-loam soil. In addition to soil type, it appears that temperature might also be an important factor in formation of these compounds. Gamliel and Stapleton (1993) heated cabbage-amended soil in a solarisation simulation system with a maximum temperature of 45°C for 4 hours daily for 24 days. Methanethiol and dimethyl sulphide were detected only in the

heated treatment while, in the non-heated treatment, these compounds were not detected. Wang *et al*. (2009) also detected dimethyl disulphide and methyl sulphide in glass house experiments, 24 hours post-incorporation of chopped brassica tissues; head space concentrations for dimethyl disulphide and methyl sulphide were found up to 283.2 µg g^{-1} and 346.4 µg g^{-1} respectively. These compounds have shown biocidal properties against pests and pathogens including nematodes and fungi (Curto *et al*., 2014; Lewis and Papavizas, 1971). In a field experiment (Wang *et al*., 2009), incorporation of *S. alba* produced methyl sulphide and dimethyl sulphide at concentrations of 5.2 and 1.2 µg g^{-1} plant dry weight respectively. Approximately, a 70% reduction of nematodes (*Tylenchulus semipenetrans*) was observed post brassica incorporation. Additionally, the inoculum of *Fusarium* spp. was also significantly reduced (58 cfu g^{-1}) when compared to the control plots (172 cfu g^{-1}).

Isothiocyanates and other sulphurous compounds of biofumigation may have a synergistic effect on pathogen suppression. In a study, methyl ITC (5 ppm) and carbon disulphide (3000-4000 ppm) were found to be sub-lethal individually against wood-colonising fungi. However, when the two compounds were combined, they caused almost complete inhibition of most of the fungal pathogens tested, including, *Gloeophyllum trabeum* and *Irpex lacteus* (Canessa and Morell, 1995).

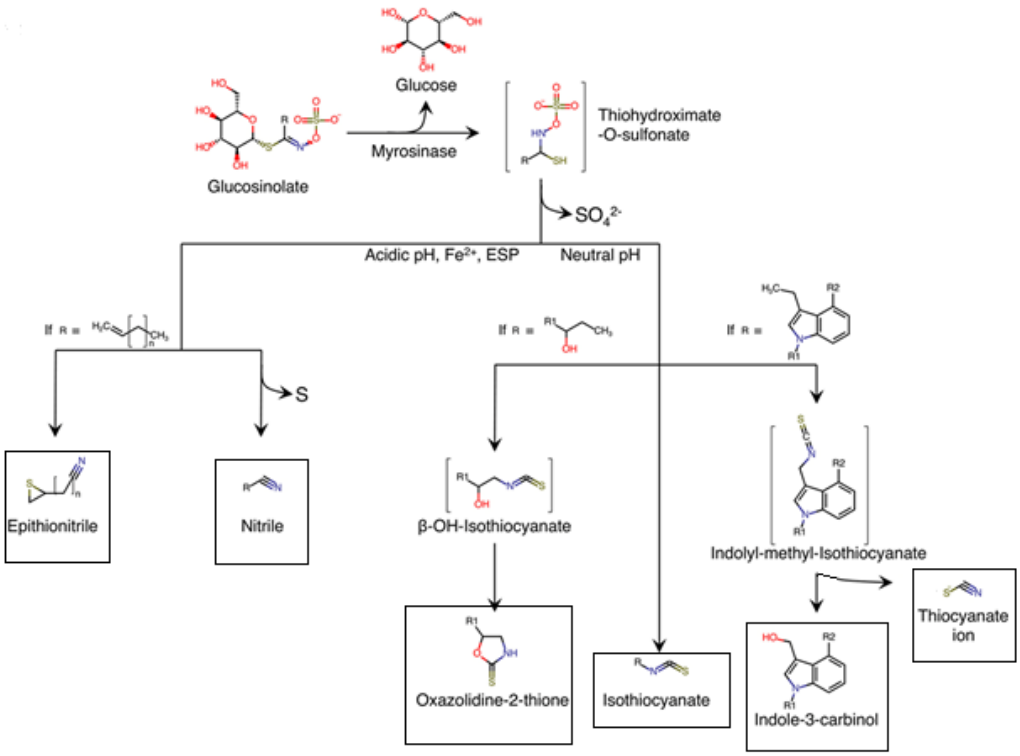


Figure 1.7 Glucosinolate break down products (shown in boxes) released as a result of myrosinase action; different compounds are produced depending on different factors. Molecules shown between brackets represent the unstable intermediates. ESP = epithiospecifier protein (Adapted from Barba *et al*., 2016)

1.5.8 Methods applied for biofumigation

Biofumigation can be achieved by using different methods of application, such as, the incorporation of fresh plant tissues (green manure crops) or application of preserved biofumigant material. Incorporation of fresh plant tissue generally aims for high biomass and high GSL content to achieve optimal release of ITC. Processed biofumigant products including seed meals and dried plant material, are used for ease of application and to avoid waiting for a whole crop cycle. The methods are discussed below.

1.5.8.1 Complete biofumigation

Brassica plants are usually grown for 10 to 14 weeks followed by thorough chopping of the plant material to ensure maximum release of ITC. The chopped tissues are then immediately incorporated into soil (Kirkegaard *et al*., 1993). This is the most recognised method of biofumigation application. Larkin and Griffin (2007) successfully used brassica crops for suppressing fungal pathogens of potato and achieved 15-80% reductions in powdery scab, common scab and black scurf diseases. Previously, this method has also shown efficacy against Verticillium wilt of cauliflower (Subbarao *et al*., 1999) and potato cyst nematode (Ngala *et al*., 2015a).

This technique provides additional benefits such as enhancing soil organic matter content, nutrient cycling and microbial antagonistic interactions in soil (Cohen *et al*., 2005; Motisi *et al*., 2010; Talgre *et al*., 2012).

1.5.8.2 Cover crop/partial biofumigation

Managing disease and pests through partial biofumigation under growing brassica crop is another way of exploiting biofumigants. In this technique, plant parts above the ground are harvested as food crops or allowed to mature in the field. This approach is dependent on root exudation containing GSL, and ITC formation from these GSL probably by myrosinase or myrosinase-like activity of soil microorganisms (Borek *et al*., 1996; Rakariyatham *et al*., 2005; Sakorn *et al*., 1999). Suppression of the wheat disease pathogen *G. graminis* (take all) has been achieved using this form of biofumigation (Kirkegaard *et al*., 2000). These authors selected brassica crops such as *B. juncea* and *B. napus* on the basis of their root

GSL profiles. Fertilisers and irrigation were used to enhance the root growth. In the majority of fields where this biofumigation approach was practised, disease incidence was reduced by approximately 85%. Moreover, partial biofumigation under growing brassicas such as *B. juncea* and *R. sativus* have caused suppression of the potato cyst nematode *Globodera pallida* under field conditions (Ngala *et al*., 2015a). Therefore, brassicas used for biofumigation may affect soil-borne pests and pathogens not just after incorporation of the crop residues, but also during active growth of these plants.

1.5.8.3 Preserved biofumigants

Seed meals and dried tissues

Seed meal, produced by extracting the oil from brassica seeds, is known to contain high levels of GSL and intact myrosinase (Furlan *et al*., 2010; Vaughn *et al*., 2006). Various studies have assessed the efficacy of brassica-derived seed meals to suppress plant diseases (Lazzeri *et al*., 2009; Mazzola *et al*., 2007; Mazzola *et al*., 2015; Morales-Rodríguez *et al*., 2016). Seed meal and seed meal extract of *B. juncea* were shown to effectively suppress potato-parasitic nematodes *G. pallida* and *G. ellingtonae* by almost completely inhibiting egg hatch (Dandurand *et al*., 2017*).* In contrast, Rodríguez-Molina *et al*. (2016) failed to demonstrate significant suppression of *Phytophthora nicotianae* in field experiments using seed meal pellets of *B. carinata.*

Other processed products that could be used for biofumigation include dried parts of brassica plant in which sufficient GSL and myrosinase activity are preserved. The plants are oven-dried ensuring minimum loss of GSL and myrosinase, so when watered, the dried parts are able to produce biotoxic compounds in soil. The antifungal activity of pellets prepared from oven-dried GSL-containing plant parts against *R. solani* and *Pythium irregulare* was demonstrated under laboratory conditions by (Lazzeri *et al*., 2004). The effectiveness index of the dried tissues ranged from 22-100% for *Rhizoctonia* and 12-100% for *Pythium.*

The benefits of the above mentioned processed products could come from the fact that these products are available throughout the year and they could be easily and uniformly incorporated in fields (Rodríguez-Molina *et al*., 2016), however, higher costs of this approach are a constraint.

Liquid formulations

Another category of preserved biofumigants is liquid formulations. These liquid formulations are derived from brassica seed meals and have been investigated for pest and pathogen suppression (Baldi *et al*., 2015; De Nicola *et al*. 2013; Piccinini *et al*., 2015). A liquid formulation of the product BioFence™ (*B. carinata* seed meal) proved to be >60% efficient in reducing the inoculum densities of *Verticillium dahliae* in mini-plots under field conditions (Wei *et al*., 2016). Another liquid formulation also based on *B. carinata* seed meal was found to be suitable for drip irrigation. This liquid formulation significantly suppressed the root-knot nematode *Meloidogyne incognita* under glasshouse conditions (De Nicola *et al*., 2013). Even though the liquid formulations are derived from plant-based products, these alternatives may face obstacles in implementation under the green approach category due to high concentrations of the biocidal substances (Kirkegaard, 2009).

There are different methods of application of biofumigants, but while opting for a particular method it should be taken into account that biofumigation efficacy depends on factors including the pathogen, host plant and geographical, climatic and economical aspects. Therefore, a single protocol of application method may not be suitable in every agricultural scenario.

1.5.9 Factors affecting biofumigation

The successful implementation of biofumigation is dependent on a number of factors, such as, brassica species and their GSL profile, soil conditions and incorporation method (Figure 1.8). These factors can generally be categorised as genetic, climatic, edaphic and agronomic factors.

1.5.9.1 Genetic factors: brassica species and cultivar choice

There is a great range of variation in the total content and type of GSL found between species of brassica plants. Consequently the hydrolysis products and type of ITC produced also vary between the brassica species. For example, *B. juncea* shoots are rich in sinigrin comprising >90% of the total GSL content of this brassica

(Doheny-Adams *et al*., 2018; Ngala *et al*., 2015b). Hydrolysis of sinigrin would hence lead to the production of allyl ITC. Previously allyl ITC was found to be the predominating compound (>90%) in the volatiles emitted from macerated leaves of *B. juncea* (Charron and Sams, 1999). On the other hand, the predominant GSL in the shoots of *R. sativus* is glucoraphanin (Ngala *et al*., 2015b), the precursor of sulforaphane (Zhen-xin *et al*., 2012). The hydrolysis products also vary in their toxicity with different pathogens responding differently to these products. For instance, in a field simulation study, *B. juncea* amendment in soil significantly suppressed *V. dahliae* microsclerotia whereas *R. sativus* treatment was not effective compared to untreated (Neubauer *et al*., 2014). Moreover, difference between cultivars of the same species can produce different outcome of biofumigation. Previously, different cultivars of broccoli (Wang *et al*., 2012) and pakchoi (Zhu *et al*., 2013) showed difference in the predominant type and concentration of GSL when the cultivars of the same species were grown under same conditions. Different cultivars are also reported to produce different biomass when grown under same conditions. The above-ground biomass of *B. juncea* at 50% flowering for the three cultivars, Vitasso, ISCI99 and Scala, were 50, 40 and 33 t ha^{-1} respectively (Doheny-Adams *et al*., 2018).

Glucosinolate concentration and type varies in different tissues. Additionally, the type and concentration of GSL in brassica tissues varies at different stages of the plant's growth cycle such as rapid growth, early-bud and flowering (Bhandari *et al*., 2015). Glucosinolates are generally found to be in highest concentrations during the flowering stage (Malik *et al*., 2010). Root tissue contains higher GSL content in earlier stages of root growth and the levels decrease during the root growth cycle (Kruger *et al*., 2013). Doheny-Adams *et al*. (2018) found sinigrin concentrations to be higher in leaves compared to stems of *B. juncea*. In addition to these factors, the pattern in which ITC are released also have varying effect on pathogens. The release pattern of a certain type of ITC from the same parent GSL differs among different brassica species. The production efficiency of 2-phenylethyl ITC from the respective GSL (gluconasturtiin) was found to be lower in *B. juncea* than *S. alba* (Hossain *et al*., 2014). The ITC/GSL ratio for *S. alba* was 15.51 and that for *B. juncea* was 11.51. Moreover, ITC were liberated faster from hydrolysis of *S. alba* GSL than *B. juncea* GSL, but the latter produced ITC over a longer time with increasing rate which comparatively rendered more effect on *Aphanomyces euteiches in vitro.*

1.5.9.2 Agronomic factors

Although GSL content of a plant is determined by the species and cultivar used, external factors can also significantly influence the efficacy of biofumigation. In order to exploit maximum potential of a biofumigant, utilising the highest possible achievable concentrations of the biocidal compounds is one of the most important factors to consider. This can be carried out by thorough crushing of the tissues using suitable mechanisms. This aspect together with the biomass portion should be considered while aiming for maximum ITC production from hydrolysis. Researchers have emphasised the importance of thorough shredding prior to incorporation for higher ITC release efficiency (Gimsing and Kirkegaard, 2006). Morra and Kirkegarrd (2002) reported up to 26% increase in ITC release efficiency with better tissue disruption of brassica leaves using freeze-thaw technique as compared to fresh leaves. In another study (Ojaghian *et al*., 2012), fresh macerated tissues of *B. juncea* inhibited radial growth of *Sclerotinia sclerotiorum* as much as twice when compared to irradiated dried tissues. *Brassica campestris* macerated tissues were also four times more effective than the dried tissues.

Additionally, dose and exposure time of the biocidal compounds are also critical factors for effective biofumigation. Losses by volatilisation or sorption by organic matter in soil may decrease the required effective concentration of ITC which otherwise could be achieved in closed systems in laboratory experiments. To prevent escape of these volatile ITC, it is preferable to smear the soil with a roller (Kirkegaard and Matthiessen, 2004).

1.5.9.3 Edaphic and climatic factor

In addition to the above mentioned parameters, others factors, such as soil temperature, moisture content and nutrient levels are known to affect the success of biofumigation.

Soil temperature can have a significant impact on ITC production (Price *et al*., 2005). Previously, soil amended with cabbage residues was analysed for volatile production (Gamliel and Stapleton, 1993). It was reported that the concentration of volatiles in the headspace were higher in heated, amended soils than in non-heated amended soils. Very low soil temperatures are not suitable when aiming for optimal myrosinase activity. Hence, incorporation of biofumigants at soil temperature near

0°C should be avoided (Kruger *et al*, 2013). Moisture content of soil may also affect the efficacy of biofumigation. Previously, soil amended with *B. juncea* and *S. alba* seed meal were evaluated for allyl ITC emission in the headspace (Wang and Mazzola, 2019). Allyl ITC production was found to be elevated (~0.05-0.265 μg g^{-1} soil) with an increase in soil temperature from 10°C to 30°C and increase in moisture level from -1000 kPa to -40 kPa. Presence of sufficient moisture is important to enable myrosinase activity for production of GSL hydrolysis products (Mora and Kirkegaard, 2002). In a field study, addition of 42 mm of water to *B. juncea* plant material resulted in a 7- to 10-fold increase in ITC concentrations in soil compared to where no water was added (Matthiessen *et al*., 2004).

Soil texture can impact the activity of ITC produced. Price *et al*. (2005) investigated ITC production from *B. juncea* tissues incorporated in soil under controlled conditions. Of the two types of soil used, ITC levels were found to be 38% higher in sandy loam soil than clay loam soil. The authors argued that the lower ITC concentration found in clay loam soil could be due to sorption of ITC to the organic matter in clay loam soil. Gimsing *et al*. (2009) have demonstrated that organic matter content in soil is the main sorbent of ITC. Higher organic matter content could reduce the activity of ITC hence, negatively affecting the outcome of biofumigation.

The application of nitrogen and sulphur fertilisers to brassica crops can affect the GSL concentrations in brassica tissues (Aires *et al*., 2006; Omirou *et al*., 2009; Rosen *et al*., 2005; Schonhof *et al*., 2007). In a field experiment, the effect of nitrogen and sulphur application on GSL concentrations in cabbage (*B. oleracea* var. *Capitata* L) was investigated (Maršic *et al*., 2021). Nitrogen and sulphur when applied at a 4.5:1 ratio (nitrogen=180 kg ha^{-1}, sulphur=40 kg ha^{-1}), total GSL concentrations in cabbage heads were highest (30 μmol g^{-1} dry weight); sinigrin concentration was also highest at 13.2 μmol g^{-1} dry weight. In comparison, total GSL concentrations (23.9-25.9 μmol g^{-1} dry weight) and sinigrin concentrations (10.8-11.6 μmol g^{-1} dry weight) were lower in the other treatments: nitrogen alone at 180 and 240 kg ha^{-1}, nitrogen 240 kg ha^{-1} + sulphur 40 kg ha^{-1}, and control (no nitrogen or sulphur).

Seasonal variations are also known to affect the GSL concentration in brassica tissues. Biofumigants grown during summer conditions are exposed to higher UV intensity, longer daylight hours and higher temperatures. These factors are known to increase the production of GSL in brassica tissues (Björkman *et al*., 2011). Summer grown brassica crops such as *B. juncea*, *E. sativa* and *R. sativus* produced higher

concentrations of GSL in the summer when compared to being overwintered (Ngala *et al*., 2015a). The high biomass biofumigants, when chopped and incorporated, are then likely to produce ITC at sufficiently effective concentrations, hence improving the efficacy of biofumigation.

1.5.9.4 Influence of soil microorganisms

Some soil microorganisms are also known to enhance biofumigation effect by displaying antagonistic behaviour towards pathogens. *Trichoderma* spp. was reported to show resistance to ITC and when combined with *B. carinata* seed meal under controlled conditions, an increase in the effectiveness of biofumigation was observed (Galletti *et al*., 2008). *Trichoderma* combined with the seed meal reduced disease incidence in sugar beet plants caused by *Pythium ultimum* by about 75%, compared to seed meal treatment alone which reduced disease incidence around 30%. In another study (Handiseni *et al*., 2016), pasteurised and non-pasteurised soils were amended with brassica tissues and placed in closed jars. In this study, analysis of the vapour samples revealed higher ITC emission (>4 μg ml^{-1}) in non-pasteurised soil than the pasteurised soil in which the ITC release was minimal (<0.5 μg ml^{-1}). This indicates a possible role of soil microbiota in GSL hydrolysis. As mentioned earlier, some microorganisms such as *Aspergillus* sp. and *Bifidobacterium* sp. are known to have the ability to degrade GSL (Cheng *et al*., 2004; Palop *et al*., 1995; Rakariyatham *et al*., 2005; Sakorn *et al*., 1999). Glucosinolates released from roots of brassica plants while still growing, are believed to be hydrolysed by soil microorganisms, thus producing biofumigation effect (Ngala *et al*., 2015c). The role of soil microbiota is hence very important for partial biofumigation.

All these factors, in combination or individually, affect the outcome of biofumigation. These factors should be considered while designing systems for biofumigation according to the target pathogen.

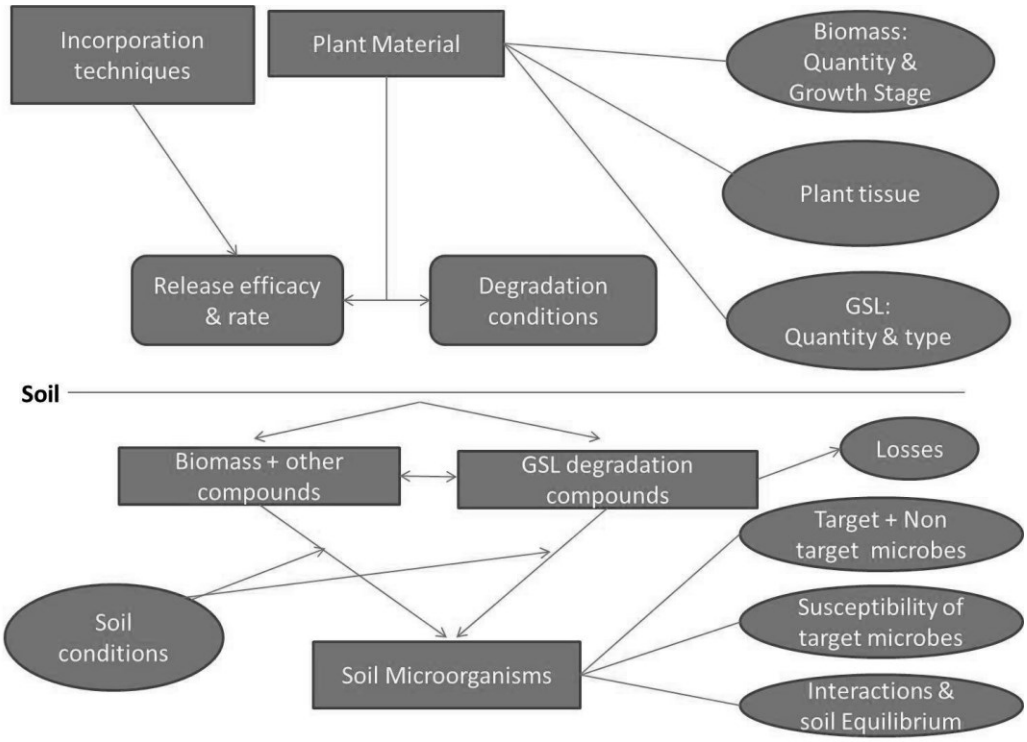


Figure 1.8 Factors affecting the outcome of biofumigation (Source: Kruger *et al*., 2013)

1.5.10 Impact of biofumigation on soil microbiota

The practice of biofumigation may affect soil microbial community. The effect of brassica biofumigants on soil microbiota was recently reviewed by Tagele *et al*. (2021). Increase in bacterial diversity of soil by biofumigation has been reported. In a greenhouse study, the effect of different rotations with eggplant on soil bacterial diversity were investigated (Li *et al*., 2017). In this study, eggplant-*B*. *napiformis* and eggplant-*B. campestris* rotations resulted in higher bacterial diversity compared to continually planted eggplant and chemical disinfection treatments, hence improving soil health. The increase in soil bacterial diversity can prove beneficial for the outcome of biofumigation due to their competitive and antagonistic effects on soil-borne pathogens (Wang *et al*., 2014). In another study, the effect of ITC on soil microbiota was investigated in glasshouse experiments (Taylor, 2013). It was found that the addition of ITC to soil does not have adverse effects on bacterial diversity in soil during the time period after incorporation (30 days), suggesting ITC incorporation does not negatively impact soil health. In *in vitro* experiments, macerated tissue of *B. juncea* was found to completely inhibit fungal pathogens including *R. solani* and *Phytophthora erythroseptica* whereas common soil fungi *Penicillium* sp. and *Trichoderma* sp. were comparatively less affected (30-45% inhibition) (Larkin and Griffin, 2007). In another study (Hu *et al*., 2015), the influence of pure ITC in combination with flax seed meal on soil microbial community was shown to be dependent on the type of ITC. qPCR assays showed that allyl ITC temporarily suppressed soil fungal populations whereas butyl ITC increased bacterial (initially decreased) and fungal abundance. Benzyl and phenyl ITC on the other hand showed no effect on soil microbial community.

Even if biofumigant incorporation alters soil microbial diversity immediately post-incorporation, the microorganisms may later flourish and recover from the alteration effects. Moreover, other factors such as, addition of organic matter by green manuring may lower the risks to beneficial soil microbes. However, considering the biotoxic nature of ITC, the possibility of mild collateral damage cannot be ruled out. Nevertheless, the effect of biofumigation on soil microbiota may vary depending on a range of factors such as the brassica plant, the type and concentration of GSL and the hydrolysis products and the microbial community structure in the soil.

1.6 Biofumigation-related studies on fungal pathogens

The biocidal effects of brassicaceous amendments have been well documented long before the term biofumigation was coined in the 1990s. The fungitoxic properties of ITC have been known about since at least 1937 (Walker *et al*., 1937), while suppression of soil-borne pathogens by brassica tissues was reported in the 1970s and 1980s. For example, Chan and Close (1987) successfully demonstrated suppression of *Aphanomyces euteiches* using air-dried chopped plant materials of kale, cabbage, foder radish, white mustard and rape in glasshouse trials. These brassica amendments reduced the disease severity index of Aphanomyces root rot of peas when added to *A. euteiches*-infested soil at a rate of 0.5% dry weight of soil. Cabbage amendment resulted in 50% and rape- and fodder radish-amendment resulted in 40% reduction in disease severity index when compared to control treatments. Lewis and Papavizas (1974) also reported suppression of *R. solani* using cabbage tissues in laboratory experiments. Colonization of buck-wheat stem segments in *R. solani*-infested soil was reduced by 75% when exposed to vapours from decomposing cabbage tissues. Since these initial studies, biofumigation has been investigated for its suppressive effects against fungal pathogens, nematodes and weeds. The effect of biofumigation has been examined under different experimental conditions including *in vitro* assays, glasshouse experiments and field based studies.

1.6.1 *In vitro* studies on biofumigation related volatiles

In vitro assays have mostly focused on the use of pure ITC due to their importance as the main toxic product of GSL hydrolysis. For example, ITC-amended media has been utilised to determine the *in vitro* effect on mycelial growth in a number of studies. In one study, Kurt *et al*. (2011) demonstrated that *Sclerotinia sclerotiorum* was suppressed by pure ITC. Allyl ITC (72 µmol l^{-1}) and benzyl ITC (60 µmol l^{-1}) had the lower EC_{50} values for mycelial growth when determined by volatile phase (exposing the pathogen to ITC-glass filter in media plates). However, when the pathogen was exposed to ITC-amended media, the lowest EC_{50} value was recorded for methyl ITC (168 µmol l^{-1}), but overall, aromatic ITC were effective in inhibiting the mycelial growth. This could be attributed to different volatility of these ITC. Previously, aromatic ITC when incorporated in to media showed stronger toxicity against fungal pathogens but were less toxic in volatile form due to their lower

volatility (Sarwar *et al*., 1998). Kurt *et al*. (2011) reported that overall, EC_{50} values of ITC for mycelial inhibition of *S. sclerotiorum* were lower when determined by volatile phase as compared to when dissolved in the agar. Sarwar *et al.* (1998) also reported a similar trend in similar ITC experiments with pathogens such as, *R. solani*, *G. graminis*, *F. graminearum*, *Pythium irregulare* and *Bipolaris sorokiniana*. Pure ITC (benzyl, allyl, 3-butenyl, 4-pentenyl, 2-phenylethyl) when introduced to headspace of flask were more suppressive to mycelial growth of these fungal pathogens. In the headspace experiments, lethal concentrations of allyl ITC for *R. solani*, *G. graminis* and *F. graminearum* were 1.6 µmol l^{-1}. In comparison, lethal concentrations of ITC when dissolved in agar increased to 20, 30 and 40 µmol l^{-1} for *G. graminis*, *R. solani*, and *F. graminearum* respectively. As more mycelial surface area is potentially exposed to the headspace volatiles at any point in time, this could explain the stronger effect of ITC in the headspace experiments (Sarwar *et al.*, 1998).

Some studies have demonstrated that different fungal isolates respond differently to ITC. As discussed earlier, sensitivity to ITC also varies with the life cycle stage of the fungal pathogen. In one study (Ramos-Garcia *et al*., 2012), allyl, benzyl, propyl, phenyl and phenylethyl ITC were tested against *F. oxysporum*. The effect of the ITC varied across the isolates from different sources (mango leaves, jatropha seeds, coahuayote fruit, papaya, gladiolus corms). Benzyl ITC was found to be the most toxic, showing complete mycelial inhibition of two isolates at all concentrations tested (0.1, 0.3, 0.5, 1.0, 1.5, 2 µl l^{-1}). Conidia were found to be more sensitive than mycelium. Overall, 100% inhibition of conidial germination was observed with all ITC at all concentrations. However, the ITC-treated conidia and mycelia showed normal germination and growth when transferred to unamended PDA media, suggesting a fungistatic i.e., a temporary fungitoxic, effect. Similarly, Smolinska *et al*. (2003) also reported complete inhibition of germination of *F. oxysporum* conidia with pure ITC. Among the ITC tested, ethyl and allyl ITC significantly suppressed mycelial growth and chlamydospore germination. However, this effect was also fungistatic. The fungistatic effect observed in these two studies could have resulted due to the lower concentrations used. As discussed by Ramos-Garcia *et al*. (2012), a fungicidal effect was achieved in higher concentrations in their further studies. The higher sensitivity of *F. oxysporum* conidia to ITC could be due to higher penetrability of these ITC.

Sotelo *et al*. (2015) evaluated the effects of pure GSL, ITC and indoles against bacterial and fungal pathogens *in vitro* by disc diffusion assay. They found that *S.*

sclerotiorum was most susceptible to 2-phenylethyl GSL and its respective ITC (~9.5 mm growth inhibition zone) followed by allyl ITC (8.9 mm growth inhibition zone). On the other hand, 3-butenyl GSL, 2-hydroxy 3-butenyl GSL and indole-3-carbinol had the highest inhibitory effect (~11.5 mm growth inhibition zone) on *Alternaria brassicae.* The findings of this study are quite unexpected because intact GSL are non-toxic unless hydrolysed by myrosinase enzyme (Donkin *et al*., 1995). In addition to investigations on the effect of ITC on fungal growth, there are also some reports on inhibition of mycotoxin production. Nazareth *et al*. (2016) tested the efficacy of pure ITC to reduce mycotoxin production by *Aspergillus* and *Fusarium* in wheat flour. In this study, wheat flour was infected with *F. poae* and *A. parasiticus* which produced 183 mg kg^{-1} of beauvericin and enniatins, and 126 mg kg^{-1} of aflatoxins respectively in control samples. Filter papers containing allyl ITC at different concentrations (0.1, 1, 10 µl l^{-1}) were placed with infected flour in closed jars. Allyl ITC at 10 µl l^{-1} completely inhibited mycotoxin production. At the lowest concentration tested (0.1 µl l^{-1}), volatiles from the filter paper were overall more effective against aflatoxins (16-23% reduction) than enniatins (6-16% reduction), whereas 1 µl l^{-1} of allyl ITC was able to inhibit around 50% production of all mycotoxins analysed in the study.

1.6.2 Laboratory studies on brassica tissue

Brassica tissues can also be used in *in vitro* assays to investigate the impact of the volatiles on fungal growth. Such assays involve the use of realistic quantities of brassica tissue to simulate biofumigation under field conditions. The release of volatile compounds is achieved by tissue maceration or the use of 'freeze-thaw'. Brassica tissues particularly those of *B. juncea* have shown a pronounced effect against a wide range of soil-borne fungal pathogens. In one such *in vitro* assay by Kirkegaard *et al*. (1996), freeze-dried tissues of *B. napus* and *B. juncea* were tested for their suppressive effect against fungal pathogens of cereals. In Petri dishes, agar plugs of *G. graminis*, *B. sorokiniana*, *P. irregulare* and *R. solani* were exposed to brassica root, shoot and seed meal tissues. *Brassica juncea* shoots harvested at flowering stage were found to be the most suppressive. At the highest rate, 500 mg tissue, *B. juncea* shoot completely inhibited *G. graminis* growth while *B. juncea* shoot and root tissues caused ~50% suppression of *F. graminearum*. This relates to the ITC profile detected in the headspace where *B*. *juncea* tissues at flowering contained allyl ITC and only *B. napus* root tissues contained 2-phenylethyl ITC.

These two ITC were not detected from tissues at maturity which relates to the low suppression (less than 25%) observed for all the fungal species tested in the study. Seed meal of *B. juncea*, on the other hand, had the most suppressive effect with almost 100% suppression for all the fungal species at a rate of 25 mg of tissue added and this was also related to the 10 times higher levels of ally ITC detected in the *B. juncea* seed meal than in the shoot tissue. Overall, *B. juncea* was found to be more suppressive than *B. napus*, and *G. graminis* and *R. solani* were the most sensitive pathogens to the volatiles while *F. graminearum* had intermediate sensitivity. In another *in vitro* study (Charron and Sams, 1999), macerated leaves of different *Brassica* species were tested against *P. ultimum* and *R. solani*. *Brassica juncea* (10 g macerated tissue in 500 ml jar) completely suppressed radial growth of *P. ultimum* and reduced *R. solani* radial growth by 73% in the inverted Petri dishes. They found allyl ITC to be the predominating compound (>90%) in the volatiles measured from *B. juncea* tissues. When the fungal pathogens were exposed to pure allyl ITC (3.3 µmol l^{-1}), an almost similar effect was observed with 100% and 55% inhibition of mycelial growth of *P. ultimum* and *R. solani* respectively.

In laboratory studies, Neubauer *et al*. (2014) investigated the antifungal potential of ITC and brassica tissues against *V. dahliae*. When pure ITC were added to sterilised quartz sand, artificially infested with *V. dahliae* microsclerotia, benzyl ITC and 2-phenylethyl ITC were found to be the most suppressive with LD_{90} values of 8.4 and 23 nmol g^{-1} sand, respectively, whereas for methyl ITC and allyl ITC, higher LD_{90} values (89 nmol g^{-1} sand) were determined. In another part of this study, where freeze-dried, macerated shoot tissues of brassicas were mixed with 200 g artificially infested sterile quartz sand (200 *V. dahliae* microsclerotia g^{-1} sand) in sealed flasks, *B. juncea* tissue (0.6 g) was found to be the most effective causing up to 80% reduction. However, *B. juncea* caused 10-66% reduction of viable microsclerotia in soils naturally infested with *V. dahliae.* Similarly, allyl ITC at 150 nmol g^{-1} sand showed 100% reduction of microsclerotia in artificially infested quartz sand whereas in natural soils, suppression levels ranged from 9 to 92%. The varying efficacy in artificial and natural infestation was associated with sorption of ITC in organic matter and microbial degradation of ITC. Therefore, it should be kept in mind that much higher concentrations of ITC than those determined in laboratory bioassays may be necessary to achieve sufficient biofumigation effect against *V. dahliae*. Similarly, in another bioassay with seed meals (Neubauer *et al*., 2015), the group of researchers found that suppression of *V. dahliae* was lower in naturally infested soils. Complete suppression was observed in artificially infested quartz sand with *B. juncea* seed meal at an application rate of 0.4% (vol/vol), while in natural soils, the suppression

was lower (15 to 80% suppression). They also determined the GSL content of these seed meals detecting up to 110 µmol g^{-1} (tissue) of sinigrin. In addition, ITC release efficiency (based on sinigrin content) of 100% and 95% was determined in *B. juncea* seed meals derived from the cultivars Energy and Terrafit respectively.

Ojaghian *et al*. (2012) tested tissues of three *Brassica* species, *B. juncea*, *B. napus* and *B. campestris*, against *S. sclerotiorum*. *Brassica juncea* was found to be the most effective, inhibiting mycelial growth and sclerotia formation up to 90% *in vitro*. Handiseni *et al*. (2016) also successfully demonstrated antifungal potential of brassica against *R. solani*. *Brassica juncea* tissue (3 g in Petri dish) was found to be the most effective causing >90% inhibition of mycelial growth. In another part of this study, *R. solani* mycelial plugs in Petri dishes were exposed to *B. juncea*-amended soil in sealed bags. Approximately 50% inhibition was achieved at the highest incorporation rate tested, 3.2% (wt/wt). Usually for storage, brassica tissues are recommended to be frozen using dry ice (-80ºC) or liquid nitrogen (-196°C) (Wathelet *et al*., 2004). This allows to stop the enzymatic activity, preventing GSL conversion to ITC. In this study, brassica biomass were stored at -20ºC before maceration, which might have reduced the effectiveness of the brassicas as some hydrolysis could have already occurred during storage. However, despite the non-ideal storage conditions, a higher suppression was achieved.

1.6.3 Glasshouse/microcosm studies

In a glasshouse study (Mazzola *et al*., 2007), *B. juncea* seed meal suppressed *R. solani* but the effect was dependent on the timing of inoculum introduction. When orchard soil was amended with the seed meal the same time the inoculum was added, apple root infection by *R. solani* was reduced by 84%. In contrast, when the inoculum was introduced after 24 hours of seed meal incorporation, the infection was similar to untreated soils. This could be explained by the immediate release of toxic volatiles following biofumigant amendment. As evident from a previous report (Gimsing and Kirkegaard, 2006), following incorporation of *B. juncea* and *B. napus* (500-700 g m^{-2} dry biomass), the highest concentration of ITC (10-80 nmol g^{-1}) were detected immediately (30 min) and decreased rapidly during the first 4 days (<5 nmol g^{-1}). Nevertheless Mazzola *et al*. (2007) observed that after seed meal amendment, when *R. solani* inoculation was further delayed for 4 weeks, the suppression was restored and it was significantly similar to the treatment where pathogen infestation and application of amendment occurred at the same time. The

effect was attributed to proliferation of soil bacteria during the 4-week delay period. The increase in soil microflora could be related to the effect arising from addition of organic matter to the soil. Soil microflora enhanced by brassica incorporation has been linked to suppression of fungal pathogens. This is evident from a previous study (Cohen *et al*., 2005), where incorporation of *B. napus* seed meal having a low total GSL content (22 µmol g^{-1}), increased *Streptomyces* spp. numbers from 10^5 to $6x10^6$ cfu g^{-1}. The suppression of *R. solani* observed after 4 weeks could be due to antagonism and competition by soil microflora such as *Streptomyces* spp., which are known to produce antibiotics against *R. solani* (Rothrock and Gottlieb, 1981). The suppression observed after the delay could also be explained by the production of other toxic substances that are released later compared to ITC production. For instance, in a laboratory-based study (Bending and Lincoln, 1999), a variety of non-GSL compounds such as dimethyl sulphide, dimethyl disulphide, carbon disulphide and methanethiol were produced at concentrations ranging from 39-992 nmol g^{-1} (leaf dry weight) after 2-12 days of *B. juncea* incorporation in soil. These compounds have shown biocidal properties against pests and pathogens including nematodes and fungi (Curto *et al*., 2014; Lewis and Papavizas, 1971) as discussed earlier.

In another green manure simulation study, Lazzeri and Manici (2001) investigated the effect of the GSL-containing plants *Iberis amara, Rapistrum rugosum* and *Cleome hassleriana*- against *Pythium* spp. At the stage of full flowering, the above-ground parts of these plants were chopped and incorporated to naturally infested soil in pots. This decreased populations of *P. deliense* and *P. ultimum* from a starting level of about 300 cfu g^{-1} (dry soil) to ≤ 40 cfu g^{-1} (dry soil). The reduction in *Pythium* spp. was lower or similar to that of untreated soil. However, it was not evident from the results whether the levels of *Pythium* spp. population resulting from the biofumigant treatment was significantly lower than that of the untreated soil. Although the suppressive effect of these biofumigant plants on *Pythium* spp. might not be too different from that of the untreated soils, these biofumigant plants were found to be comparatively more efficient in increasing the soil microflora. In contrast to the three biofumigant treatments, the two green manure controls, *Crambe abyssinica* (GSL plant with low fungitoxic activity) and *Helianthus annuus* (non-GSL plant) increased *Pythium* spp. populations to about 3000 cfu g^{-1} dry soil. In terms of soil microflora, *Cleome hassleriana*, increased total soil fungal population by as high as 600% in contrast to the untreated soil where the total soil fungal population either remained the same or decreased after 10 weeks. The total soil fungal population mostly comprised of pathogenic and non-pathogenic saprophytes such as species of *Fusarium*, *Aspergillus* and *Trichoderma*. These saprophytes may have competed

with *Pythium* spp. providing an additional suppressive effect of the biofumigant plants. In this study, all green manure treatments increased the total soil fungal population, but the two green manure controls (*Crambe abyssinica, Helianthus annuus*) also stimulated *Pythium* spp., indicating these cover crops provided a good substrate for *Pythium*. This indicates that the three biofumigant crops tested, could be used as cover crops without the risk of increasing *Pythium* populations. Whilst the biofumigants tested in this study may be useful for suppressing *Pythium* spp., they may also increase other fungal pathogens such as *F. oxysporum* and *F. equiseti*. Unfortunately, the data on total soil fungi cannot be used to assess the suppression of particular fungal pathogens.

1.6.4 Field studies

Several field experiments have also been conducted to investigate the antifungal potential of different brassica plants. Different effects have been observed on inoculum and disease levels depending on the pathogen and the brassica species used. Researchers have also used additional approaches such as tarping the field following brassica incorporation to prevent loss of volatiles. In a field study, Subbarao *et al*. (1999) reported suppression of Verticillium wilt in cauliflower by broccoli residue incorporation in comparison to metam sodium, chloropicrin and other control treatments. Prior to cauliflower drilling, broccoli residues were incorporated at a rate of 205 kg plot^{-1}. At crop maturity, *V. dahliae* was recovered from 5 out of 8 sampled plants in broccoli treatments (with and without tarp) and metam sodium treatment. The chloropicrin and control treatments showed higher infection as observed in 6 and 7-8 plants (out of a total of 8 sampled plants) respectively. The soil population of *V. dahliae* microsclerotia was reduced by 50-75% by broccoli incorporation treatments, compared to pre-treatment levels, with most reduction occurring in the first 4 weeks. There was no difference in suppression levels between tarped and non-tarped broccoli treatments. In contrast, the synthetic fungicides initially decreased microsclerotia levels but later increased the number of microsclerotia; an increase as high as 130% in case of metam sodium treatment and 200% in chloropicrin treatment. The authors suggest that the increase in the microsclerotia levels could be due to soil chemical treatments having a reduced suppressive effect against inoculum held in larger chunks of debris. However, as microsclerotia are survival structures (Huisman and Ashworth, 1974), the increase could have also resulted as a stress-response to the chemical

treatment (Georgiou *et al*., 2006). In one of the two field experiments in this study (Subbarao *et al*., 1999), the number of harvestable cauliflower heads in the broccoli residue treatments was significantly higher than control treatments but not significantly different to the number of harvestable heads in the two conventional fungicides.

In another study, Larkin and Griffin (2007) investigated the efficacy of brassica crops including *B. juncea, B. napus, B. rapa, R. sativus* and *S. alba* to control soil-borne pathogens of potato. In the *in vitro* bioassays, macerated tissues of brassicas were tested against soil-borne pathogens including *R. solani*, *Phytophthora erythroseptica*, *S. sclerotiorum*, *F. oxysporum* and *F. sambucinam*. *Brassica juncea* leaf tissue resulted in 70-80% radial growth inhibition of *Fusarium* species and 90-100% inhibition of other pathogens. Moreover, leaves of *B. napus* and *B. rapa* caused 40-45% inhibition of *P. erythroseptica*. Overall, macerated leaf material was more effective than root tissues. In the field experiments of this study, *B. juncea* reduced *Spongospona subterranea* (powdery scab) inoculum by 26%. *Brassica juncea* also reduced disease incidence and severity of powdery scab and common scab (caused by *Streptomyces scabiei*) by 40% and >20% respectively. On the other hand, *B. napus* effectively showed >70% reduction of black scurf incidence and severity (caused by *R. solani*). However, non-brassica crops such as ryegrass also showed suppressive effects (20-40%). This may be due to factors such as, enhanced soil microbial communities, possibly due to increased organic matter content. This is evident from studies where organic amendment to the soil increased soil microbial mass (El-Sharouny, 2015).

In some cases, brassica amendments have been found to stimulate plant pathogens. For instance, Njoroge *et al*. (2008) observed that amending soil with *B. juncea* and *B. napus* stimulated *F. oxysporum* and *Pythium* spp. growth in field experiments. The investigators used plastic mulch which also did not make the amendment more efficacious. One possible reason could be low levels of sinigrin (0.22-0.31 μmol g^{-1}) detected in *B. juncea* tissues in this study. Sinigrin, the parent GSL of allyl ITC, is mostly found to be the predominant GSL in *B. juncea*, and allyl ITC has been previously shown to suppress *F. oxysporum* in *in vitro* assays (Smolinska *et al*., 2003). Lack of suitable ITC profile among other factors may result in inefficacy of biofumigants.

A number of fungal pathogens have shown sensitivity to brassica biofumigants. In some cases brassica incorporation showed similar or better suppression than synthetic fungicides. *Brassica juncea* with sinigrin as the predominant GSL, has

shown good suppression activity against a wide range of soil-borne pathogens in these studies. Different fungal species have been shown to respond differently to brassica biofumigants. Effect of ITC also varied between the isolates of the same species. Selection of brassica plant with the most suitable GSL profile is a major factor affecting the outcome of biofumigation. In the field, to achieve a successful biofumigation effect, the biofumigant crop establishment and growth should be considered. Moreover, the approach needs to be optimised based on the target pathogen while also considering environmental factors, such as, temperature and moisture content of soil.

1.7 Identifying the research gap and objectives

The main aim of this research was to investigate the biofumigation potential of brassicas to suppress *Fusarium graminearum* inoculum in soil and thus suppression of *F. graminearum* disease and mycotoxin production in subsequent cereal crop. *Fusarium graminearum* mainly overwinters as mycelium in infected crop debris which serves as the primary inoculum for head blight disease in cereals. Currently, *F. graminearum* is managed through triazole fungicides, however, there is a serious concern due to their limited effectiveness and the high selection pressure for fungicide resistance. Additionally, evidence suggesting endocrine-disrupting potential of triazoles has raised serious concerns which may lead to restrictions on the use of triazoles. Therefore, there is a need to explore an alternative safer management strategy. Whilst biofumigation has attracted significant interest, research on its potential application for reducing the inoculum of *Fusarium* species affecting cereals is scarce. The existing literature suggested that there is a need for a comprehensive study to understand the potential of the biofumigation system for the management of *F. graminearum*.

Objectives:

1. Develop a selective media for *Fusarium graminearum*
2. Determine the efficacy of isothiocyanates to inhibit *Fusarium graminearum* isolates *in vitro*
3. Determine the efficacy of brassica species to inhibit *Fusarium graminearum* isolates *in vitro*
4. Determine the efficacy of brassica species to inhibit *Fusarium graminearum* in debris in microcosm experiments
5. Evaluate whether biofumigation can inhibit *Fusarium graminearum* colonised debris under field conditions in a wheat-maize rotation

Chapter 2

Chapter 2

2 Screening of fungicides and comparison of selective media for isolation of *Fusarium graminearum* from soil and plant material

2.1 Abstract

Culture media recommended for the isolation and enumeration of *Fusarium* spp. are not very selective for *Fusarium graminearum*. In the present study, five fungicides - Amistar® (250 g l^{-1} azoxystrobin), Filan® (500 g kg^{-1} boscalid), Comet® 200 (200 g l^{-1} pyraclostrobin), Imtrex® (62.5 g l^{-1} fluxapyroxad), Poraz® (450 g l^{-1} prochloraz) - were investigated for their potential as selective inhibitors in culture media for the isolation of *F. graminearum* from soil and plant material. Based on the screening, Imtrex® was further tested for selective inhibition for the isolation of *Fusarium graminearu*m from soil. Additionally, selective media were compared for isolation of *Fusarium graminearum* from plant material. The fungicides did not prove to be effective inhibitor for the development of selective media. For detection of *Fusarium graminearum* in plant materials, modified Czapek Dox iprodione dichloran agar was found to be better medium than Komada's media, as the former resulted in colonies with darker pigmentation over a shorter incubation time.

2.2 Introduction

A number of selective media have been developed for the isolation and enumeration of *Fusarium* species. The most widely used selective medium for *Fusarium* is the Nash and Snyder (1962) medium which contains pentachloronitrobenzene (PCNB) as the selective agent. It partially inhibits a variety of fungal species while allowing the growth of *Fusarium* species along with a few other fungal species. Some modifications of this medium have been published. For example, modification of the Nash and Snyder medium through the addition of benomyl, increased the selectivity for *F. solani* (Hall, 1981). Other PCNB-containing media selective for *Fusarium* are the Papavizas medium (Papavizas, 1967) and Komada's medium (Komada, 1975), the latter being preferred when the target organism is *F. oxysporum*. Rose bengal-glycerine-urea medium developed by van Wyk *et al.* (1986) which also contains PCNB, is as selective for *Fusarium* as the Komada and Nash and Snyder media but with the advantage that the colonies are easier to distinguish due to being transparent. Malachite green agar 2.5 is reported to be a better selective medium for *Fusarium* as it is restrictive to other fungal genera (Castella *et al*., 1997). The concentration of malachite green dye used in the medium is important because a previously higher concentration of malachite green (15-50 mg kg^{-1}) used in the Singh-Nene medium restricted the germination of many *Fusarium* spores (Singh and Nene, 1965). Malachite green agar maintains the morphological characteristics of the colonies eliminating the necessity to subculture the colonies onto other media for identification. Bragulat *et al*. (2004) also found malachite green agar 2.5 to be a useful selective medium for isolation of *Fusarium* spp. from naturally contaminated samples, when compared with different media like Nash and Snyder, modified Czapek Dox agar and potato dextrose iprodione dichloran agar. Similarly, for the isolation and enumeration of *Fusarium* species from grains, Alborch *et al*. (2010) recommended the use of malachite green agar 2.5 as an alternative to Nash and Snyder medium as no differences were observed in the recovery of *Fusarium* species in both media. Malachite green is used to replace PCNB in culture media but carcinogenic properties of malachite green are evident in experimental animals (Culp *et al*., 2006; Rao and Fernandes, 1996). Moreover, previous studies suggest that malachite green is a multi-organ toxin (Srivastava *et al*., 2004). However, malachite green has recently been classified in Group 3 "not classifiable as to its carcinogenicity to humans" by the International Agency for Research on Cancer (IARC, 2021). A drawback of this dye is that it is deactivated upon exposure to light which might affect its antifungal activity (Vujanovic *et al*., 2002).

Isolation of *F. graminearum* on Nash and Snyder medium requires subculture of the fungus onto potato dextrose agar (PDA) medium as the former medium often fails to maintain the morphological characteristics of *F. graminearum.* To avoid the requirement of more than one medium which might lead to identification errors, Segalin and Reis semi-selective medium for *F. graminearum* detection in seed samples was developed (Segalin and Reis, 2010). This medium, which contains triadimenol among other inhibitory compounds, was as selective and sensitive for *F. graminearum* as Nash and Snyder medium but with the advantage of maintaining colony morphology, thus facilitating *F. graminearum* identification. In another study (Thompson *et al*., 2013), *F. graminearum* showed poor growth in malachite-containing media except for malachite green agar medium supplemented with carnation leaf pieces, in which better growth and sporulation of the fungus was observed. A selective media for *F. graminearum* was developed using the bacterial toxin, toxoflavin, as the selective agent (Jung *et al*., 2013). The toxin which is produced by rice pathogen, *Burkholderia glumae*, is inhibitory against many fungi including *Aspergillus*, *Colletotrichum* and *Penicillium* but *Fusarium* species, particularly, *F. graminearum* was found to be highly resistant to this toxin. However, *F. verticillioides* and *F. oxysporum* were also found to be resistant to the bacterial toxin, thus the toxoflavin-based medium may not prove very selective for *F. graminearum* in natural samples contaminated with the two *Fusarium* species. The important characteristics of various selective media developed for *Fusarium* species are summarised in Table 2.1.

These different types of media are useful either for identifying *Fusarium* at the genus level or as a semi-selective media for *Fusarium* spp. *Fusarium graminearum* is a globally important cereal pathogen that causes head blight in wheat, resulting in yield losses and mycotoxin contamination (McMullen *et al*., 2012). There is a need to isolate *F. graminearum* from soil and plant debris and because these types of samples have a very diverse and large number of fungal flora, a highly selective medium for *F. graminearum* is needed. This fact prompted the need to investigate the potential of a range of fungicides as selective inhibitors in culture media for the isolation of *F. graminearum* from soil and plant material. The efficacy of selective media was also compared for the isolation of *F. graminearum* from wheat debris.

Table 2.1 Selective media for isolation of *Fusarium* species

Medium	Selectivity	Inhibitor	Source	Reference
Nash and Snyder	*Fusarium* sp.	PCNB	soil	Nash and Snyder, 1962
Papavizas	*Fusarium* sp.	PCNB	soil	Papavizas, 1967
Komada	*Fusarium* sp., *F. oxysporum*	PCNB	soil	Komada, 1975
Rose bengal-glycerine-urea	*Fusarium* sp.	PCNB	soil	van Wyk *et al.,* 1986
Dichloran-chloramphenicol peptone agar	*Fusarium* sp., dematiaceous hyphomycetes	dichloran	seed	Andrews and Pitt, 1986
Czapek Dox iprodione dichloran agar	*Fusarium* sp.	iprodione, dichloran	seed	Abildgren *et al*., 1987
Malachite green agar 2.5	*Fusarium* sp.	malachite green dye	cereal substrates, feedstuff	Castella *et al*., 1997
Myclobutanil agar	*Fusarium* sp.	myclobutanil	soil	Vujanovic *et al*., 2002
Segalin and Reis	*F. graminearum*	iprodione, nystatin, triadimenol	seed	Segalin and Reis, 2010
Toxoflavin-based medium	*F. graminearum*	toxoflavin	seed, air	Jung *et al*., 2013

2.3 Materials and Methods

2.3.1 Fungal isolates

Three strains of *F. graminearum* (FG2556, FG2498, FG2481), supplied by Dr Phil Jennings, Fera Sciences Ltd, were isolated from wheat samples collected in 2016.

2.3.2 Preparation of *Fusarium graminearum* conidial suspension

Conidial suspensions were prepared following the method reported by Edwards and Seddon (2001) with some modifications. The three isolates of *F. graminearum* were sub-cultured onto PDA (Merck) using 10 plates for each isolate and incubated at room temperature (ca. 20°C). After 14 days, conidia were harvested by adding 20-25 ml sterile distilled water to each plate and dislodging conidia using a sterile spreader. The suspension was filtered through Miracloth (EMD Millipore Corp., Billerica MA, USA) to remove mycelium. The filtrate was centrifuged at 3000 *g* for 10 min. Supernatant was removed and conidia re-suspended in 15 ml sterile distilled water and centrifuged at 3000 *g* for 10 min. Conidia were washed twice and re-suspended in 15 ml sterile distilled water. Conidia of all three isolates were mixed in the final conidial suspension. Conidia were counted using an Improved Neubauer counting chamber (Weber 99, Scientific International, Teddington, UK) and the concentration adjusted to 10^5 spores ml^{-1}.

2.3.3 Media preparation

2.3.3.1 Czapek Dox propiconazole dichloran agar

Czapek Dox propiconazole dichloran agar (CZPD) was prepared as described by Hofgaard *et al*. (2016). CZPD media contained (per L distilled water): 48 g Czapek Dox agar (Sigma-Aldrich), 1 ml of 0.2% dichloran (Aldrich) solution in ethanol, 1 ml of 5% chloramphenicol (Sigma) solution in ethanol, 1 ml of trace metal solution (1 g $ZnSO_4*7H_2O$ [Fisher Scientific] + 0.5 g $CuSO_4*5H_2O$ [Fisher Scientific] per 100 ml distilled water), 10 ml of filter-sterilised 0.5% chlortetracycline hydrochloride (Sigma) solution, 1 ml of 0.3% Bumper® suspension (containing 750 µg propiconazole).

Chlortetracycline and Bumper® solution were added after autoclaving and tempering the media to 55°C.

2.3.3.2 Komada's media

Komada's media (Komada, 1975) contained (per L distilled water): 1 g K_2HPO_4 (Acros Organics), 0.5 g $MgSO_4.7H_2O$ (Fisher Scientific), 0.5 g KCl (Acros Organics), 0.01 g Fe-Na- ethylenediamine tetraacetic acid (EDTA) (Sigma-Aldrich), 2 g L-asparagine (Sigma-Aldrich), 20 g D(+)-galactose (Sigma-Aldrich), 15 g agar (Lab M Ltd),1 g PCNB (Aldrich). After autoclaving and tempering to 55°C, the following were added after filter sterilisation: 1 ml of Oxgall solution (5 g Oxgall powder [Sigma-Aldrich] + 10 g $Na_2B_4O_7.10H_2O$ [Fisher Scientific] per 100 ml sterile distilled water), 5 ml of 6.5% streptomycin sulfate (Sigma-Aldrich) solution.

2.3.4 Screening of fungicides for selectivity

2.3.4.1 Incorporation of fungicides into media

Five fungicides - Amistar® (250 g l^{-1} azoxystrobin), Filan® (500 g kg^{-1} boscalid), Comet® 200 (200 g l^{-1} pyraclostrobin), Imtrex® (62.5 g l^{-1} fluxapyroxad), Poraz® (450 g l^{-1} prochloraz) - were used. PDA media was amended with the fungicides to achieve the concentrations of the active ingredients (a.i.) at 0.001, 0.01, 0.1, 1, 10, 100, 1000 mg l^{-1}. For uniform distribution of the fungicide within the medium, fungicide suspensions in sterile distilled water were first vortexed. This was then immediately transferred to molten PDA agar (at 50°C) and then shaken gently before pouring. Control plates contained PDA treated with sterile distilled water in place of fungicide.

2.3.4.2 Inoculation of fungicide-amended media plates

(a) Mycelial plugs: Agar plugs of 7 mm diameter were cut from outer margin of the three actively growing *F. graminearum* isolates growing on PDA. These were transferred to the centre of 9 cm Petri dishes of the untreated and fungicide-amended media plates with mycelium facedown. The plates were incubated at room temperature (ca. 20°C) and radial colony growth was measured after 5 days.

(b) Conidial suspensions: A 10^5 spores ml^{-1} stock of *F. graminearum* was prepared as described above and diluted to 10^4 and 10^3 spores ml^{-1} in sterile distilled water. Plates of the control and fungicide-amended media were spread with 100 µl of the stock and the two dilutions. The plates were incubated at room temperature (ca. 20°C) and *F. graminearum* colony forming units (cfu) were enumerated after 24-48 hours.

(c) Soil suspension: A 10% stock soil suspension in sterile distilled water was prepared and diluted to 1% and 0.1%. Plates of the control and fungicide-amended media were spread with 100 µl of the stock and the two dilutions. The plates were incubated at room temperature (ca. 20°C) and observed for colony growth.

2.3.5 Further testing of selected fungicides with CZPD

1st screening

Based on section 2.3.4., two fungicides - Comet® 200 and Imtrex were selected for further screening. CZPD media was prepared as described above and amended with (i) Comet® 200 a.i. 10 mg l^{-1}, 100 mg l^{-1} and (ii) Imtrex® a.i. 10 mg l^{-1} and inoculated as described in 2.3.4.

2nd screening

Comet® 200 and Imtrex® at lower concentrations were tested. CZPD media was amended with the two fungicides to achieve the concentrations of the a.i. at 0.001, 0.01, 0.1, 1 mg l^{-1} and inoculated as described in 2.3.4.

2.3.6 Attempts at isolation of *Fusarium graminearum* from soil using potential selective inhibitor

Based on 2.3.5., Imtrex® a.i 1 mg l^{-1} CZPD was selected for further testing. CZPD media was prepared as described above and another set of CZPD media was amended with Imtrex® a.i. at 1 mg l^{-1} concentration after autoclaving and cooling the media to 50°C. A 10^5 spores ml^{-1} stock of *F. graminearum* was prepared as described above and was diluted using sterile distilled water and soil suspension. The soil suspension was prepared by adding 5 g fresh soil (flower bed soil collected

from Harper Adams University, UK premises) to sterile distilled water to give a final volume of 50 ml and mixed well. Aliquots of the (a) soil suspension and (b) sterile distilled water were used to dilute the conidia stock solution to 10^4, 10^3 and 10^2 spores ml^{-1}. Triplicate plates of CZPD and Imtrex®-amended CZPD were spread with 100 µl of the dilutions. Later cfu were counted on the plates after 5-8 days incubation at room temperature (ca. 20°C). Growth of *F. graminearum* in plates spread with dilutions from soil suspension was recorded based on the characteristic reddish pink pigmentation. Assumed *F. graminearum* colonies were subcultured on PDA media plates and incubated at room temperature (ca. 20°C) for 14 days. The conidia were harvested as described above and confirmed as *F. graminearum* based on spore morphology (Leslie and Summerell, 2006). As it was not possible to confirm all the assumed *F. graminearum* colonies, about 5-10% representative sample colonies were used for confirmation.

2.3.7 Comparing selectivity of media using *Fusarium graminearum*-infected wheat debris

Four types of media were compared for their selective efficacy to isolate *F. graminearum*. (a) CZPD (b) Imtrex® a.i. 1 mg l^{-1} of CZPD (c) Imtrex® a.i 5 mg l^{-1} of CZPD and (d) Komada's media were prepared as described above. Wheat debris (chaff, straw, rachis) were collected post-harvest from a *F. graminearum*-inoculated wheat experiment at the research facilities of Harper Adams University, Newport, Shropshire, UK. The debris were surface sterilised with sodium hypochlorite (1.2% available chlorine) containing 0.05% Tween 20 for 3 min and washed three times with sterile distilled water. Five debris were placed per plate in five plates of each of the four media types. Plates were incubated at room temperature (ca. 20°C) and *F. graminearum* growth was observed after 7-14 days. *Fusarium graminearum* colonies were confirmed as described above.

2.4 Results

2.4.1 Selectivity of fungicides

Preliminary results from screening of the five fungicides (Amistar®, Filan®, Comet® 200, Imtrex®, Poraz®) with soil suspension (Figure 2.1), and *F. graminearum* (Table

2.2) suggested that Comet® and Imtrex® should be assessed further. Comet® 200 a.i. at 10 mg l^{-1}, 100 mg l^{-1} and Imtrex® a.i. at 10 mg l^{-1} were comparatively suppressive for the non-target fungi and had a weak effect on *F. graminearum* growth (Figure 2.2). Poraz® at the two highest tested concentrations also hindered the growth of the non-target fungi, however, it was inhibitory for *F. graminearum* too.

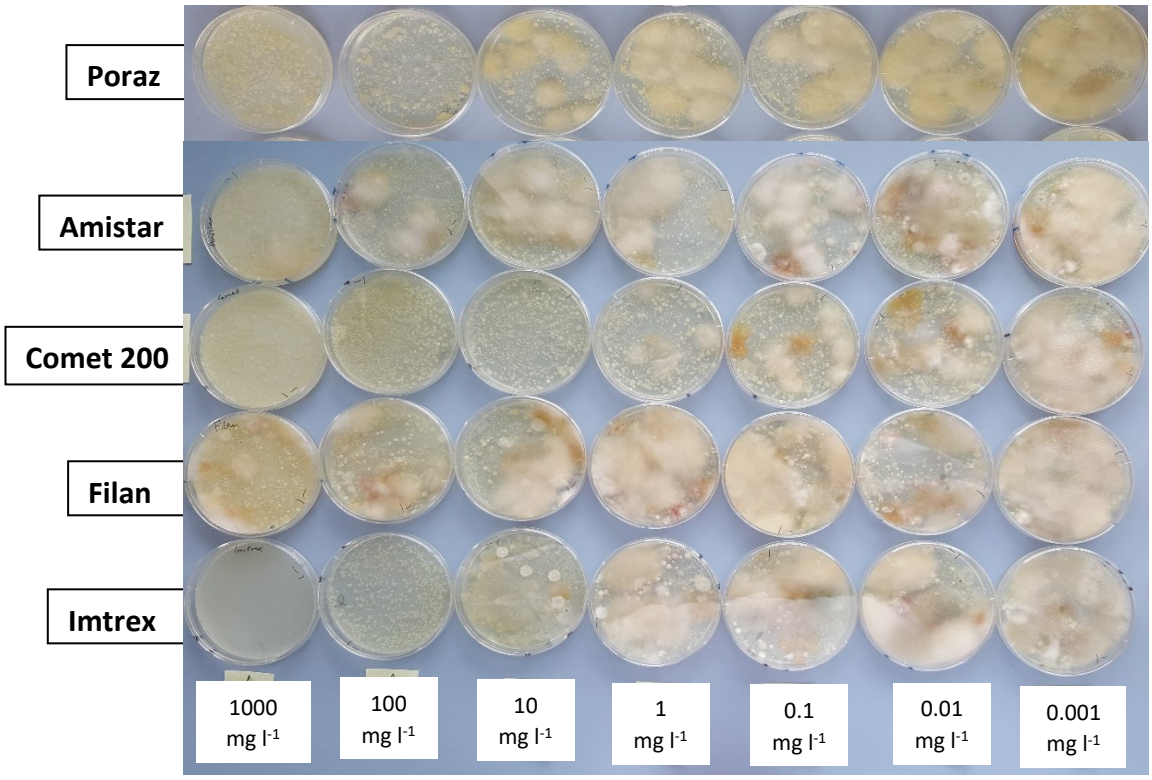


Figure 2.1 1% soil suspension (containing fungal and bacterial species which were not identified) on potato dextrose agar amended at (from right) 0.001, 0.01, 0.1, 1, 10, 100, 1000 mg l^{-1} concentrations of active ingredient of five fungicides (from top) – Poraz®, Amistar®, Comet® 200, Filan®, Imtrex® - after 5 days incubation at room temperature (ca. 20°C)

Table 2.2 Radial colony growth of *Fusarium graminearum* (FG2498) and colony forming units of *F. graminearum* (FG2556, FG2498, FG2481) on potato dextrose agar amended with different concentrations of active ingredient of five fungicides: Imtrex®, Comet® 200, Amistar®, Filan®, Poraz®. The fungicides were tested for their potential as selective inhibitor for the development of selective medium for *Fusarium graminearum*

Fungicide concentration (mg l^{-1})[a]	**0**	**0.001**	**0.01**	**0.1**	**1**	**10**	**100**	**1000**
	F. graminearum radial colony (mm)[b]							
Imtrex	58[c]	72	61	68	34	21	3	0
Comet 200	58	77	68	37	30	26	13	2
Amistar	58	77	77	67	39	30	38	40
Filan	58	65	65	74	77	77	69	75
Poraz	58	44	45	34	13	18	0	0
	F. graminearum cfu $plate^{-1}$ [d]							
Imtrex	121[e]	110	110	120	106	88	0	0
Comet 200	121	162	99	106	115	96	65	92
Amistar	121	107	113	137	123	151	106	112
Filan	121	136	134	144	171	156	121	54
Poraz	121	128	122	179	0	0	0	0

[a] Concentrations of active ingredient of fungicides in potato dextrose agar (PDA)

[b] Radial colony growth of mycelial plug from *Fusarium graminearum* culture (FG2498) after 5 days at room temperature (ca. 20°C)

[c] Mean of triplicate of unamended PDA used as control

[d] Colony forming units (cfu) per plate of *Fusarium graminearum* from conidial suspension of mixed isolates FG2556, FG2498, FG2481 (10^4 spores ml^{-1}), after 48 hours at room temperature (ca. 20°C)

[e] Mean of triplicate of unamended PDA used as control

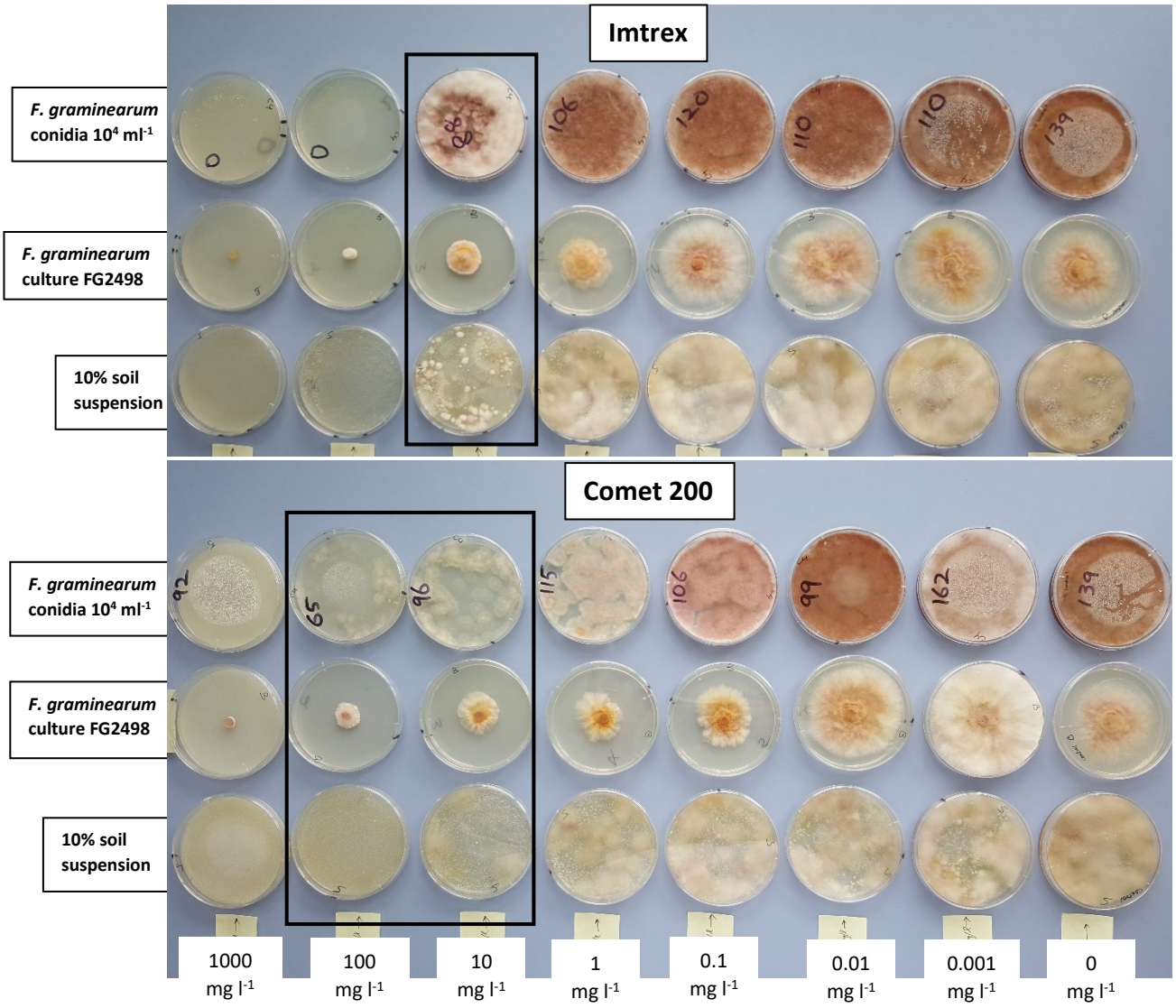


Figure 2.2 Different concentrations of active ingredient (a.i.) of Imtrex® and Comet® 200 added to potato dextrose agar (PDA) and tested for their potential as selective inhibitor for the development of selective medium for *Fusarium graminearum*. The growth of *Fusarium graminearum* from a conidial suspension (10^4 ml^{-1}), a mycelial plug from culture FG2498 and growth from 10% soil suspension on (from right) PDA-unamended, fungicide a.i at 0.001, 0.01, 0.1, 1, 10, 100, 1000 mg l^{-1} PDA after 5 days incubation at room temperature (ca. 20°C). Fungicide concentration of treatments in rectangles were selected for further screening

2.4.2 Selectivity of Comet® 200 and Imtrex®

Comet® 200 at the two concentrations (a.i. 10 mg l^{-1} and 100 mg l^{-1}) was found to be too inhibitory for *F. graminearum* (Figure 2.3). Data from media amended with lower concentrations of these two fungicides suggested that Imtrex® a.i. at 1 mg l^{-1} could be a potential inhibitor to isolate *F. graminearum* from soil and plant material (Figure 2.4).

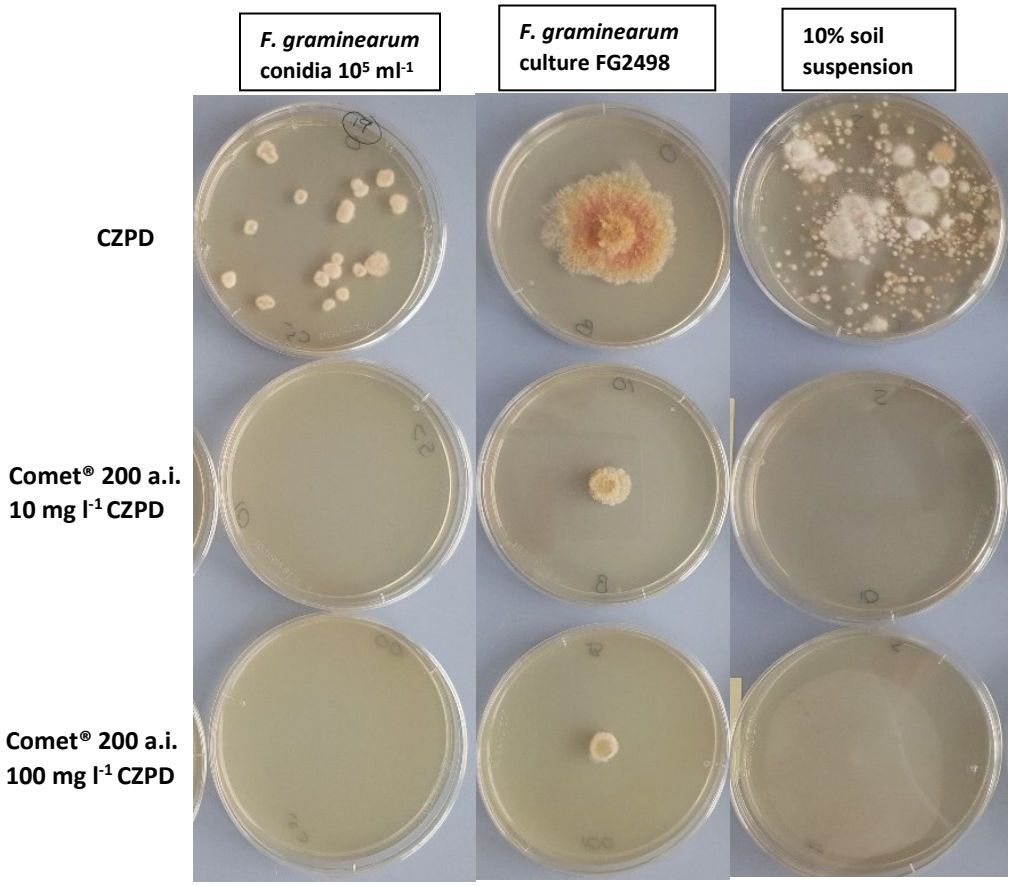


Figure 2.3 The growth of *Fusarium graminearum* from a conidial suspension (10^5 ml^{-1}) and mycelial plug from culture FG2498, and growth from 10% soil suspension on (from top) CZPD (Czapek Dox propiconazole agar), Comet® 200 a.i. 10 mg l^{-1} CZPD and Comet® 200 a.i. 100 mg l^{-1} CZPD, after 5 days incubation at room temperature (ca. 20°C)

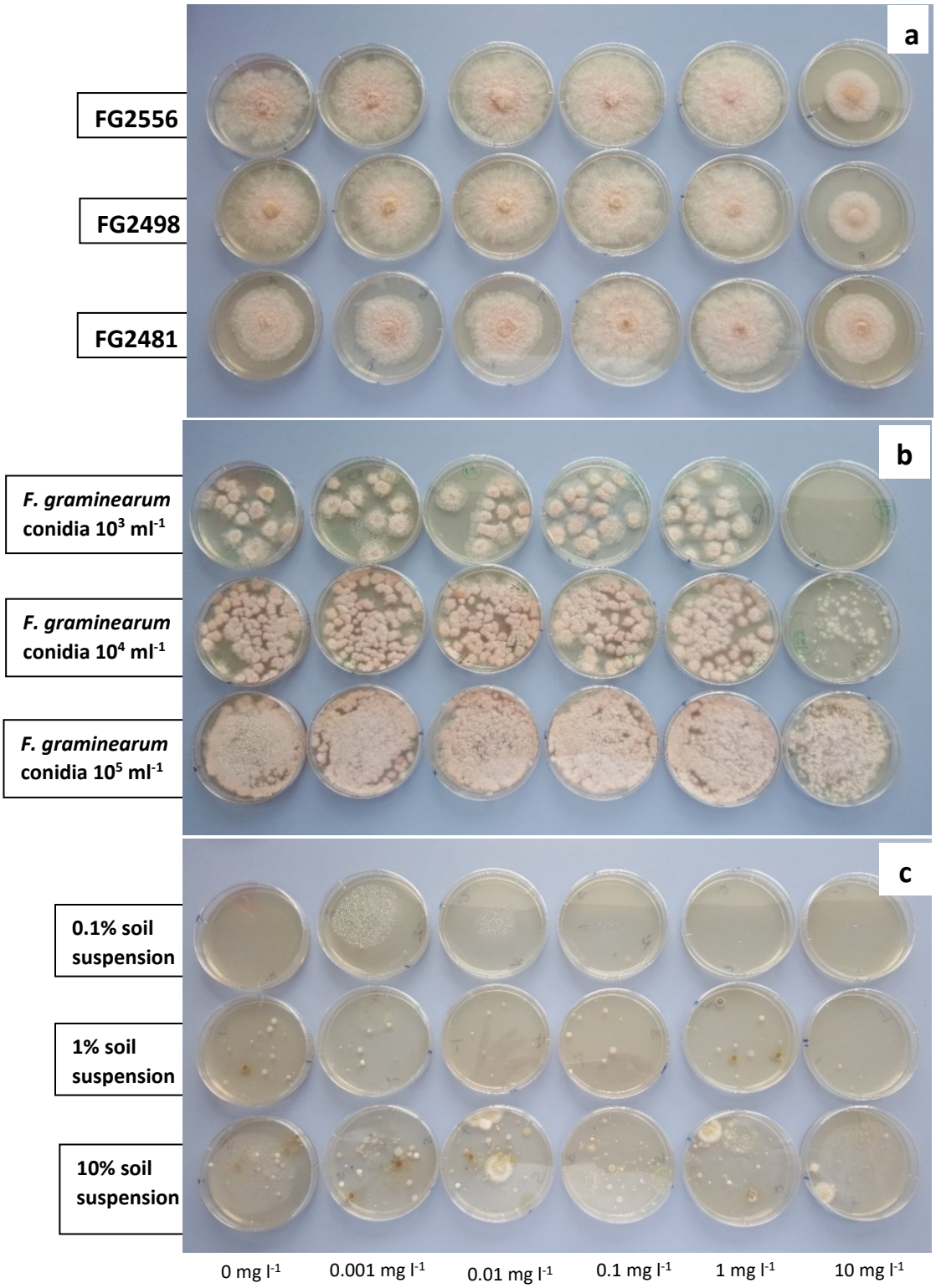


Figure 2.4 Different concentrations of Imtrex® active ingredient (a.i.) fluxapyroxad added to Czapek Dox propiconazole dichloran agar (CZPD). (a) Growth of *Fusarium graminearum* culture from mycelial plug, (b) growth of *Fusarium graminearum* from conidial suspension and (c) growth from soil suspension on (from left) CZPD, Imtrex® a.i. 0.001 mg l^{-1} CZPD, Imtrex® a.i. 0.01 mg l^{-1} CZPD, Imtrex® a.i. 0.1 mg l^{-1}CZPD, Imtrex® a.i. 1 mg l^{-1} CZPD, Imtrex® a.i. 10 mg l^{-1} CZPD after 5 days incubation at room temperature (ca. 20°C)

2.4.3 Isolation of *Fusarium graminearum* from soil using Imtrex®

Mean of values for colony counts of *F. graminearum* on unamended CZPD and Imtrex®-amended CZPD media after 8 days of incubation are presented in Table 2.3. *Fusarium graminearum* colonies on both types of media were identified by the characteristic reddish pink pigmentation. Colony counts of *F. graminearum* was not very different between the two types of media used. *Fusarium graminearum* colony count from sterile distilled water were similar to those from soil suspension on both types of media. However, the two media did not prove to be very selective as growth of soil mycobiota was more than *F. graminearum*.

Table 2.3 Colony-forming units of *Fusarium graminearum* from three dilutions of *F. graminearum* conidia in sterile distilled water and soil on media. Data given are the mean from triplicate plate counts and numbers in parentheses represent the standard error of the mean

		***F. graminearum* (cfu plate^{-1})**					
		10^4		**10^3**		**10^2**	
Media	**Diluent**	**Fg**[a]	**Other**[b]	**Fg**	**Other**	**Fg**	**Other**
CZPD[c]	Sterile distilled water	24 (3)[d]	0	2 (1)	0	1 (1)	0
	Soil suspension	28 (2)	48 (2)	7 (2)	58 (7)	3 (1)	55 (5)
Imtrex®-CZPD[d]	Sterile distilled water	32 (4)	0	3 (1)	0	1 (1)	0
	Soil suspension	26 (2)	49 (5)	10 (2)	61 (7)	2 (1)	70 (3)

a Fg, *Fusarium graminearum*

b Other, other fungal species

c CZPD, Czapek Dox propiconazole dichloran agar

d Imtrex®-CZPD, Imtrex® a.i. (fluxapyroxad) 1 mg l^{-1} of CZPD

2.4.4 Selectivity of media for isolation of *Fusarium graminearum* from wheat debris

When wheat debris used, there was not much difference among CZPD, Imtrex® amended-CZPD and Komada's media in terms of selectivity. *Fusarium graminearum* colonies became identifiable and produced dark pigmentation on CZPD (both unamended and amended) after 9-10 days (Figure 2.5). The colour of *F. graminearum* colonies on Komada's media became darker and identifiable after 17-18 days, yet not as dark as observed on CZPD after 10 days.

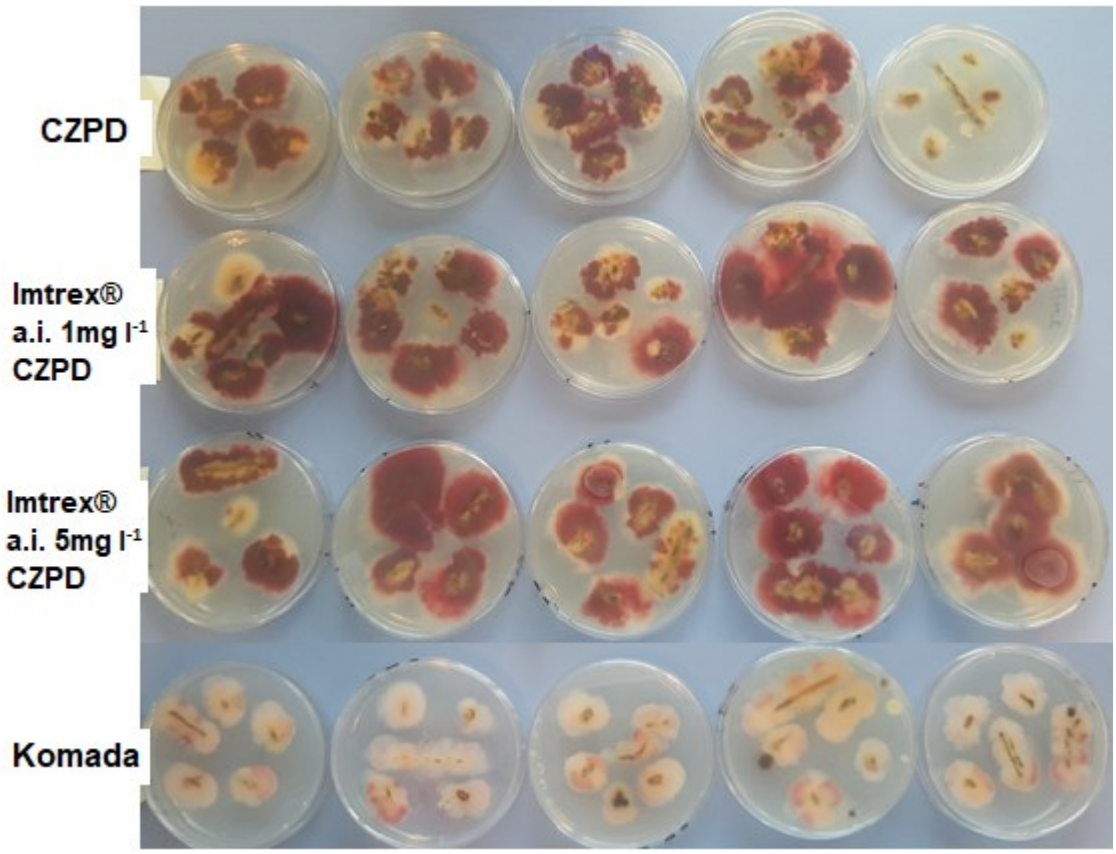


Figure 2.5 *Fusarium graminearum*-infected wheat debris on different media after 10 days (from top- Czapek Dox propiconazole dichloran agar (CZPD); Imtrex® a.i. 1 mg l^{-1} of CZPD; Imtrex® a.i. 5 mg l^{-1} of CZPD; Komada's media)

2.5 Discussion

The available selective media were introduced based on old fungicide chemistry, however, the newer fungicide chemistry reveals that strobilurins and succinate dehydrogenase inhibitor (SDHI) fungicides have poor activity towards *F. graminearum* (Frąc *et al*., 2016; Masiello *et al*., 2019). Hence it was of interest to investigate if these fungicides would improve selectivity of *Fusarium* selective media. However, the fungicides used in the present study did not prove to be effective inhibitors in culture media for the isolation of *F. graminearum* from soil. CZPD and Imtrex®-amended CZPD allowed growth of *F. graminearum* conidia in soil suspension, however they were not suppressive enough for other fungi in the soil samples.

An ideal selective medium for a specific group of fungi should promote the growth of all viable propagules of this specific fungal group and facilitate its identification and restrict the growth of unwanted microbiota. Moreover, the stability and toxicity of the added inhibitors are important factors that should be considered for the efficacy and safety of the culture medium (Bragulat *et al*., 2004). Widely used media such as Nash and Snyder (1962) and Komada's medium (Komada, 1975), contain PCNB as a fungal inhibitor. Although PCNB has been categorised as "not classifiable as to its carcinogenicity to humans" by the International Agency for Research on Cancer (IARC, 1987), the US Environmental Protection Agency has classified it as possible human carcinogen (Group C) (US EPA-OPP, 2018). Due to concern about the safety of PCNB, other alternative selective inhibitors for *Fusarium* isolation are reported such as dichloran in dichloran-chloramphenicol peptone agar (Andrews and Pitt, 1986) or a combination of iprodione and dichloran in Czapek Dox iprodione dichloran agar (CZID) (Abildgren *et al*., 1987) and potato dextrose iprodione dichloran agar (Thrane *et al*., 1992). CZID agar was recommended by Thrane (1996) to be used for detection of *Fusarium* in flour samples, as the colony morphology of *Fusarium* on this medium allowed easier identification of different *Fusarium* species. The originally published CZID (Abildgren *et al*., 1987) is here modified to CZPD (Hofgaard *et al*., 2016) where iprodione is replaced with propiconazole to make the media more stable and long lasting. Abildgren *et al*. (1987) used 3 mg of iprodione per L of CZID media, whereas in CZPD, 750 µg propiconazole per L of media has been used instead.

In the present study, when isolating *F. graminearum* from plant material, a higher concentration of Imtrex® a.i. (5 mg l^{-1}) was also tested to see if better selectivity

could be achieved. However, no observable difference was seen between the selective effect of the two concentrations of Imtrex® (1 mg l^{-1}, 5 mg l^{-1}) and unamended CZPD media. The widely recommended media, Komada's, was also included for this part of the study. CZPD (both unamended and amended) was better in terms of pigmentation and growth rate, requiring shorter incubation time of 9-10 days compared to Komada's media. Moreover, the colonies on CZPD were easily identifiable due to darker pigmentation. CZPD medium has been successfully used during the project when detecting *F. graminearum* from plant material. Their use has provided easier monitoring than the recommended Komada's media, and therefore their use for monitoring *F. graminearum*-infected plant material is recommended.

Chapter 3

Chapter 3

3 *In vitro* activity of isothiocyanates against *Fusarium graminearum*

3.1 Abstract

Isothiocyanates are biotoxic degradation products formed as a result of enzymatic hydrolysis of glucosinolates present in brassicas. The application of biofumigant brassica crops, as an alternative crop protection method for soil-borne pathogens and pests is increasingly gaining interest. However, little is known of the potential of biofumigation to reduce the inoculum of *Fusarium* species affecting cereals. The aim of this study was to evaluate the antifungal activity of five isothiocyanates, namely allyl, benzyl, ethyl, 2-phenylethyl and methyl isothiocyanates, against germination and growth of *Fusarium graminearum* under *in vitro* conditions. Aromatic isothiocyanates were more inhibitory than the aliphatic isothiocyanates against mycelial growth whereas the reverse was observed for conidial germination. Among the tested isothiocyanates, allyl and methyl isothiocyanates were overall more efficient, showing lower ED_{50} values (35-150 mg l^{-1}) for conidial germination and mycelial radial growth. The findings suggest that brassica plants containing allyl and methyl glucosinolates could have a suppressive effect on reducing the inoculum of *F. graminearum* in soil prior to cereal production.

3.2 Introduction

Historically, soil fumigation with synthetic pesticides has been used to manage soil-borne pathogens. For example, methyl bromide was widely used as soil fumigant until it was phased out under The Montreal Protocol and Clean Air Act in order to reduce damage to the ozone layer (Enebak, 2007). Metam sodium, which breaks down to methyl isothiocyanate (MITC), has been used for the control of Verticillium wilt of potato, caused by the soil-borne fungus *Verticillium dahliae* (Tsror (Lahkim) *et al*., 2005). However, fumigants are not used for treating Fusarium pathogens in cereal rotations, mainly due to cost constraints. The use of fungicides, particularly triazoles, has been the main method of *Fusarium graminearum* management. However, there is a serious concern due to their limited effectiveness and the high selection pressure for fungicide resistance (Becher *et al*., 2010; Klix *et al*., 2007; Yerkovich *et al*., 2020). Additionally, evidence suggesting endocrine-disrupting potential of triazoles (Lv *et al*., 2017; Poulsen *et al*., 2015) has prompted the quest to find alternative safer management strategies. Currently, control strategies such as biopesticides and biofumigation have been gaining interest due to their safer effect on environment and health. As mentioned earlier, the focus of interest in biofumigation is the isothiocyanates (ITC) owing to their biopesticidal properties. Isothiocyanates have toxic effects against a broad range of noxious organisms including nematodes (Dahlin and Hallmann, 2020; Wood *et al*., 2017), weed (Norsworthy and Meehan, 2005) and fungi (Smolinska *et al*., 2003). Each brassica species has a specific GSL profile, which results in the corresponding ITC profile. Studies have linked glucosinolates (GSL) content with the biotoxic effects on pathogens (Charron and Sams, 1999). Previous studies on individual ITC have shown promising fungitoxic potential against fungal pathogens such as *Rhizoctonia solani*, *Gaeumannomyces graminis* var. *tritici* and *F. oxysporum* (Sarwar *et al*., 1998; Smolinska *et al*., 2003; Ramos-Garcia *et al*., 2012). Isothiocyanates such as allyl isothiocyanate (AITC), benzyl isothiocyanate (BITC), propyl isothiocyanate and phenyl isothiocyanate have been shown to inhibit conidial germination and mycelial growth of *F. oxysporum* (Ramos-Garcia *et al*., 2012). Similarly, Smolinska *et al.* (2003) reported complete inhibition of germination of *F. oxysporum* conidia with pure ITC such as ethyl ITC (EITC) and AITC. Whilst biofumigation has attracted significant interest, research on its potential application for reducing the inoculum of *Fusarium* species affecting cereals is scarce. The present study was undertaken to investigate the inhibitory effect of pure ITC against growth and germination of *F. graminearum* under *in vitro* conditions.

3.3 Materials and Methods

3.3.1 Isothiocyanates

Pure AITC, BITC, EITC, MITC and 2-phenylethyl ITC (PEITC) were obtained from Sigma Aldrich (Dorset, UK). Sterile distilled water was used to prepare the desired stock solutions of ITC which were diluted to the final concentration in media. Appropriate concentrations were determined, and varied from 0 to 5000 mg l^{-1}.

3.3.2 *Fusarium graminearum* isolates

Five strains of *F. graminearum* from UK wheat isolated in 2016 (FG2556, FG2498, FG2560, FG2502, FG2481) were provided by Fera Science Ltd. These isolates were confirmed as *F. graminearum* by species-specific PCR (Waalwijk *et al*., 2004).

3.3.3 Assay on mycelia

The effect of each ITC on mycelial growth was evaluated using potato dextrose agar (PDA) (Merck, KGaA, Germany) media amended with ITC at 1.2, 4.9, 19.5, 78, 312.5, 1250, 5000 mg l^{-1}. For uniform distribution of the ITC within the medium, ITC suspensions in sterile distilled water were first vortexed. This was then immediately transferred to molten PDA agar (at 50°C) and then shaken gently before pouring. Control plates contained PDA treated with sterile distilled water in place of ITC. Agar plugs of 7 mm in diameter were cut from the outer margin of the five actively growing *F. graminearum* isolates growing on PDA. These were transferred to the centre of 9 cm Petri dishes of all treatments with the mycelial part facing the media. Three replicates were used per treatment. The plates were incubated at 15°C in the dark and radial colony growth was measured after 9 days, just before untreated mycelia had reached the edge of the plates. Data were expressed as mycelium growth compared with the control. The experiment was conducted twice. The experiment was repeated with the calculated ED_{50} values and hyphal morphology was observed using a light microscope (x100 and x400 magnification).

3.3.4 Assay on conidia

3.3.4.1 Preparation of *Fusarium graminearum* conidial suspension

Conidial suspensions were prepared following the method reported by Edwards and Seddon (2001) with some modifications. The five isolates of *F. graminearum* were sub-cultured on PDA using 10 plates for each isolate and incubated at room temperature (ca. 20°C). After 14 days, conidia were harvested by the addition of 20-25 ml sterile distilled water to each plate and conidia were dislodged using a sterile spreader. The suspension was filtered through Miracloth (EMD Millipore Corp., Billerica MA, USA) to remove mycelium. The filtrate was centrifuged at 3000 *g* for 10 min. Supernatant was removed and conidia re-suspended in 15 ml sterile distilled water and centrifuged at 3000 *g* for 10 min. Conidia were washed twice and re-suspended in 15 ml sterile distilled water. Conidia of all five isolates were mixed in the final conidial suspension. Conidia were counted using an Improved Neubauer counting chamber (Weber 99, Scientific International, Teddington, UK) and the concentration adjusted to 10^5 spores ml^{-1}.

3.3.4.2 Assessment of conidial germination

The five ITC under investigation were tested for their efficacy at inhibiting the germination of *F. graminearum* conidia. Four-fold dilutions of each ITC were prepared by mixing 500 µl of stock solution in 1500 µl sterile distilled water in sterile 2 ml Eppendorf tubes. The suspensions were vortexed and immediately pipetted for the serial dilution. One ml of each ITC dilution was then mixed with 900 µl conidial suspension and 100 µl potato dextrose broth (Oxoid, Basingstoke, UK) to give final ITC concentrations of 5000, 1250, 312.5, 78, 19.5, 4.9 and 1.2 mg l^{-1}. The suspensions were vortexed each time and immediately transferred to the tubes. For controls, sterile distilled water was used instead of ITC. Twenty µl of each treatment were pipetted on flame sterilised microscope glass slide and placed in a 9 cm Petri dish containing two 85 mm diameter filter paper (Fisherbrand, Fisher Scientific UK) moistened with 2 ml sterile distilled water. Four replicates were used for each treatment and the plates were incubated for 16-17 hours at 12°C in the dark. The slides were observed under x100 magnification and a minimum of 100 conidia per replicate were counted to calculate percentage germination. Conidia were considered germinated if the length of germ-tube was equal to or greater than conidia width (Manners, 1966). The experiment was conducted twice.

3.3.5 Data analysis

Genstat (18th edition) was used to determine ED_{50} of each ITC for the percentage of inhibition of mycelial growth and conidia germination. Each treatment in the mycelium assay, is represented by a mean of the three replicates taken for each of the five isolates. The concentrations were converted to log10 scale to fit non-linear curves. Data was best fitted with logistic curves with separate lines for each ITC, while the ED_{50} and 95% confidence interval values were generated from the fitted curve. All data was fitted using ITC as a group.

3.4 Results

3.4.1 *In vitro* effects of isothiocyanates on mycelial growth

Different responses of *F. graminearum* were observed according to type and concentration of ITC. All tested ITC except EITC showed 100% inhibition of the radial growth at the highest two concentrations, 5000 and 1250 mg l^{-1}. 2-phenylethyl ITC and BITC showed complete inhibition at 312.5 mg l^{-1}. Allyl ITC (Figure 3.1) and MITC at 78 mg l^{-1}, on average, inhibited the radial growth by 20-60% and 45-65% respectively. Ethyl ITC showed the weakest inhibition (ED_{50}, ca. 1964 mg l^{-1}) and at concentrations ranging from 1.2 to 312.5 mg l^{-1} showed 10-20% stimulation compared to the control. Allyl ITC and PEITC also showed slight stimulation at the lowest concentrations. The calculated mean ED_{50} values for the tested ITC from the two experiments showed some variability, they are however, within a four-fold order of magnitude (Table 3.1). As evident from the ED_{50} values, BITC (ca. 5 mg l^{-1}) and PEITC (ca. 26 mg l^{-1}) showed the strongest inhibition. The fitted curves accounted for 91.6% and 85.8% of the observed variance (p<0.001) within the data from experiments 1 (Figure 3.2) and 2, respectively. The five isolates showed little variation in their responses to ITC, apart from one isolate (FG2481), which showed greater variation in response to EITC (Figure 3.3).

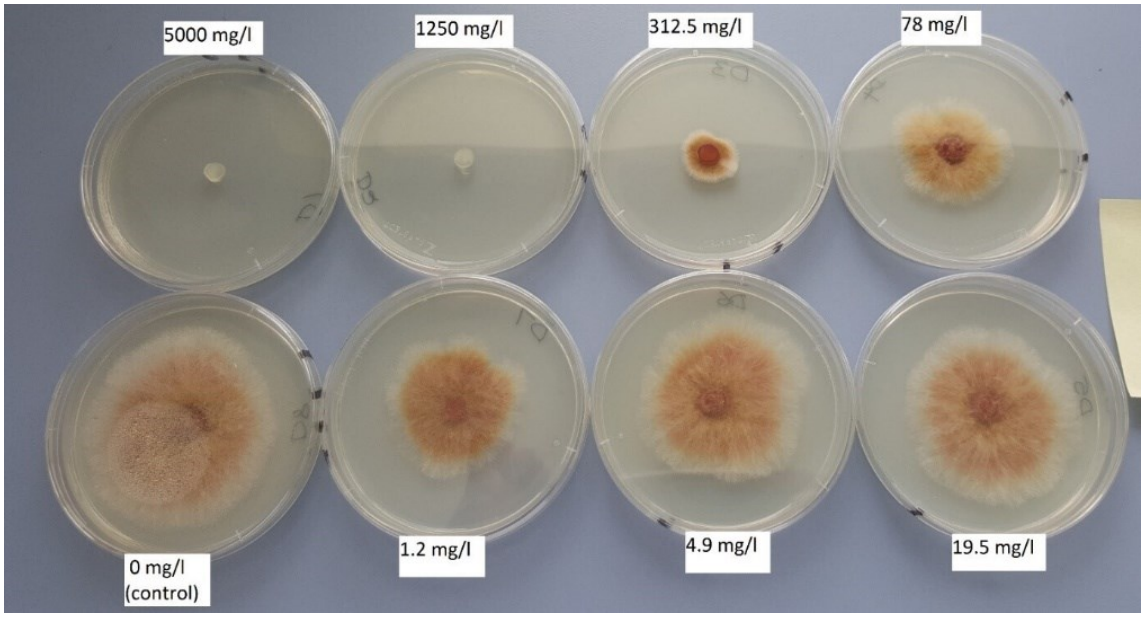


Figure 3.1 *Fusarium graminearum* cultures exposed to different concentrations of allyl isothiocyanate after 9 days at 15°C

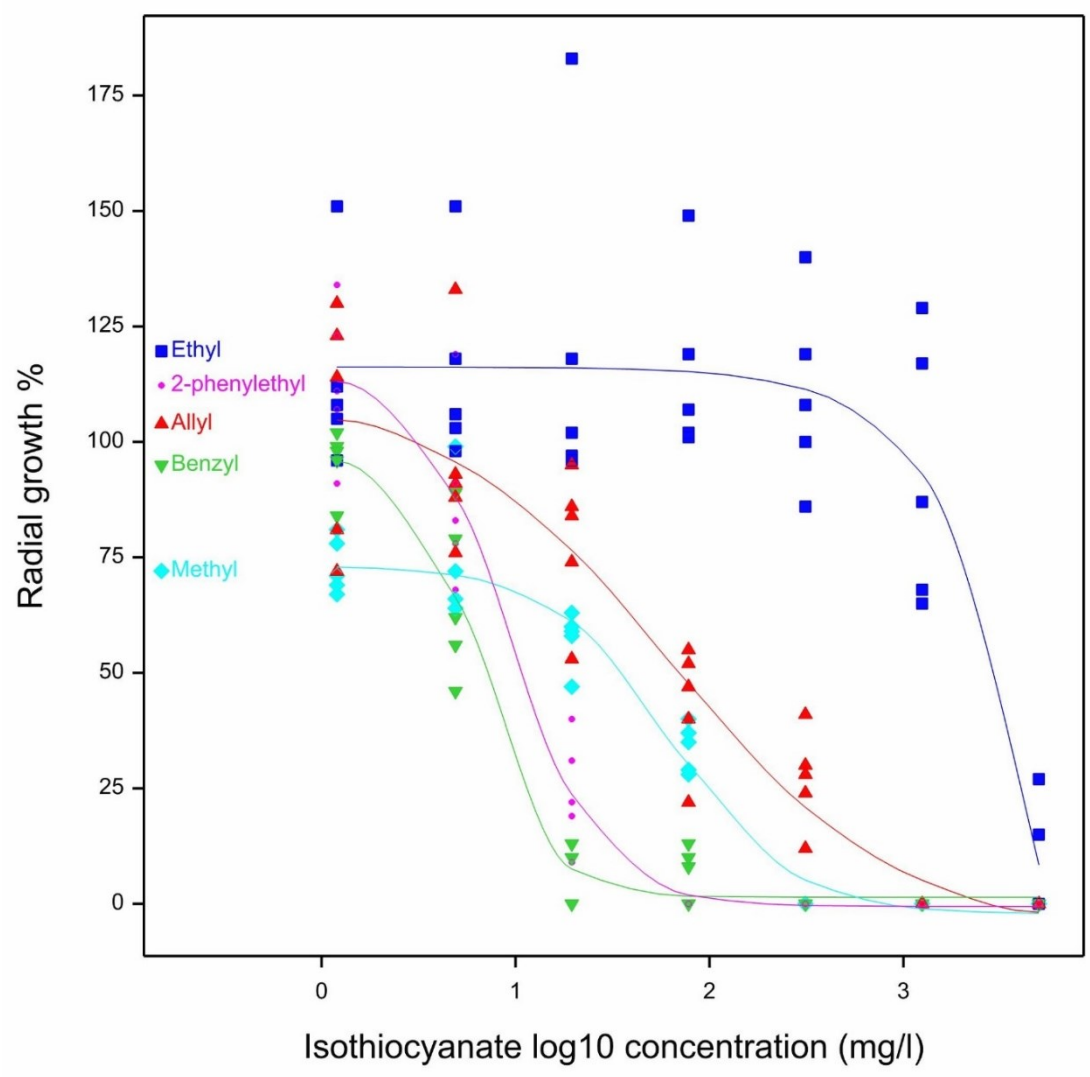


Figure 3.2 Dose-response curves for ethyl, 2-phenylethyl, allyl, benzyl and methyl isothiocyanates at concentrations 5000, 1250, 312.5, 78, 19.5, 4.9 and 1.2 mg l^{-1} (presented on log10 scale) on mycelial growth of *Fusarium graminearum* isolates when compared with control. Data from experiment 1 is presented here

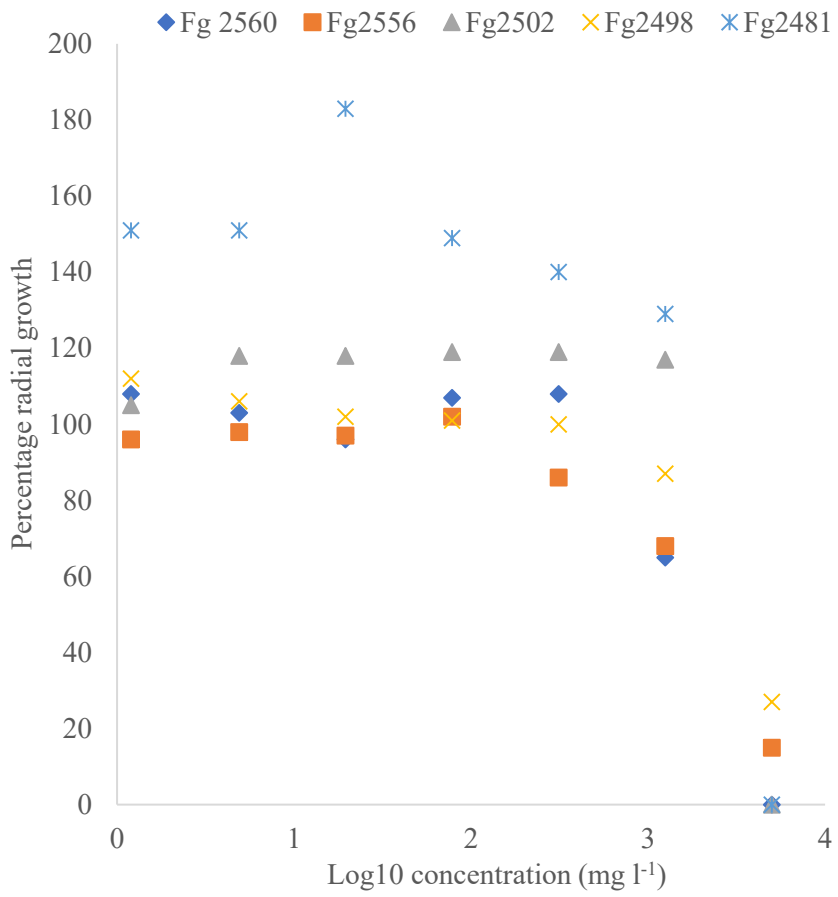


Figure 3.3 Mycelial growth of *Fusarium graminearum* isolates (FG2556, FG2498, FG2560, FG2502, FG2481) exposed to ethyl isothiocyanates at concentrations 5000, 1250, 312.5, 78, 19.5, 4.9 and 1.2 mg l^{-1} (presented on log10 scale) when compared with control (Experiment 1)

Table 3.1 Effective dose values at 50% (ED_{50}) of isothiocyanates for *Fusarium graminearum*

	ED_{50} (mg l^{-1}) [a]			
Isothiocyanate	**Mycelial growth**		**Conidial germination**	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
Allyl	69 (19-269)	129 (56-240)	135 (129-141)	62 (58-66)
2-phenylethyl	11 (7-20)	41 (21-110)	>5000	>5000
Methyl	35 (<1-110)	91 (20-224)	151 (148-155)	45 (43-47)
Benzyl	7 (3-12)	2 (<1-13)	3020 (2951-3090)	2951 (2884-3020)
Ethyl	2951 (1905-4169)	977 (138-3631)	1288 (1202-1413)	1549 (1413-1660)

[a] For mycelial growth and conidia germination, inhibitory activity was evaluated at 15°C and 12°C respectively; 95% confidence interval for each ED_{50} value are included in parentheses

Microscopic examination (x100 and x400) of the mycelial growth of *F. graminearum*, when exposed to the five ITC at their respective calculated ED_{50} values, showed no observable difference in the hyphal morphology when compared to the control (Appendix Figure 1).

3.4.2 *In vitro* effects of isothiocyanates on conidial germination

The fitted curves accounted for 99.8% and 99.9% of the deviance within the data from experiments 1 (Figure 3.4) and 2, respectively. All tested ITC (except PEITC) showed 100% inhibition at the highest concentration, 5000 mg l^{-1}, when compared to the control treatment (100% germination). At 1250 and 312.5 mg l^{-1}, AITC (Figure 3.5) and MITC showed complete inhibition of conidial germination whereas 1250 mg l^{-1} of EITC was found to be comparatively less effective with around 50% inhibition. However, at 19.5 mg l^{-1} or lower concentrations, none of the tested ITC appeared to have any effect on conidial germination. 2-phenylethyl ITC showed no inhibitory response on conidial germination at any concentration, therefore no ED_{50} values were determined for PEITC. For the other ITC, as with the mycelial experiments, the calculated mean ED_{50} values from the two experiments showed some variability, they are however, within a four-fold order of magnitude (Table 3.1). Among the tested ITC, AITC and MITC were the most efficient, showing lower ED_{50} values, ca. 99 mg l^{-1} and ca. 98 mg l^{-1} respectively (Table 3.1). As evident from the ED_{50} values, BITC (ca. 2986 mg l^{-1}) and EITC (ca. 1419 mg l^{-1}) were the least effective for inhibition of conidial germination.

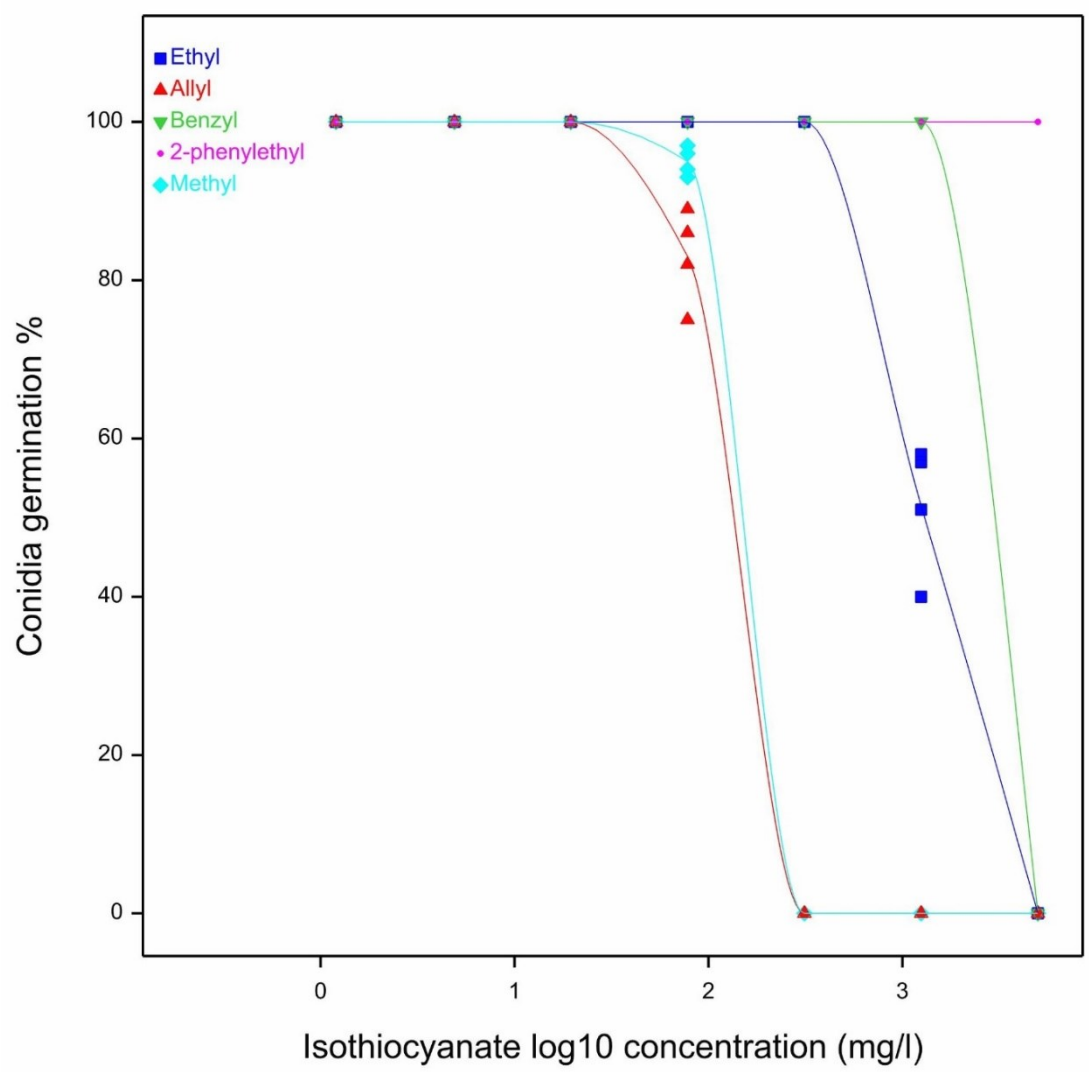


Figure 3.4 Dose-response curves for ethyl, 2-phenylethyl, allyl, benzyl and methyl isothiocyanates at concentrations 5000, 1250, 312.5, 78, 19.5, 4.9 and 1.2 mg l^{-1} (presented on log10 scale) on conidia germination of *Fusarium graminearum* when compared with control. Data from experiment 1 is presented here

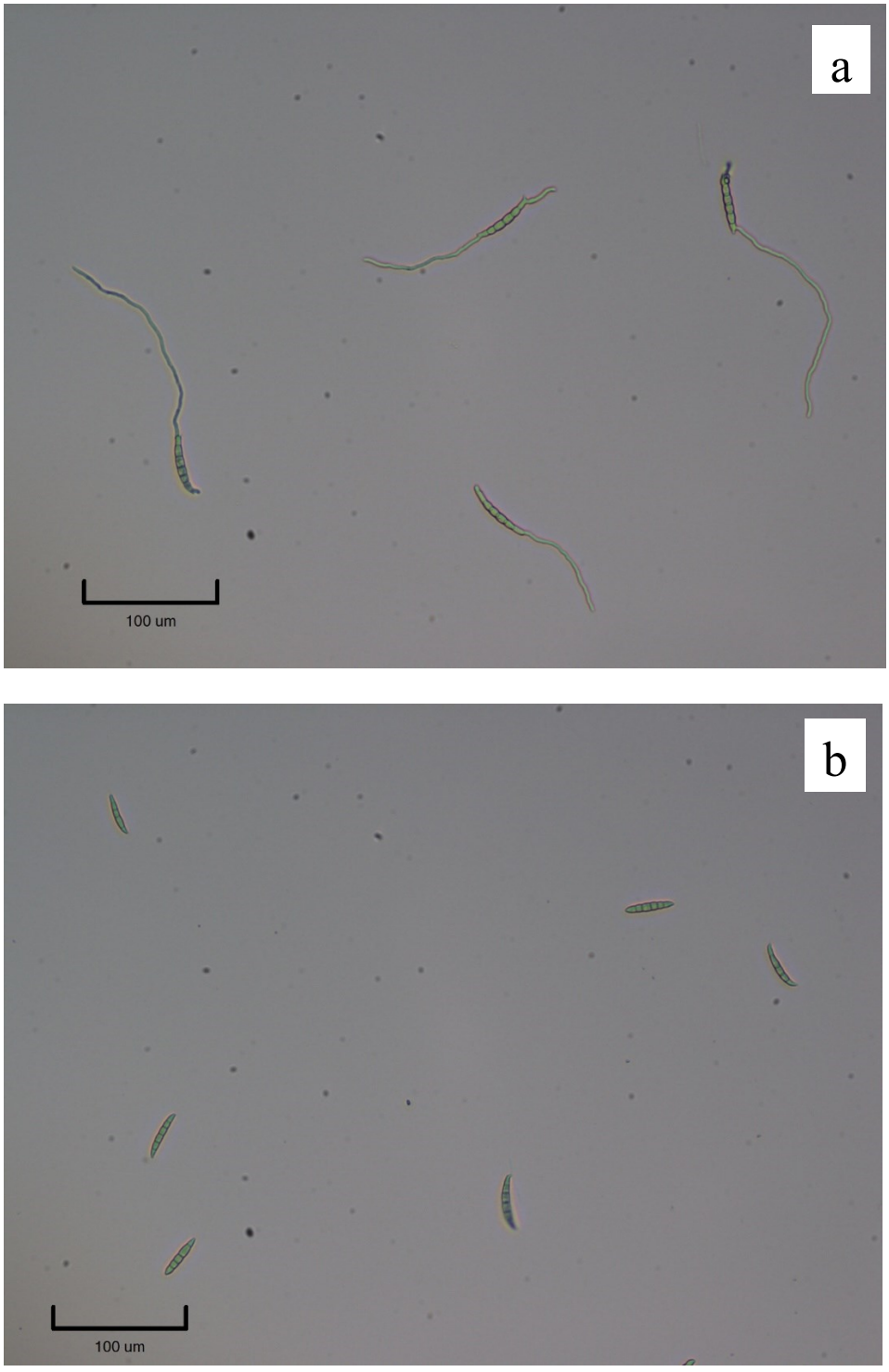


Figure 3.5 *Fusarium graminearum* conidia exposed to (a) water control and (b) 312.5 mg l^{-1} of allyl isothiocyanate after 16 hours incubation in diluted potato dextrose broth at 12°C. Images captured at x100 magnification

3.5 Discussion

The present study has demonstrated that the sensitivity of *F. graminearum* varied for ITC type and concentration tested. Aliphatic ITC (AITC, MITC, EITC) were more toxic than the aromatic ITC (PEITC, BITC) in the present conidial assay, AITC and MITC caused complete inhibition at 312.5 mg l^{-1}. Methyl ITC is generally considered to be a synthetic ITC although it has been detected in tissues of *Brassica napus*, *B. juncea* and *B. campestris* (Ojaghian *et al*., 2012). In a previous study (Ramos-Garcia *et al*., 2012), ITC at lower concentrations (0.1 - 2 mg l^{-1}) were completely inhibitory to *F. oxysporum* conidia in a shorter duration (7 hours) which is in contrast to the present results. This suggests that the response of *F. graminearum* to ITC differs from other *Fusarium* species. Mari *et al*. (2008) also reported low ED_{50} values of AITC (0.17 mg l^{-1}) and BITC (0.12 mg l^{-1}) for conidial germination of *Monilinia laxa*.

In the conidial assay, the aliphatic ITC showed lower ED_{50} values than the aromatic ones whereas the reverse was seen in the mycelial assay. Sarwar *et al*. (1998) reported that aromatic ITC when incorporated in to media showed stronger toxicity against fungal pathogens but were less toxic in volatile form due to their lower volatility. The slightly higher ED_{50} value for AITC (ca. 99 mg l^{-1}) recorded in the mycelial assay was unexpected. As AITC is highly volatile, some of the volatiles might have been lost when the media plates were prepared, which may have resulted in a higher ED_{50} value than the ones for aromatic ITC and MITC (ca. 63 mg l^{-1}). Previously, Ojaghian *et al*. (2012) found MITC and AITC to be the most effective in inhibiting mycelial growth of *Sclerotinia sclerotiorum*. The two ITC were completely inhibitory at ca. 500 µg l^{-1}. Neubauer *et al*. (2014) observed that microsclerotia of *V. dahliae* were more sensitive to aromatic ITC than the aliphatic ITC which is consistent with the present mycelial assay. They recorded lower LD_{50} values for BITC (0.36 µg l^{-1}) and PEITC (0.46 µg l^{-1}) than MITC (0.72 µg l^{-1}) and AITC (1.45 µg l^{-1}). Smolinska *et al*. (2003) reported that the inhibitory effect of various ITC on mycelial growth of *F. oxysporum* was fungistatic rather than fungicidal. However, in the present study, mycelial plugs from the treatments which showed complete inhibition when transferred to fresh PDA media did not grow (data not shown), indicating a fungicidal effect. The toxicity of ITC depends on the molecular structure but is not strictly dependent on the aliphatic or aromatic structure (Smolinska *et al*., 2003). Moreover, the varying responses of different ITC

observed in different studies could be due to their mode of action, different side chains and penetrability, and sensitivity of the target fungal species.

In the present study, the extent of mycelial growth inhibition varied slightly among isolates, when subjected to ITC. However, greater variation was observed among isolates when exposed to EITC. Previously (Smolinska *et al*., 2003), differences in mycelial growth inhibition of different isolates of the same species in the presence of ITC had been observed for *F. oxysporum*. In the mycelial assay, AITC and PEITC at very low concentrations appeared to stimulate growth of *F. graminearum* as the developing colonies were slightly larger than the control plates. Such stimulation has been observed in other studies with fungicides. For example, fosetyl-aluminium at the recommended rate, stimulated radial growth of *Lecanicillium longisporum* (Shah *et al*., 2009). The assays have shown inhibition of mycelial growth and conidial germination of the pathogen after exposure to ITC, although the susceptibility of conidia in comparison to mycelium was inconsistent. In other studies conidia, compared to mycelium, were more sensitive to ITC (Mari *et al*., 2008; Ramos-Garcia *et al*., 2012). However, in an *in vitro* assay with fungicides (Masiello *et al*., 2019), the ranking of *F. graminearum* sensitivity of conidia and mycelia differed for different fungicides. A study on *Alternaria brassicicola* suggests that ITC alter mitochondrial function, trigger production of reactive oxygen species and possibly disrupt metabolic pathways in fungal cells (Calmes *et al*., 2015). The variation in sensitivity at the different development stages could be due to differences in structure and physiology and mode of action of the antifungal compounds.

The results from the conidial and mycelial assays suggest that AITC and MITC are the most inhibitory ITC against *F. graminearum* under laboratory conditions. The precursor GSL of AITC is sinigrin. This GSL comprises about 98-99% of the total GSL content of some *Brassica* species such as *B. juncea* and *B. nigra* (Matteo, 2017; Ngala *et al*., 2015b). Charron and Sams (1999) detected volatile compounds emitted from macerated leaves of *B. juncea* placed in a jar and determined that AITC was the predominating compound (>90%) in the volatiles measured by gas chromatography. Since, sinigrin (AITC-precursor) is the dominant GSL in *B. juncea*, this species would be a strong candidate for biofumigation against *F. graminearum* under field conditions. In a field experiment (Larkin and Griffin, 2007), incorporation of *B. juncea* reduced *Spongospona subterranea* (powdery scab) inoculum by 27% and also reduced disease incidence and severity of powdery scab and common scab (caused by *Streptomyces scabies*) by 40% and >20% respectively. In the

same study, an *in vitro* assay showed that macerated leaf tissues of *B. juncea* resulted in 73% inhibition of *F. oxysporum* and 80% inhibition of *F. sambucinum*.

Higher ED_{50} values recorded in the present study might be achievable in field. Researchers have detected GSL at concentrations high enough to produce ITC concentrations comparable to the ED_{50} values determined in this study (Gimsing and Kirkegaard, 2006). High content of sinigrin, the parent GSL of AITC, has been detected in different *Brassica* species. Sinigrin at concentration of 90 µmol g^{-1} and 147 µmol g^{-1} have been detected in *B. juncea* leaves (Ngala *et al*., 2015b) and *B. nigra* defatted seed meal (Matteo, 2017) respectively. In contrast, lower concentrations of sinigrin ranging from 15 to 27 µmol g^{-1} tissue (freeze-dried) has also been observed previously (Charron and Sams, 1999; Neubauer *et al*., 2014). However, it is indicated that a GSL concentration of around 13 µmol g^{-1} (dry weight) would be able to produce at least 50 mg l^{-1} of AITC (Wood *et al*., 2017) suggesting that the ED_{50} value for AITC determined in the present study (ca. 99 mg l^{-1}) might be achievable in field. Gimsing and Kirkegaard (2006) also reported high ITC release efficiency in field following incorporation of *Brassica* species. They recorded ITC release efficiency as high as 56% for *B. juncea* and 26% for *B. napus*. However, it should be kept in mind that such higher ITC concentrations were achievable by following practices, such as thorough pulverisation and heavy watering, to maximise ITC release under field conditions.

In the present study, ITC were mixed with water and not dissolved in a solvent such as ethanol because *F. graminearum* cultures when exposed to ethanol alone resulted in abnormal colony growth of the fungus. Unlike the assay on mycelium where treatments were incubated at 15°C, the conidial assay was performed at 12°C. This temperature was selected for logistic reasons because at 15°C, germination tubes grew longer which were no longer distinguishable as individual conidium after overnight incubation. As the response of all five isolates was broadly similar in the assay on mycelia, conidia of all five isolates were mixed in the final conidial suspension in the conidial assay. Moreover, during the preliminary runs for the conidial experiment, the five isolates when treated separately with ITC, a similar response was noted among the isolates. Data from mycelial and conidial assays were broadly similar in the repeated experiments with the aromatic ITC. However, the ED_{50} values determined for the aliphatic ITC were less consistent among the two experiments for each assay. Due to these ITC being highly volatile, slight differences in temperature may have affected their volatility. Vapour pressure of

AITC was reported to increase rapidly with temperature, 136 to 2267 Pa from 5 to 50°C (Lim, 1999), indicating its high volatile properties. Similarly, the vapour pressure of EITC is estimated to increase from 1150 Pa at 20°C to around 1500 Pa at 25°C (Yaws and Satyro, 2015), suggesting this ITC may be sensitive to slight differences in temperature. Moreover, the vapour pressure of EITC can reach as high as 5000 Pa at 50°C (Yaws and Satyro, 2015), a volatilisation rate more than double that of AITC at the same temperature (Lim, 1999), indicating the highly volatile nature of EITC. This may explain the discrepancies observed for EITC in the mycelial assay where the ITC were added to molten media at 50°C. Delaquis and Sholberg (1997) have argued that due to high volatility and weak water solubility of ITC, the test concentrations may not remain constant throughout the experiment. Variation in volatilisation may explain the discrepancies observed for ITC between the two experiments.

The present findings have demonstrated the antifungal potential of ITC particularly, MITC and AITC against *F. graminearum*. During the ongoing issue of the use of synthetic fumigants where some fumigants are already banned and others are likely to be banned in the near future, biofumigation could prove a "green" alternative solution. Higher ED_{50} values of ITC were recorded in the present study which might be achievable in the field depending on factors such as agronomic practices and brassica species used. Nevertheless, the outcome suggests that brassicas rich in sinigrin and glucocapparin, such as *B. juncea* and *B. napus*, respectively, could have a suppressive effect on reducing the inoculum of *F. graminearum* in soil prior to cereal production.

Chapter 4

Chapter 4

4 Biofumigation potential of brassica tissues against *Fusarium graminearum*

4.1 Abstract

Fusarium graminearum, a globally important cereal pathogen, causes head blight in wheat, resulting in yield losses and mycotoxin contamination. Currently, triazole fungicides are used to suppress *Fusarium graminearum*, however, limited effectiveness of triazoles and concerns over safety of pesticides have led to the pursuit of safe alternative crop protection strategies such as biofumigation. In the present study, species belonging to Brassicaceae (*Brassica juncea*, *Raphanus sativus*, *Eruca sativa*) were assessed for their biofumigation potential against *F. graminearum*, and the glucosinolate profile of the brassicas were determined. Mycelial plugs of *Fusarium graminearum* were exposed to frozen leaf discs of brassicas collected at early-leaf, stem-extension and early-bud stages. Additionally, *F. graminearum* inoculum was incubated in soil amended with chopped tissues of brassicas in a microcosm experiment. *Brassica juncea* (Indian mustard) leaf discs were effective against mycelial growth showing up to 100% suppression, while the sinigrin content in the leaf tissue corresponded with the level of suppression. In the microcosm experiment, each brassica species significantly suppressed *F. graminearum* inoculum by 41-55%. The findings suggest that the brassica species investigated in the present study could be effective in reducing the inoculum of *F. graminearum* in soil prior to cereal production.

4.2 Introduction

As discussed in section 1.6, several studies have successfully demonstrated the suppressive potential of brassica tissue as biofumigants against a range of soil-borne pathogens e.g. *Rhizoctonia solani* and *Verticillium dahliae* (Handiseni *et al*., 2016; Neubauer *et al*., 2014). Previously, isothiocyanates (ITC) were tested against mycelial radial growth and conidial germination of *Fusarium graminearum* under *in vitro* conditions (Chapter 3). Among the tested ITC, allyl and methyl ITC were overall more efficient, showing lower ED_{50} values (35-150 mg l^{-1}), suggesting volatiles released from damaged brassica tissues could have suppressive effect on *F. graminearum.* Thus, the present study was performed to investigate the potential of brassicas to suppress *F. graminearum*. The aim of this study was to evaluate the effect of leaf tissue of brassicas on mycelial growth of *F. graminearum in vitro* and to investigate the effect of shredded brassica tissues on *F. graminearum* inoculum in a microcosm experiment.

4.3 Materials and Methods

4.3.1 Brassicas and fungal cultures

Seeds of *Brassica juncea* 'Brons' (Indian mustard), *Raphanus sativus* 'Bokito' (oilseed radish) and *Eruca sativa* 'Trio' (rocket) were supplied by RAGT seeds, UK and *B. juncea* 'Caliente Rojo' (Indian mustard) by Tozer Seeds, UK. The four cultivars were grown in glasshouse in 10 cm pots with mean temperatures of 12°C (night) and 17°C (day). For experiment 1, plants were grown between October 2019-January 2020 and for experiment 2, plants were grown between September-November 2020. *Fusarium graminearum* strain FG2502 from UK wheat isolated in 2016 was provided by Fera Science Ltd. The strain was confirmed as *F. graminearum* by species-specific PCR using a previously published assay (Waalwijk *et al*., 2004).

4.3.2 Leaf disc assay

Leaves of three cultivars (*B. juncea* 'Brons', *R. sativus* 'Bokito', *E. sativa* 'Trio') were sampled at the early-leaf stage (4-5 true leaves unfolded). Using a 15 mm cork borer, 45 discs were cut from leaves of each cultivar. The leaf discs, separated by Miracloth (EMD Millipore Corp., Billerica MA, USA) in stacks of fives and held together by a paper clip, were immediately flash frozen in liquid nitrogen and stored at -80°C until required for the assay. Agar plugs of 7 mm diameter were cut from outer margin of actively growing *F. graminearum* (FG2502) on potato dextrose agar (PDA) (Merck, KGaA, Germany) media. These were transferred to the centre of 9 cm Petri dishes containing PDA with the mycelium facedown. The frozen leaf discs were transferred in dry ice from freezer to the work station to prevent discs defrosting prematurely. Leaf discs at doses of 1, 2, 4 and 8 discs were placed with forceps onto the upturned lid of the Petri dishes while the inverted bottom containing the fungal plug was held aside. The plates in inverted position were immediately sealed with parafilm. Control plates did not contain leaf discs. Three replicates were used per treatment. The plates were incubated at room temperature (ca. 20°C) and radial colony growth was measured after 5-6 days, just before untreated mycelia had reached the edge of the plates. Experiments to investigate the effect of brassica leaves at stem-extension and early-bud stages were performed as above.

The assay was repeated independently, using *B. juncea* 'Caliente Rojo' in addition to the other brassica species tested in the first experiment.

4.3.3 Microcosm experiment

4.3.3.1 Preparation of inoculum bags

Conidial suspensions were prepared following a previous method (Edwards and Seddon, 2001) with some modifications. *Fusarium graminearum* (FG2502) was sub-cultured on PDA using 20 plates and incubated at room temperature (ca. 20°C). After 14 days, conidia were harvested by the addition of 20-25 ml sterile distilled water to each plate and conidia were dislodged using a sterile spreader. The suspension was filtered through Miracloth to remove mycelium. The filtrate was centrifuged at 3000 *g* for 10 min. Supernatant was removed and conidia re-suspended in 15 ml sterile distilled water and centrifuged at 3000 *g* for 10 min.

Conidia were washed twice and re-suspended in 15 ml sterile distilled water. Conidia were counted using an Improved Neubauer counting chamber (Weber 99, Scientific International, Teddington, UK) and the concentration adjusted to 3 x 10^4 spores ml^{-1}.

One kg of oat screenings (Morning Foods, Crewe, UK), which were predominantly blind oat spikes, were soaked in distilled water for 12 hours before autoclaving in a 78 x 40 cm autoclavable bag [VWR (129-0581)]. The oats were re-autoclaved after 24 hours and allowed to cool. A 100 ml of the above prepared spore suspension was added to the bag, mixed well and incubated for 10-12 days in dark. Five pieces of inoculum (blind oat spikes) were added to perforated nylon sachets (7 x 5.5 cm) and sealed with plastic bag sealing machine (Impulse heat sealer, 200mm). Additionally, wheat chaff was randomly collected post-harvest from a *F. graminearum*-inoculated wheat experiment at the research facilities of Harper Adams University, Newport, Shropshire, UK. Five pieces of chaff were added to nylon bags and sealed as described above. Both types of inoculum (Figure 4.1a) were confirmed for *F. graminearum* infection on PDA prior to using in the experiment.

4.3.3.2 Evaluating effect of biofumigants on inoculum

Two litre glass jars with rubber seal clips (22 x 12 x 12 cm) were filled with John Innes No.2 loam based compost [composition: loam, peat, coarse sand, hoof & horn meal, superphosphate, potassium sulphate, calcium carbonate; pH: 5.5-6 (provided by supplier); 46% moisture] to a depth of 10 cm. At early-bud stage, shoots of *B. juncea* 'Brons', *R. sativus* 'Bokito' and *E. sativa* 'Trio' were harvested and chopped in a food processer for 15 s. Chopped tissue (Figure 4.1b) at two quantities, 65 g and 15 g, were added to soil-filled jars and mixed well. Jars without brassica amendments served as controls. One inoculum bag (blind oat spikes) was buried in each jar and lid closed hermetically. Five replicates were used per treatment and the jars were incubated in glasshouse with mean temperatures of 12°C (night) and 17°C (day) (Figure 4.1c). After 8 weeks, the bags were extracted and the inoculum substrates were tested for *F. graminearum* growth on Petri dishes containing modified CZID media (CZPD) (Hofgaard *et al*., 2016). Briefly, the media was prepared by adding dichloran solution (0.2% in ethanol), chloramphenicol solution (5% in ethanol) and trace metal solution (1% $ZnSO_4.7H_2O$ + 0.5% $CuSO_4.5H_2O$)

each 1 ml l^{-1} of Czapek Dox agar (Sigma-Aldrich) media. Once autoclaved and cooled down to 55°C, 10 ml of chlortetracycline solution (0.5%) and 1 ml of Bumper® suspension (0.3%) containing 750 µg propiconazole were added per L of media, before pouring into plates. The five inoculum pieces were removed from each bag, placed on a media plate and incubated at room temperature (ca. 20°C) for 12-15 days. *Fusarium graminearum* incidence in the inoculum pieces was recorded as presence of *F. graminearum* colony growth based on the characteristic reddish pigmentation. For confirmation, assumed *F. graminearum* colonies were sub-cultured on PDA and incubated at room temperature (ca. 20°C) for 14 days. The conidia were harvested as described above and identified according to morphological characteristics (Leslie and Summerell, 2006).

The experiment was independently repeated using *B. juncea* 'Caliente Rojo' in addition to the other brassicas tested in the first experiment. Additionally, a bag of the chaff inoculum (five pieces per bag) was also buried in each jar.

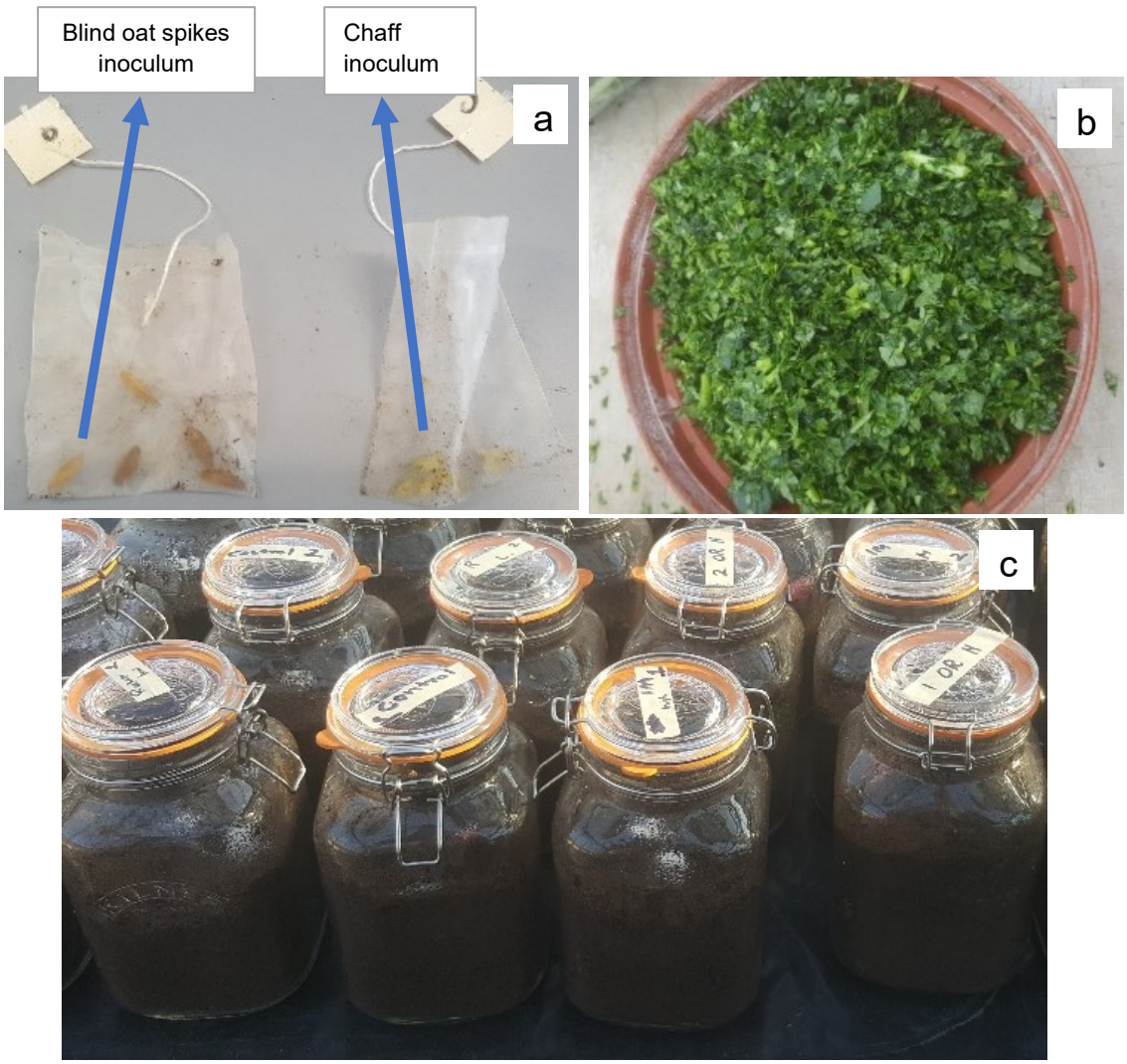


Figure 4.1 Experimental setup of microcosm biofumigation experiment (a) Blind oat spikes inoculated with *Fusarium graminearum* and chaff from *F. graminearum*-inoculated wheat in nylon sachets (b) brassica shoots chopped in food processor (c) sealed jars containing the two types of inoculum sachets buried in loam based compost incorporated with chopped brassica tissue

4.3.4 Glucosinolate analysis

Approximately 10 g leaf tissue of the brassica plants were collected at the three developmental stages (early-leaf, stem-extension, early-bud), flash frozen in liquid nitrogen and stored at -80°C until freeze dried. Freeze-dried (GVD6/13 MKI freeze dryer; GIROVAC Ltd, North Walsham, UK) samples were milled (IKA® M 20 Universal Mill) and stored at -18°C before sending to NIAB Labtest, Cambridge, UK where the samples were analysed following the ISO 9167 "Rapeseed and rapeseed meals – Determination of glucosinolates content – Method using HPLC".

4.3.5 Statistical analysis

Data were subjected to general analysis of variance using Genstat® (20th edition) statistical software. The microcosms were arranged in the glasshouse in a completely randomised design. In the leaf disc assay, there were three factors - brassica species, brassica growth stage, number of leaf discs. In the microcosm experiment, the three factors were: brassica species, biomass quantity, inoculum type. Where necessary, data were angular transformed to improve normality of residuals. Significant differences between treatments were determined using post hoc Tukey's test (p=0.05).

4.4 Results

4.4.1 Glucosinolate content of brassica leaf tissue

Concentrations of GSL occurring in the leaf tissue of the brassicas is shown in Table 4.1. The GSL profile found in the leaves varied both qualitatively and quantitatively among the cultivars. The predominant GSL of *B. juncea* was sinigrin (allyl ITC-precursor) and that of *R. sativus* was glucoraphanin (sulforaphane-precursor). The total GSL concentration of *B. juncea* 'Brons' increased with advancing growth stage (24.5-51.9 μmol g^{-1}). However, in the other three brassicas tested, the total GSL concentrations in leaf tissue were lower at early-bud stage compared to stem-extension. Sinigrin comprised 91-94% of the total GSL content of the leaf tissue of *B. juncea* 'Caliente Rojo', occurring at highest concentration (59.5 μmol g^{-1}) at stem-extension stage. The total GSL concentration of *E. sativa* ranged from 12.9 to 17.2 μmol g^{-1}.

Table 4.1 Type and concentration of glucosinolates in freeze-dried leaf tissue of brassica plants used in the leaf disc assay to investigate the effect of volatiles released from defrosted leaf discs of brassicas on mycelial growth of *Fusarium graminearum in vitro*

Glucosinolate	***Brassica juncea*** Brons			***Brassica juncea*** Caliente Rojo			***Raphanus sativus*** Bokito			***Eruca sativa*** Trio		
	I[a]	**II**[b]	**III**[c]	**I**	**II**	**III**	**I**	**II**	**III**	**I**	**II**	**III**
Glucoberin	2.47 (0.6)[d]	3.15 (0.49)	2.01 (0.41)	1.29 (0.2)	1.81 (0.3)	1.33 (0.37)	2.28 (0.4)	1.16 (0.18)	2.15 (0.6)	2.54 (0.6)	2.55 (0.33)	2.37 (0.75)
Progoitrin	0.38 (0.1)	0.34 (0.02)	0.30 (0.10)	0.25 (0.04)	0.23 (0.11)	0.26 (0.09)	0.35 (0.08)	0.37 (0.12)	0.46 (0.08)	0.30 (0.1)	0.00	0.00
Sinigrin	21.44 (1.7)	24.02 (2.94)	48.52 (3.03)	22.72 (1.88)	59.54 (2.32)	50.9 (3.8)	-	-	-	-	-	-
Gluconapin	0.00	0.00	0.35 (0.09)	0.27 (0.07)	0.35 (0.03)	0.25 (0.09)	-	-	-	-	-	-
Glucobrassicin	0.14 (0.04)	0.03 (0.01)	0.11 (0.02)	0.11 (0.02)	0.14 (0.02)	0.23 (0.12)	1.62 (0.26)	3.87 (1.9)	2.62 (0.6)	0.22 (0.03)	0.54 (0.08)	0.58 (0.08)
Gluconasturtiin	0.11 (0.01)	0.39 (0.05)	0.58 (0.08)	0.24 (0.08)	0.97 (0.19)	0.76 (0.12)	0.32 (0.13)	1.13 (0.2)	0.96 (0.2)	0.77 (0.12)	0.42 (0.15)	0.24 (0.10)
Neoglucobrassicin	0.00	0.00	0.00	0.05 (0.01)	0.04 (0.01)	0.01 (0.007)	0.00	0.00	0.00	0.00	0.06 (0.02)	0.00
Glucoraphanin	-	-	-	-	-	-	2.90 (0.5)	23.56 (3.2)	7.29 (2.1)	2.42 (0.51)	2.84 (0.64)	2.75 (0.6)
Glucoraphenin	-	-	-	-	-	-	0.18 (0.05)	1.34 (0.4)	0.44 (0.07)	-	-	-
4 hydroxy glucobrassicin	-	-	-	0.05 (0.01)	0.37 (0.1)	0.4 (0.15)	0.00	0.04 (0.01)	0.03 (0.01)	-	-	-
Glucoraphasatin	-	-	-	-	-	-	1.02 (0.03)	8.10 (1.1)	11.09 (3.1)	-	-	-
Glucoalyssin	-	-	-	-	-	-	-	-	-	0.86 (0.18)	0.51 (0.2)	0.45 (0.14)
Glucoerucin	-	-	-	-	-	-	-	-	-	0.57 (0.14)	1.20 (0.01)	1.50 (0.28)
4-mercaptobutyl	-	-	-	-	-	-	-	-	-	3.58 (0.72)	5.70 (0.44)	3.21 (0.58)
unknown	-	-	-	-	-	-	-	-	-	0.61 (0.2)	0.83 (0.23)	0.69 (0.19)
unknown	-	-	-	-	-	-	-	-	-	1.04 (0.12)	2.59 (0.66)	2.83 (0.74)
Total glucosinolates	24.54 (2.26)	27.94 (4.18)	51.87 (3.34)	24.98 (4.59)	63.45 (3.46)	54.15 (2.94)	8.67 (2.33)	39.56 (1.25)	25.05 (3.69)	12.90 (1.20)	17.23 (2.47)	14.63 (2.57)

a I=early-leaf growth stage (4 to 5 true leaves unfolded); b II=stem-extension stage; c III=early-bud stage; d GSL conc. μmol g^{-1} freeze-dried leaf tissue, mean (SE) values from two replicates

4.4.2 Effect of brassica leaf discs on *Fusarium graminearum*

The effect of *B. juncea* 'Brons' on *F. graminearum* varied slightly between first and second experiment. For example, *F. graminearum* when exposed to 2 leaf discs collected at the stem-extension stage of *B. juncea* 'Brons', showed 9% reduction in the first experiment in contrast to 60% reduction in second experiment. *Raphanus sativus* and *E. sativa* showed similar effects in both experiments. Results of the second experiment are presented here.

Different responses of *F. graminearum* were observed according to the brassica species, dosage of leaf discs and growth stage (Figure 4.2, 4.3). The interaction between brassica growth stage, brassica species and number of leaf discs was very highly significant ($p<0.001$). The fungal growth measured 5 days after exposure to *B. juncea* leaf discs indicated a decline in the mycelial growth of *F. graminearum*. At the early-leaf stage experiment, the highest dosage of *B. juncea* 'Brons' (8 leaf discs) inhibited the radial growth by 41%. At stem-extension and early-bud stages, the efficacy of *B. juncea* 'Brons' showed 87-90% suppression with 4 leaf discs, and complete suppression with 8 leaf discs. The suppressive effect of all doses at early-bud stage of *B. juncea* 'Brons' was significantly higher ($p<0.05$) than the control. *Brassica juncea* 'Caliente Rojo' leaf discs collected at the stem-extension stage showed 20% inhibition with the lowest dose (1 disc) and 94% inhibition with 8 discs. When compared to control, no significant difference in the radial growth of *F. graminearum* was observed when exposed to leaf discs of *R. sativus* and *E. sativa* collected at each of the growth stages.

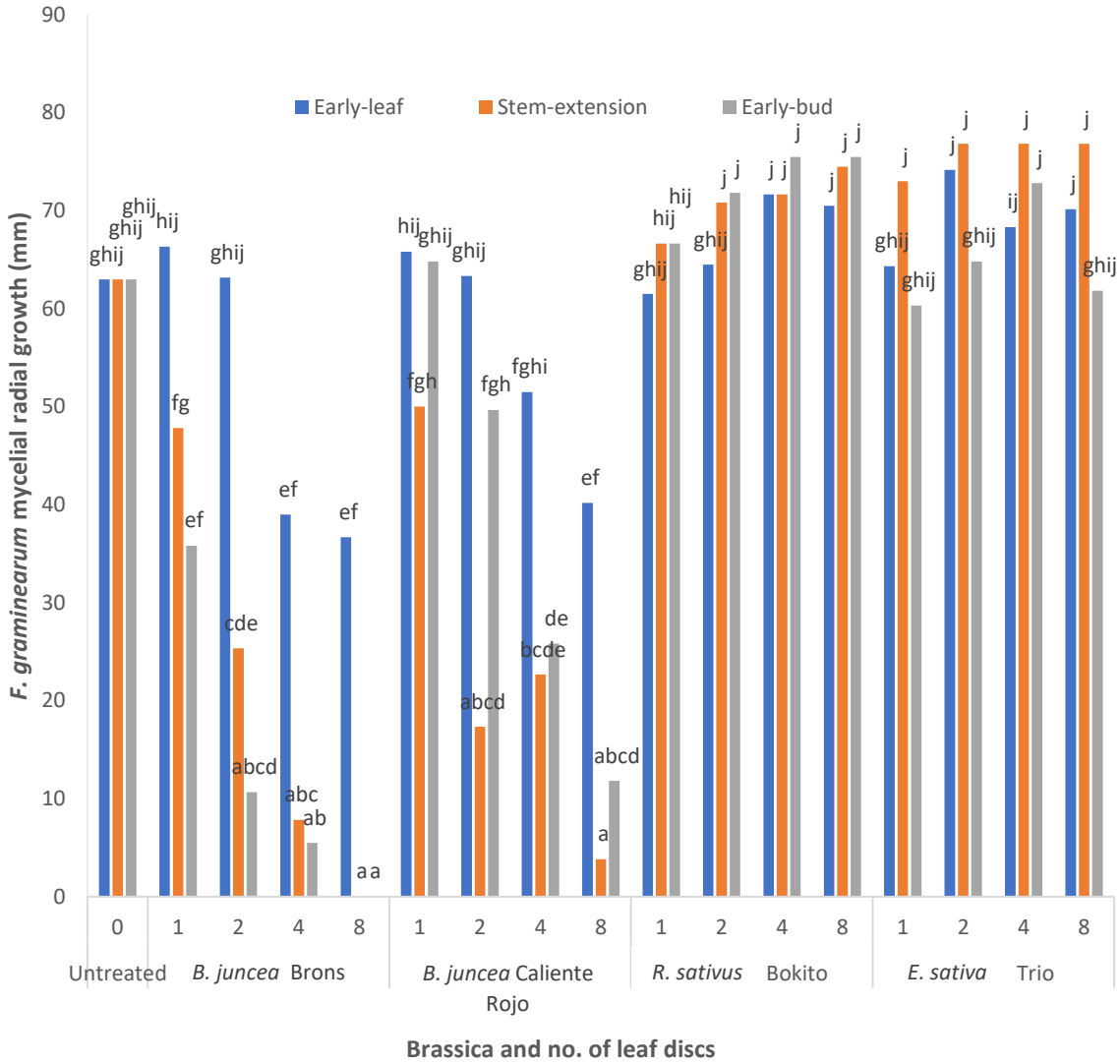


Figure 4.2 Mycelial colony growth of *Fusarium graminearum* FG2502 in Petri dishes after 5 days untreated and exposed to 1, 2, 4, 8 leaf discs collected at three growth stages of *Brassica juncea* 'Brons', *B. juncea* 'Caliente Rojo', *Raphanus sativus* 'Bokito' and *Eruca sativa* 'Trio'. Different letters indicate significant differences according to post hoc Tukey's test (p=0.05, CV%=10, SED=4.276)

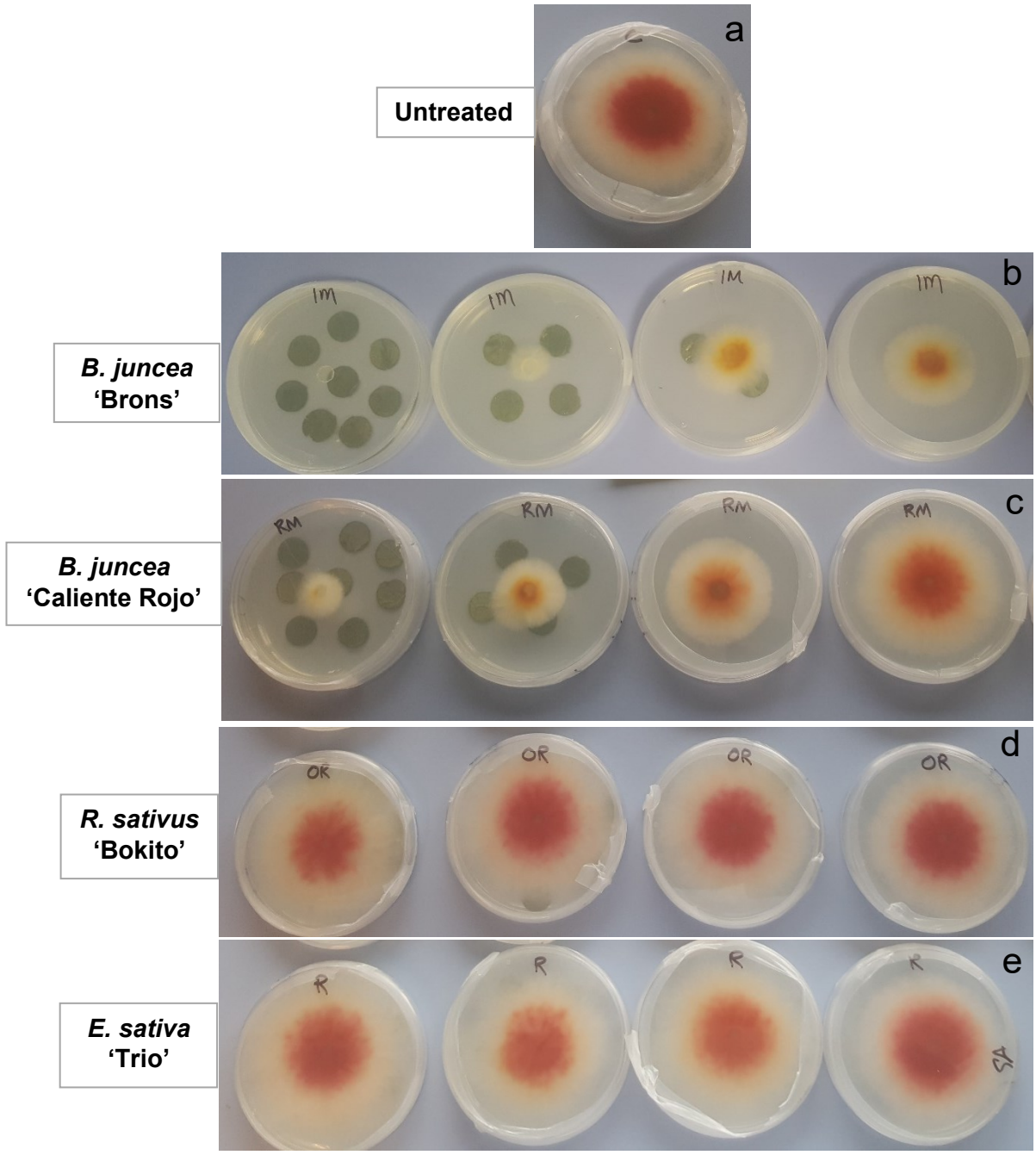


Figure 4.3 *Fusarium graminearum* FG2502 agar plugs exposed to (a) untreated and 1, 2, 4, 8 leaf discs (right to left) collected at early-bud stage of (b) *Brassica juncea* 'Brons' (c) *Brassica juncea* 'Caliente Rojo' (d) *Raphanus sativus* 'Bokito' (e) *Eruca sativa* 'Trio', incubated at room temperature (ca. 20°C) after 5 days

4.4.3 Biofumigation effect of brassicas in microcosm experiment

Data from the three brassica species (*B. juncea* 'Brons', *R. sativus* 'Bokito', *E. sativa* 'Trio') was consistent between the repeated experiments and the results of the second experiment are presented here.

There was no significant interaction between the brassica species, biomass quantity or inoculum type. The suppressive effect of the chopped shoots of brassicas on *F. graminearum* inoculum was very highly significant (p<0.001) (Figure 4.4). On average, inhibition efficiency between 41-55% was determined for the brassica treatments tested. The effect of biomass quantity at the two doses (15 g and 65 g) was not significant.

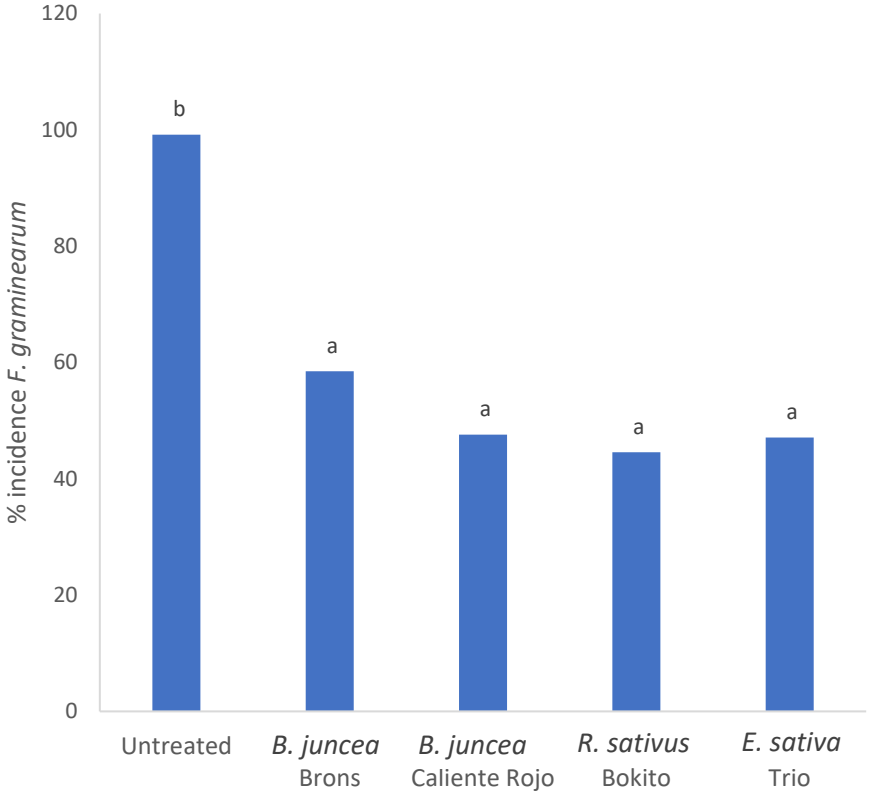


Figure 4.4 Percentage incidence of *Fusarium graminearum* recovered in inoculum incubated in microcosms filled with chopped brassica shoots and soil after 8 weeks. Different letters indicate significant differences according to post hoc Tukey's test (p=0.05, CV%=60.3, SED=9.93)

4.5 Discussion

Variation in the efficacy of Brassicaceae plants in inhibiting *F. graminearum* mycelium in the leaf disc assay could be associated with the respective glucosinolate (GSL) profile. Results highlight that the defrosted leaves of *Brassica juncea* 'Brons', collected at the three development stages, could cause significant inhibition of the mycelial growth of *F. graminearum*. Meanwhile, the results of the GSL analysis indicate that sinigrin content increases with advanced stages in this cultivar. This could be related to the inhibition of *F. graminearum*, as the level of suppression increased with the advancing growth stage of *B. juncea* 'Brons'. Similarly, the effect of *B. juncea* 'Caliente Rojo' could be related to the sinigrin content detected in the leaf tissue, with the greatest suppression of *F. graminearum* observed with leaves collected at the stem-extension stage. Hence, effective inhibition of *F. graminearum* mycelium by *B. juncea* leaf discs could be attributed to high levels of sinigrin. Sinigrin is the parent-GSL of allyl ITC and this GSL comprises about 98-99% of the total GSL content of some *Brassica* species such as *B. juncea* and *B. nigra* (Matteo, 2017; Ngala *et al*., 2015b). Allyl ITC has been found to be the predominating compound (>90%) in volatiles released from macerated leaves of *B. juncea* (Charron and Sams, 1999). Correlations of mycelial inhibition with the release of allyl ITC from brassica leaf tissues have been observed (Mayton *et al*., 1996). In a previous study (Ojaghian *et al*., 2012), *B. juncea* was found to be most effective in inhibiting *Sclerotinia sclerotiorum* radial growth by 74-90%, when agar plugs were exposed to fresh macerated tissues of *B. juncea*, *B. campestris* and *B. napus*. An *in vitro* assay (Larkin and Griffin, 2007) showed that macerated leaf tissues of *B. juncea* resulted in 73% and 100% inhibition of *F. oxysporum* and *Rhizoctonia solani* respectively.

If we assume a sinigrin content of 48.5 µmol g^{-1} leaf tissue (Table 4.1) and an ITC release efficiency of 1% (Morra and Kirkegaard, 2002), 8 leaf discs would yield allyl ITC concentrations of 49 mg kg^{-1} suggesting this concentration was sufficient to completely inhibit *F. graminearum* mycelial growth as shown by *B. juncea* 'Brons' leaves from early-bud stage. Morra and Kirkegaard (2002) recorded 14-26% efficiency of ITC release from *B. juncea* leaf discs frozen at -19°C prior to incubation with soil in bottles, whereas <1% release efficiency was noticed using fresh leaf discs. The higher efficiency was attributed to extreme membrane disruption due to freezing and thawing of tissues, allowing greater contact between GSL and

myrosinase. Hence, allyl ITC release efficiency from the frozen leaf discs in the present assay might be higher than 1% due to a greater GSL/ITC conversion. Previous *in vitro* work has shown suppressive effect of allyl ITC on *F. graminearum* at ED_{50} concentrations of 62-135 mg l^{-1} (Chapter 3). Studies on *Alternaria* spp. suggest that ITC promote production of reactive oxygen species and disrupt mitochondrial function (Calmes *et al*., 2015) and the plasma membrane (Wang *et al*., 2020; Zhang *et al*., 2020) in fungal cells.

In the leaf disc assay, *F. graminearum* mycelial plugs which were completely inhibited by *B. juncea* 'Brons' treatments showed no subsequent growth when transferred to fresh PDA media, indicating that the effect was fungicidal rather than fungistatic (data not shown). This is in consistent with Charron and Sams (1999), who reported a fungicidal effect of *B. juncea* macerated leaves on the radial growth of *Pythium ultimum*. The radial growth of *P. ultimum*, exposed to macerated leaves, was completely inhibited after 48 hours and the *P. ultimum* plugs when transferred to fresh PDA, did not grow. The lowest dose (1 leaf disc) of *B. juncea* showed a slight stimulation of colony growth, although this was not significantly different to the control. *Raphanus sativus* and *E. sativa* also appeared to stimulate colony growth insignificantly. Such stimulation is consistent with a previous report by Kirkegaard *et al*. (1996) where colony growth of *Bipolaris sorokiniana* was stimulated when exposed to lower quantities of *B. juncea* and *B. napus* tissues. Slightly higher inhibition in *B. juncea* 'Brons' treatment was seen in the second experiment in comparison to the first leaf disc assay, as mentioned above. This could be due to plants grown at different times of the year (experiment 1: October-January; experiment 2: September-November). Slightly longer daylight hours and higher temperatures during the time period of the second experiment may have resulted in higher GSL concentrations as these factors are known to increase the production of GSL in brassica tissues (Björkman *et al*., 2011).

The two cultivars of *B. juncea* were effective in both leaf disc and microcosm experiments, however, *R. sativus* and *E. sativa* despite being ineffective in the leaf disc assay, showed significant suppression in the microcosm experiment. In addition to ITC, other less toxic compounds such as nitriles and thiocyanates are also produced as a result of GSL hydrolysis and these compounds are known to have biocidal properties (Peterson *et al*., 2000; Tsao *et al*., 2002). Moreover, other toxic compounds such as dimethyl sulphide and carbon disulphide are released during the decomposition of plant material which may also contribute to biofumigation effect

(Bending and Lincoln, 1999; Motisi *et al*., 2010). These factors may have contributed to the suppressive activity seen by *R. sativus* and *E. sativa* in the present microcosm study.

In the microcosm experiment, the two quantities of the chopped tissue added, 65 g and 15 g, were calculated as equivalent to 50 t fresh wt. ha^{-1} and 12 t fresh wt. ha^{-1} respectively. The rationale behind this rate was to mimic the biofumigation potential of brassica plants in field that would generally yield a high biomass and a low biomass. Previously, Handiseni *et al*. (2016) exposed *R. solani* mycelial plugs to *B. juncea*-amended soil in sealed bags. A dose dependent response was reported with inhibition ranging from <5% inhibition at 0.4% (wt/wt) incorporation rate to approximately 50% inhibition at 3.2% (wt/wt) incorporation rate. This is in contrast to findings from microcosm experiment where dose effect was not significant. As the present experiment was carried out in air tight jars, volatiles released from even the lower dose (15 g) were sufficient to effectively suppress *F. graminearum*. However, in agreement to the present findings, Mayton *et al*. (1996) reported >50% radial growth inhibition of *F. sambucinum* by macerated leaf tissue of *B. nigra* and *B. juncea* which was not affected by quantity of tissue (10-40 g) in their microcosm experiments.

Using a cut-and-carry approach, brassica mulch was applied to *F. graminearum*-infected wheat plots in field experiments performed over two years (Drakopoulos *et al*., 2020). Fusarium head blight incidence was significantly reduced by 58% in the first year by *Sinapis alba* and 18% by *B. juncea* in the second year. The two types of mulch also reduced DON content in wheat grain by 40-50%. Crop debris, particularly that of maize, is a primary source of inoculum for Fusarium head blight in wheat (Champeil *et al*., 2004; Vogelgsang *et al*., 2019). Crop debris is present in all shapes and sizes and to reduce the variability within an experimental system it is beneficial to have an artificial crop debris model system. Two types of substrates were used in the present study, chaff represented natural crop residue and blind oat spikes represented an artificial crop debris which is uniform in size and nutritional status, and uniformly infected with *F. graminearum*. The volatiles from chopped brassica tissue appeared to have inhibited *F. graminearum* inoculum, irrespective of inoculum type. This suggests that biofumigation could prove to be effective in reducing *F. graminearum* inoculum present in a variety of crop residues such as chaff, seed and straw, under field conditions. However, it would be useful to identify if biofumigation is equally effective against inoculum of various sizes as *F.*

graminearum within larger pieces of inoculum maybe protected from contact with the inhibitory compounds released during the process.

Brassica juncea 'Caliente Rojo', which is fairly new cultivar, was not available at the time period of the first experiments. The suppressive effect of *B. juncea* 'Caliente Rojo' seen in the present study suggests that this cultivar could be a promising biofumigant for managing *F. graminearum* in the field. The cultivar could be sown following harvest of wheat in summer, and incorporated in autumn followed by subsequent cereal production. Glucosinolate analysis of this cultivar showed that concentrations of the dominant GSL, sinigrin, were highest at stem-extension stage compared to the other two stages. A balance between biomass and GSL concentration could be achieved by identifying an appropriate growth stage between stem-extension and flowering to maximise GSL content for incorporation. The biofumigant, when chopped and incorporated, is then likely to produce ITC at sufficiently effective concentrations. Glucosinolate concentrations recorded in the present study could be achievable in field situations. Total GSL concentrations of up to 50, 69 and 61 μmol g^{-1} biomass have been detected in *B. juncea*, *R. sativus* and *E. sativa* respectively under field conditions (Ngala *et al*., 2015a). Biofumigation if applied in a cereal rotation, may therefore be useful in reduction of *F. graminearum* inoculum in the field, thus suppressing infection in the subsequent cereal crop. However, it should be considered that the present experiments were carried out under controlled conditions. In the field, various factors such as soil pH, temperature, moisture and organic content as well as the biofumigant crop establishment and growth should be considered, as these factors can affect the outcome of biofumigation. Moreover, weather conditions such as temperature, daylight hours and rainfall also influence the efficacy of biofumigation.

Chapter 5

Chapter 5

5 Application of biofumigation for the management of *Fusarium graminearum* in a wheat-maize rotation

5.1 Abstract

Fusarium graminearum is the most important causal agent of head blight in wheat, and stalk and ear rot in maize. A field experiment was conducted to investigate the effect of incorporation of Brassicaceae cover crops on *Fusarium graminearum* in a wheat-maize rotation. Five species belonging to Brassicaceae (*Brassica juncea, Eruca sativa, Raphanus sativus, B. carinata, B. oleracea* var. *caulorapa* L.) were used in the field experiment to investigate their potential to suppress *F. graminearum* inoculum in soil, disease incidence in maize and to reduce subsequent mycotoxin contamination in maize. *Brassica juncea* was found to contain the highest glucosinolate concentration in shoots (31 µmol g^{-1}). Severity of ear rot and stalk rot in maize was not significantly reduced in the amended plots. Incorporation of *R. sativus* 'Terranova' significantly decreased the amount of *F. graminearum* DNA by 58% compared with the cultivated fallow treatment, however the DNA concentration was not significantly different to fallow uncultivated. *Fusarium graminearum* DNA and deoxynivalenol in maize was 50% lower after incorporation of *B. oleracea* var. *caulorapa* L. compared to after fallow treatment but the difference was not significant. The brassica crops used in the present field experiment were not effective in suppressing *F. graminearum*, therefore further studies to optimise the current approach are recommended.

5.2 Introduction

The suppressive effect of biofumigation is generally attributed to the toxicity of isothiocyanate (ITC) which is produced as a result of glucosinolate (GSL) hydrolysis by myrosinase enzyme upon plant tissue disruption. However, the application of green manure also increases the organic matter content in the soil. This organic matter helps in nourishing and flourishing soil microorganisms, thus enhancing their competition and antagonism effects. Additionally, toxic compounds released during the decomposition of the organic matter may contribute to the suppressive activity (Kirkegaard and Matthiessen, 2004; Motisi *et al*., 2010; Tagele e*t al*., 2021).

Field research conducted on biofumigation for soil-borne pathogens has been discussed in section 1.6.4. Recently, Drakopoulos *et al*. (2020) applied the mulch of *Sinapis alba* and *Brassica juncea*, in a cut-and-carry approach, to *Fusarium graminearum*-infected wheat plots; Fusarium head blight incidence was significantly reduced by 58% in the first year by *S. alba* and 18% by *B. juncea* in the second year, in field experiments performed over two years. Following encouraging results from studies conducted under laboratory conditions (Chapters 3 and 4), a field experiment was undertaken to investigate the potential of incorporation of brassica crops on suppressing *F. graminearum* inoculum in a wheat-maize rotation.

5.3 Materials and methods

5.3.1 Experimental set up

From June 2018 to December 2019, a field experiment was conducted at 'Heaford' field, Harper Adams University, Newport, Shropshire, UK (52°47'04.0"N 2°26'04.9"W) in a 72 x 18 m area. The soil at this site is a sandy clay loam (62% sand, 18% silt, 20% clay) with organic matter content 6%, pH 5.9 and available sulphate 20.75 mg l^{-1}. The field experiment was designed for Brassicaceae cover crops incorporation in a wheat-maize rotation. The experiment comprised six replicates of eight treatments in a randomised complete block design and each plot was 27 m^2 (9 x 3 m). Treatments included *Brassica juncea* 'Brons' (Indian mustard),

Eruca sativa ‘Trio’ (rocket), *Raphanus sativus* ‘Bokito’ (oilseed radish), *R. sativus* ‘Terranova’, *B. carinata* ‘Cappucchino’ (Ethiopian mustard), *B. oleracea* var. *caulorapa* L. ‘Kolibri’ (kohlrabi), fallow cultivated and fallow uncultivated. Fallow cultivated were the plots in which no crops were grown, however flail, rotovator and roller were used on these plots similar to brassica plots. Whereas fallow uncultivated had no crops and no machinery used except roller. Seeds of all Brassicaceae were supplied by RAGT seeds, UK (except *B. oleracea* var. *caulorapa* L. ‘Kolibri’ supplied by Elsoms, UK). All crops were established and maintained according to standard crop husbandry practices at Harper Adams University, Shropshire, UK.

5.3.2 Semi-artificial infection of wheat

One kg of oat was soaked in 200 ml distilled water for 2 h, drained and autoclaved in a 78 x 40 cm autoclavable bag [VWR (129-0581)] at 124°C for 1 hour. The following day the oats were re-autoclaved at 124°C for 1 hour after 24 hours and allowed to cool. Oat broth was prepared by adding 1 g oat flour to 100 ml sterile distilled water in 250 ml flasks. A cotton plug was inserted into the neck of the flask before it was covered with tin foil and autoclaved at 121°C for 15 min. Five 7 mm mycelial plugs, taken from a 5-day old colony of *F. graminearum* growing on potato dextrose agar (PDA) (Merck, KGaA, Germany), were added to the flask containing oat broth and incubated on an orbital shaker at 120 rpm for 7 days. A 100 ml of the oat broth was added to 1.25 L potato dextrose broth (PDB) (Oxoid, Basingstoke, UK) and mixed. A total amount of 100 ml of the PDB mixture containing *F. graminearum* was added to each autoclaved oat bag, mixed well and incubated for 3 weeks before field inoculation (Figure 5.1a). Bags were agitated every few days to avoid clumping of the inoculum. All incubations were at room temperature (ca. 18°C). A total of 40 kg of *Fusarium* inoculum was prepared with an equal amount for each of five fungal strains (FG2556, FG2560, FG2481, FG2498, FG2502). All strains were isolated from wheat samples collected in 2016 and were supplied by Dr Phil Jennings, Fera Sciences Ltd. Bags were mixed to create a bulk bag of inoculum for each experimental block with an equal mix of each strain.

Winter wheat ‘Shabras’ was sown in early October 2017 and maintained using standard agronomy. Wheat at early stem-extension stage (Zadoks GS31; Zadoks *et al*., 1974) was inoculated manually by dispersing the oat inoculum at 25 g m^{-2}.

Perithecia of *F. graminearum* were observed on the oat grain inoculum five weeks after application (Figure 5.1b).

The disease incidence in the field was measured by counting the number of ears with typical Fusarium head blight symptoms of partially bleached ears, at medium milk (GS75). Wheat ears in a 60 x 120 cm quadrat were observed at 24 different locations in a central area (60 x 17 m) of the trial area at 5-12 m apart. Disease incidence was expressed as the percentage of symptomatic wheat ears.

Post-harvest, wheat debris were collected to test presence of *F. graminearum*. Chaff, rachis and straw separately were randomly collected from the field, additionally the three type of wheat debris were also collected from each block. Straw and rachis were cut into 1 cm pieces. One hundred pieces of each type of wheat debris (collected from whole field) and 100 pieces of wheat debris from each block (collected block-wise) were plated out on modified CZID media (CZPD) (Hofgaard *et al*., 2016; Chapter 2) plates with five pieces per plate. Plates were incubated at room temperature (ca. 20°C) for 12-15 days. *Fusarium graminearum* incidence in the inoculum pieces was recorded as presence of *F. graminearum* colony growth based on the characteristic reddish pigmentation. For confirmation, some of the assumed *F. graminearum* colonies were sub-cultured on PDA and incubated at room temperature (ca. 20°C) for 14 days. The conidia were harvested as described in Chapter 3 and confirmed as *F. graminearum* based on spore morphology (Leslie and Summerell, 2006).

5.3.3 Biofumigants- biomass, glucosinolate analysis, incorporation

Following wheat harvest in late July 2018, cover crops were sown on 13 August 2018 using the recommended seed rate (Table 5.1) with 100 kg ha^{-1} nitrogen and 25 kg ha^{-1} sulphur applied to the seedbed. On 19 November 2018, the brassica crops, at 25% flowering, were flailed and rotovated. Following incorporation, the soil surface was rolled to reduce soil porosity (Figure 5.1c). Flail and rotovator were used on all plots except uncultivated fallow plots.

Glucosinolate analysis: Prior to incorporation, plants for GSL analysis were sampled and processed as described by Ngala *et al*. (2015a). Briefly, three samples

from each plot were randomly selected, uprooted and processed carefully, roots and shoots separately flash frozen in liquid nitrogen, stored at -80°C until freeze dried (GVD6/13 MKI freeze dryer; GIROVAC Ltd, North Walsham, UK). Freeze-dried samples were milled and stored at -18°C. Samples collected from four blocks (2,3,4,6) were sent to NIAB Labtest, Cambridge, UK where GSL analysis was performed following ISO 9167 “Rapeseed and rapeseed meals - Determination of glucosinolates content – Method using HPLC”.

Biomass: Prior to incorporation, plants from two 0.33 m^2 quadrats (within central 1 x 5 m area of plot) were uprooted, taken to the laboratory, separated into roots and shoots and weighed fresh. The plant samples were then placed in a forced air oven, for drying at 100°C for 48 hours, to generate a dry weight.

Table 5.1 Brassica cover crops used in a field experiment to assess the effect of biofumigation on *Fusarium graminearum* in a wheat-maize rotation

Brassica species	Common name	Cultivar	Seed rate	Supplier
Brassica juncea	Indian mustard	Brons	8 kg ha^{-1}	RAGT Seeds UK
Brassica carinata	Ethiopian mustard	Cappucchino	15 kg ha^{-1}	RAGT Seeds UK
Raphanus sativus	Oilseed radish	Bokito	20 kg ha^{-1}	RAGT Seeds UK
Raphanus sativus	Oilseed radish	Terranova	20 kg ha^{-1}	RAGT Seeds UK
Eruca sativa	Rocket	Trio	10 kg ha^{-1}	RAGT Seeds UK
Brassica oleracea var. *caulorapa* L.	Kohlrabi	Kolibri	0.5 kg ha^{-1}	Elsoms, UK

Figure 5.1 Experimental set up of the study: (a) Oats inoculated with *Fusarium graminearum* conidial suspensions after incubation, (b) Perithecia of *F. graminearum* formed on the oats on the soil surface five weeks after inoculation of wheat plots, (c) Brassica crops at 25% flowering flailed, rotovated and rolled

5.3.4 Assessment of *Fusarium graminearum* inoculum buried in sachets post-incorporation of brassica biofumigants

Blind oat spikes from oat screenings (Morning Foods, Crewe, UK) were used as artificial crop debris. One kg of oat screenings was soaked in distilled water for 12 hours before autoclaving in a 78 x 40 cm autoclavable bag [VWR (129-0581)]. The blind oat spikes were re-autoclaved after 24 hours and allowed to cool. Spore suspension was prepared as described in Chapter 4 (section 4.3.3.1). A 100 ml of the spore suspension was added to the bag, mixed well and incubated for 10-12 days in dark. The inoculum was confirmed to be infected with *F. graminearum* by plating on PDA prior to using in the experiment. Square 12.5 cm^2 fine mesh (voile) sachets were prepared using a heat sealer. A 50 cm nylon string with label tag was tied to each sachet. Six grams of the above prepared inoculum, which contained more than 100 pieces of the inoculum, were added to each of the 288 sachets (six sachets per plot) and sealed with heat-sealer. Immediately after brassica incorporation, six sachets of the inoculum were buried at 10 cm depth in a central area (1 x 4 m) of each plot, at intervals of 1-2 m apart. Pegs were tied to the nylon string of the sachets to enable easy location and removal of the sachets later (Figure 5.2). From each plot, three sachets were removed 8 and 16 weeks after their burial and transported to laboratory to assess for *F. graminearum* incidence. One hundred inoculum pieces from each sachet were plated out on CZPD media (10 pieces per plate), and incubated at 20°C. After 12-15 days, *F. graminearum* growth was recorded based on the characteristic reddish pigmentation of *F. graminearum* colonies and the *Fusarium* incidence was calculated.

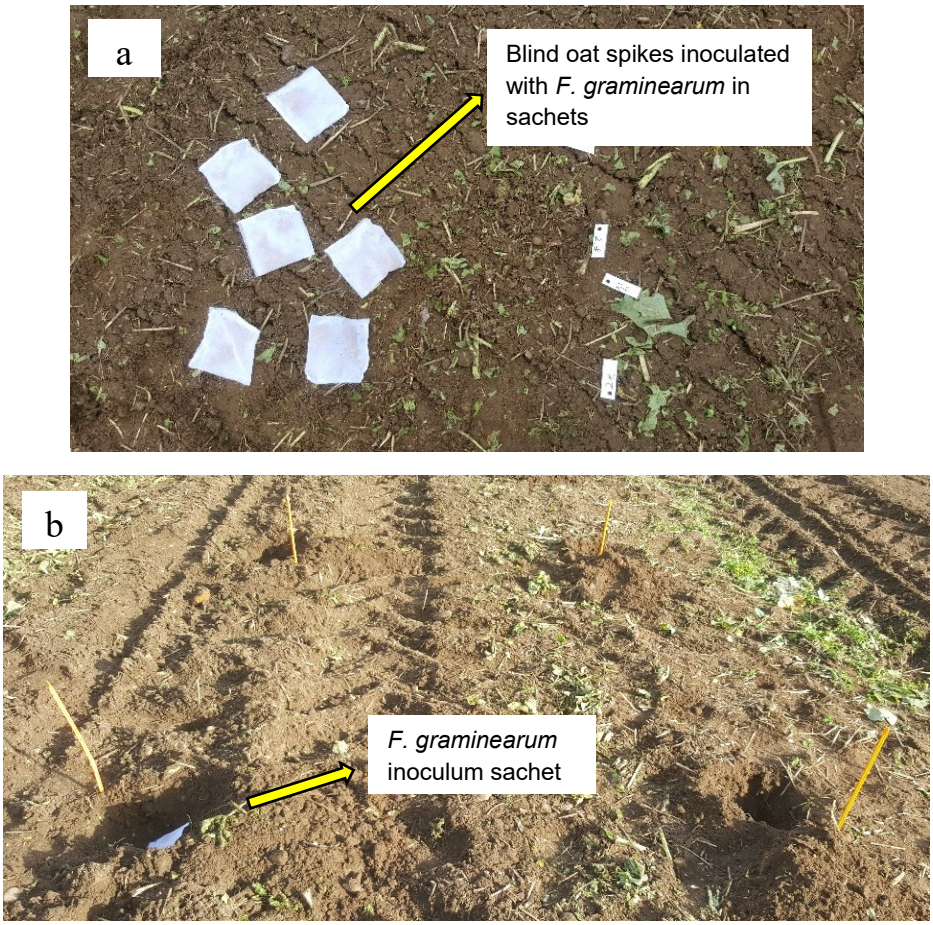


Figure 5.2 (a) Blind oat spikes inoculated with *Fusarium graminearum* in sealed sachets tied to nylon strings (b) *Fusarium graminearum* inoculum sachets placed to be buried in plots post-incorporation of brassicas

5.3.5 Maize assessment

5.3.5.1 Disease assessment and *Fusarium graminearum* incidence

Maize 'Ambition' was sown mid-April 2019. In December 2019, when the maize had a grain moisture content of 44%, 30 plants were randomly sampled from a 1 x 6 m area in the centre of each plot. Primary cobs were handpicked and stalks were cut in lateral direction above roots and above the second node from bottom. Samples were transported to laboratory, where ears were husked and the ear surface covered with mycelium was visually assessed for ear rot. Stalks were dissected and the infected area of lower node, upper node and internode was visually assessed for stalk rot (red staining). Rot which was presumably not caused by *Fusarium* was not recorded. Symptoms of *Fusarium* infection were assessed according to a seven-class disease severity rating scale of 0 (no infection) to 6 (infection symptoms close to 100% of plant surface) (Masiello *et al*., 2019) and disease severity % was calculated by McKinney Index (McKinney, 1923).

One row of kernels from each of the 30 ears were removed by hand, mixed, before 100 representative kernels were used to assess *Fusarium* incidence. The remaining kernels were placed in perforated plastic bags and dried at 60°C with forced ventilation for mycotoxin and molecular analysis. Maize kernels (100 per plot) were surface disinfected with sodium hypochlorite (1.2% available chlorine) containing 0.05% Tween 20 for 3 min, rinsed with sterile distilled water thrice and plated out on PDA plates supplemented with streptomycin sulphate (130 mg l^{-1}). Plates were incubated at room temperature (ca. 20°C) for 10-12 days and *F. graminearum* mycelial growth was recorded based on the characteristic reddish coloured colonies. *Fusarium graminearum* growth was confirmed as described above. *Fusarium* incidence was calculated as mean of the number of kernels infected.

5.3.5.2 Mycotoxin and molecular analysis

The dried maize kernels were ground with a mill (Retsch ZM200, Germany;1 mm mesh size) and samples were stored at -18°C until further processing.

Deoxynivalenol (DON) was quantified in 8 g of milled flour using a DON ELISA assay (Agraquant, Romer Lab Diagnostic, Austria) according to the manufacturer's instructions. Additionally, the flour was analysed for DNA amount of *F. graminearum*. DNA was extracted from flour using a cetyltrimethylammonium bromide (CTAB) buffer and quantified by spectrophotometry as detailed in Edwards *et al*. (2001) and diluted to 20 ng μl^{-1}. DNA was first amplified with ITS 4 and 5 primers (White *et al*., 1990) with an annealing temperature of 50°C (Imathiu *et al*., 2013). At this temperature, both fungal and plant DNA are amplified, hence it was confirmed that amplifiable DNA is present within each sample. PCR products were observed on a 2% agarose gel in Tris-acetate-EDTA (TAE) buffer using 6X GelRed loading buffer (Biotium). *Fusarium graminearum* DNA was then quantified using *Tri*5 primers as described by Edwards *et al*. (2001) by SYBR green qPCR method. EvaGreen master mix (Biotium) was used and 10-fold dilutions of 10 ng μl^{-1} of *F. graminearum* DNA were used as standards. The program used had 40 temperature cycles of 95°C for 15 s, 62°C for 15 s, 72°C for 30 s and 82°C for 10 s. The first cycle had an extra 12 min at 95°C, and the final cycle had an extra 10 s at 95°C. *Fusarium graminearum* DNA was measured relative to the total (plant and fungal) DNA extracted (pg *F. graminearum* DNA ng^{-1} total DNA).

5.3.6 Statistical analysis

Data were subjected to one-way analysis of variance using Genstat® (20^{th} edition) statistical software. Data from assessment of *F. graminearum* in inoculum (sachets) and maize kernels were logit transformed and angular transformed respectively. Data from DON and *F. graminearum* DNA in maize were log10 transformed. Significant differences between treatments were determined using post hoc Tukey's test (p=0.05).

5.4 Results

5.4.1 *Fusarium graminearum* in wheat

Fusarium head blight incidence was 2.6% in the winter wheat crop, assessed on the medium milk (GS75). Post-harvest, *F. graminearum* infection in chaff, rachis and straw were found to be 92%, 89% and 59% respectively. *Fusarium graminearum* infection was detected in wheat debris from all blocks, ranging from 90 to 98%.

5.4.2 Biomass, glucosinolate concentrations of brassicas

The biomass of brassica cover crops are shown in Table 5.2. The total above-ground biomass (fresh weight) of the brassicas ranged from 19 t ha^{-1} (*B. oleracea* var. *caulorapa* L. 'Kolibri') to 79 t ha^{-1} (*R. sativus* 'Bokito'). The two cultivars of *R. sativus* produced similar biomass (85 and 89 t ha^{-1} fresh weight; 6.5 and 7.4 t ha^{-1} dry weight). *Brassica juncea* 'Brons' produced 48 t ha^{-1} of above-ground biomass (fresh weight). Concentrations of GSL occurring in each brassica is presented in Table 5.3. The GSL profile found in the brassicas varied both qualitatively and quantitatively. *Brassica juncea* was found to contain the highest total GSL concentration among all the brassicas tested. The predominant GSL of *B. juncea* was sinigrin, precursor of allyl isothiocyanate (AITC), and it comprised 85% of the total GSL content of the shoot tissue. On the other hand, sinigrin made up 79% of total GSL content of shoot tissue of *B. carinata*, occurring at a concentration of 13.06 µmol g^{-1}. The predominant GSL found in root tissue of *R. sativus* was glucoraphasatin (4-methylsulfanyl-3- butenyl ITC or raphasatin-precursor). The GSL concentration expected per area of field was estimated (Figure 5.3). For *B. juncea*, the total GSL concentration (shoots) expected in the field was estimated to be 13 mmol m^{-2} and that for *B. carinata* was 11 mmol m^{-2}.

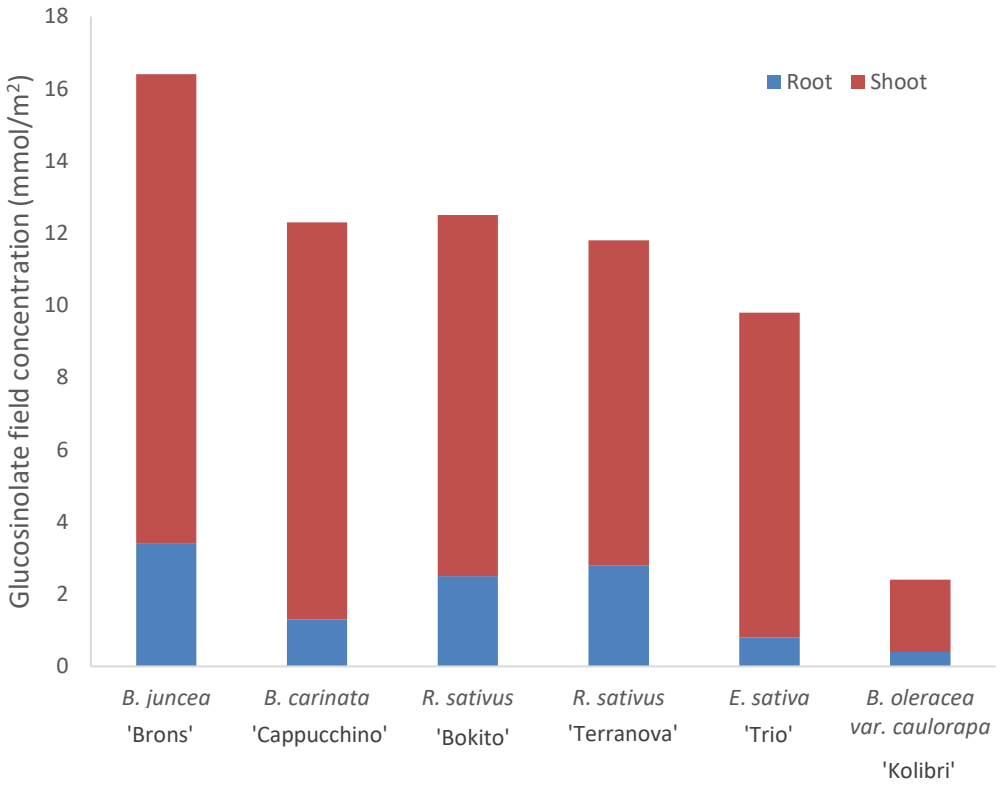


Figure 5.3 Expected glucosinolate concentration per m^2 estimated from the biomass and glucosinolate concentrations detected in brassica plants at 13 weeks after planting

Table 5.2 Shoot and root biomasses of brassica cover crops grown in a field experiment to assess the effect of biofumigation on *Fusarium graminearum* in a wheat-maize rotation

	Biomass (t ha^{-1})					
	Fresh weight			Dry weight		
Brassica	Shoot	Root	Total	Shoot	Root	Total
Brassica juncea 'Brons'	48.4 (5.6)[a]	4.8 (0.8)	53.3 (6.2)	3.9 (0.7)	0.7 (0.2)	4.6 (0.8)
Brassica carinata 'Cappucchino'	69.5 (16.5)	4.3 (0.7)	73.8 (17)	6.4 (1.6)	0.9 (0.2)	7.3 (1.7)
Raphanus sativus 'Bokito'	79.0 (14.7)	10.4 (2.4)	89.4 (15.3)	6.3 (1.3)	1.1 (0.2)	7.4 (1.3)
Raphanus sativus 'Terranova'	74.0 (22)	10.5 (3.1)	84.6 (23)	5.6 (1.8)	1.0 (0.3)	6.5 (2.0)
Eruca sativa 'Trio'	42.8 (14.4)	2.4 (0.7)	45.3 (15.0)	3.6 (1.1)	0.4 (0.1)	4.0 (1.2)
Brassica oleracea var. *caulorapa* L. 'Kolibri'	19.8 (8.0)	0.9 (0.2)	20.7 (8.2)	2.6 (1.0)	0.2 (0.1)	2.9 (1.1)

[a] n=6, numbers in parentheses represent the standard error of the mean

Table 5.3 Type and concentration of glucosinolates in freeze-dried tissue of brassicas grown in a field experiment to assess the effect of biofumigation on *Fusarium graminearum* in a wheat-maize rotation

Glucosinolate (μmol g^{-1} freeze-dried tissue)	***B. juncea*** **'Brons'**		***B. carinata*** **'Cappucchino'**		***R. sativus*** **'Bokito'**		***R. sativus*** **'Terranova'**		***E. sativa*** **'Trio'**		***B. oleracea* var. *caulorapa* L.** **'Kolibri'**	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Glucoberin	2.25 (0.32)[a]	2.17 (0.38)	2.01 (0.63)	1.89 (0.39)	2.33 (0.74)	2.22 (0.53)	2.35 (0.41)	2.25 (0.75)	2.06 (0.58)	2.18 (0.49)	2.24 (0.21)	1.83 (0.50)
Progoitrin	0.42 (0.13)	0.37 (0.08)	0.34 (0.23)	0.25 (0.17)	0.42 (0.31)	0.54 (0.39)	0.33 (0.22)	0.34 (0.27)	2.08 (1.49)	1.65 (1.02)	0.39 (0.27)	0.18 (0.13)
Sinigrin	26.39 (12.42)	19.97 (12.33)	13.06 (6.21)	4.81 (3.24)	-	-	-	-	-	-	-	-
Gluconapin	0.20 (0.14)	0.14 (0.17)	-	-	-	-	-	-	-	-	-	-
Glucobrassicin	0.17 (0.07)	0.32 (0.04)	0.65 (0.30)	0.43 (0.31)	1.43 (0.79)	1.54 (1.48)	2.05 (1.29)	0.78 (0.67)	0.38 (0.38)	0.05 (0.07)	1.19 (0.76)	0.91 (0.19)
Gluconasturtiin	1.32 (0.70)	18.04 (3.76)	0.42 (0.29)	6.17 (3.50)	1.12 (0.49)	2.47 (1.74)	1.28 (0.61)	2.38 (2.22)	0.90 (0.42)	2.96 (2.18)	0.52 (0.37)	5.42 (1.75)
Neoglucobrassicin	0.07 (0.03)	0.90 (0.17)	0.11 (0.07)	0.61 (0.44)	-	0.42 (0.38)	0.02 (0.03)	0.12 (0.13)	0.03 (0.02)	0.00	0.53 (0.36)	4.35 (1.82)
Glucoraphanin	-	-	-	-	7.84 (4.79)	1.39 (0.95)	5.24 (4.43)	1.47 (1.11)	7.36 (3.96)	3.94 (2.07)	3.45 (2.72)	1.30 (0.67)
Glucoraphenin	-	-	-	-	0.45 (0.11)	-	0.35 (0.19)	-	-	-	-	-
4 hydroxy glucobrassicin	-	0.04 (0.05)	-	-	-	-	-	0.02 (0.03)	-	-	-	0.27 (0.12)
Glucoraphasatin	-	-	-	-	2.43 (1.69)	14.90 (9.77)	3.45 (2.60)	17.60 (10.92)	-	-	-	-
Glucoalyssin	-	-	-	-	-	-	-	-	0.50 (0.21)	0.24 (0.21)	-	-
Glucoerucin	-	-	-	-	-	-	-	-	7.03 (3.65)	12.28 (7.30)	0.14 (0.16)	3.80 (1.71)
4-mercaptobutyl	-	-	-	-	-	-	-	-	4.89 (2.04)	1.26 (1.94)	-	-
unknown	-	-	-	-	-	-	-	-	0.53 (0.37)	-	-	-
unknown	-	-	-	-	-	-	-	-	0.93 (0.70)	-	-	-
Total glucosinolates	30.83 (13.76)	41.91 (15.63)	16.59 (7.62)	14.19 (7.31)	16.02 (8.66)	23.47 (14.65)	15.06 (7.33)	24.96 (15.10)	26.69 (12.33)	24.57 (12.69)	8.46 (4.55)	18.06 (6.22)

[a] n=4, numbers in parentheses represent the standard error of the mean

5.4.3 Effect of brassica incorporation on *F. graminearum* inoculum in sachets

For both sets of sachets (removal after 8 and 16 weeks after brassica incorporation), the percentage incidence of *F. graminearum* ranged from 94 to 98% for all treatments (Table 5.4). The effect of brassica incorporation on *F. graminearum* inoculum was not significant (p=0.677 and p=0.074 after 8 and 16 weeks respectively). CV% and SED for set 1 (removal after 8 weeks) were 31.8 and 0.57 respectively and that for set 2 (removal after 16 weeks) were 20.4 and 0.37.

Table 5.4 Percentage incidence of *Fusarium graminearum* recovered in oat inoculum post-incorporation of brassicas. Sachets containing *Fusarium graminearum*-inoculated blind oat spikes, representing artificial crop debris, were buried in plots post-incorporation of brassica cover crops and removed from the field plots after 8 and 16 weeks

Treatments	**% incidence *Fusarium graminearum* recovered in oat inoculum**	
	8 weeks post-incorporation	16 weeks post-incorporation
Fallow	95	98
Fallow uncultivated	95	97
Brassica juncea 'Brons'	98	97
Brassica carinata ‘Cappucchino’	96	95
Raphanus sativus ‘Bokito’	96	94
Raphanus sativus ‘Terranova’	95	95
Eruca sativa ‘Trio’	94	94
Brassica oleracea var. *caulorapa* L. ‘Kolibri’	97	97

5.4.4 Effect of brassica biofumigation on *Fusarium graminearum* diseases in a subsequent maize

5.4.4.1 Disease severity and *Fusarium graminearum* incidence in maize

Disease severity of ear rot ranged from 70% in *B. carinata*-treated plots to 80% in fallow plots (Figure 5.4). However, analysis of variance showed no significant effect of brassica incorporation on ear rot severity (p=0.579). Disease severity of stalk rot in the upper, lower and internodes of maize stalks is shown in Figure 5.5. The lowest disease severity percentage in upper nodes was found in *B. carinata* treatment (19%) and the highest (26%) in fallow uncultivated. The disease severity percentage in internodes was similar (6-9%) in all treatments. The effect of brassicas on stalk rot severity was insignificant (p=0.942). Maize kernels tested for *F. graminearum* incidence were found to be infected in all treatments ranging from 93 to 98% with no significant effect (p=0.411) between the treatments (Figure 5.6).

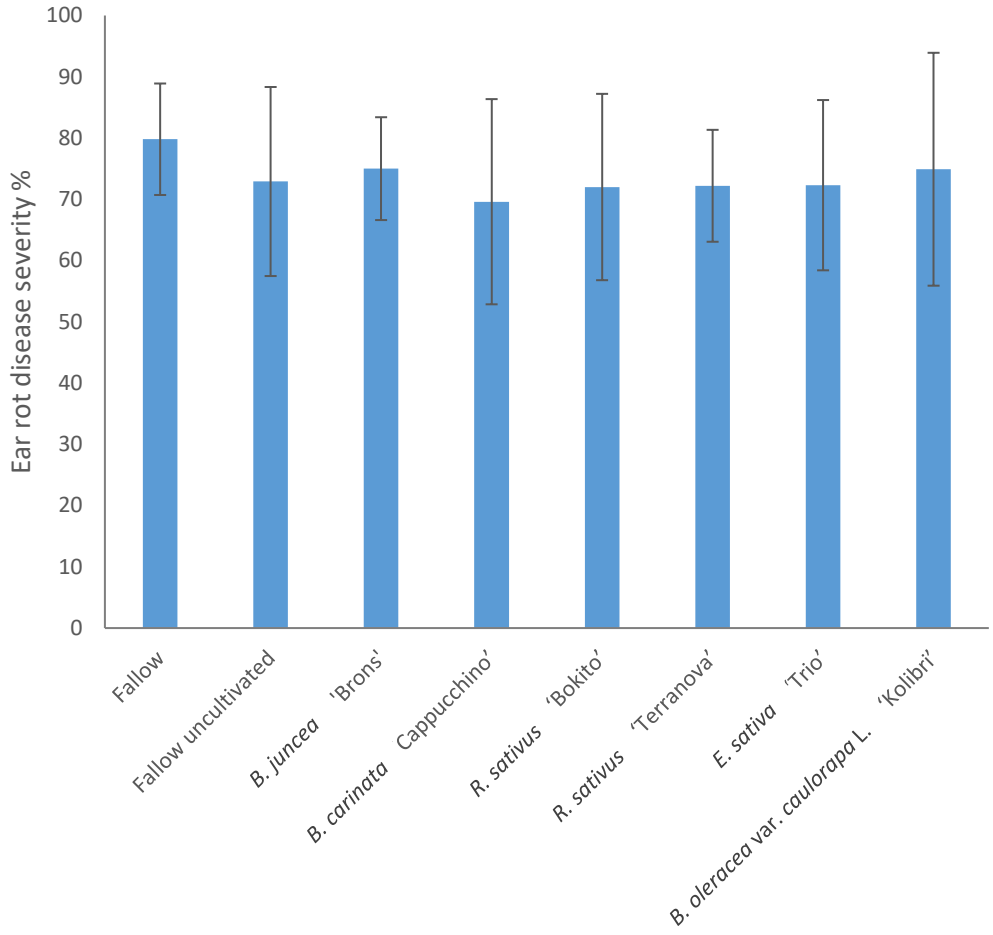


Figure 5.4 Ear rot severity in maize grown in brassica-incorporated plots preceded by *Fusarium graminearum*-inoculated wheat. Ears were visually assessed according to a 7-class disease severity rating scale of 0 (no infection) to 6 (infection symptoms close to 100% of plant surface) and disease severity % was calculated by McKinney Index. Analysis of variance showed no significant difference between treatments (p=0.579, CV%=11.2, SED=4.75)

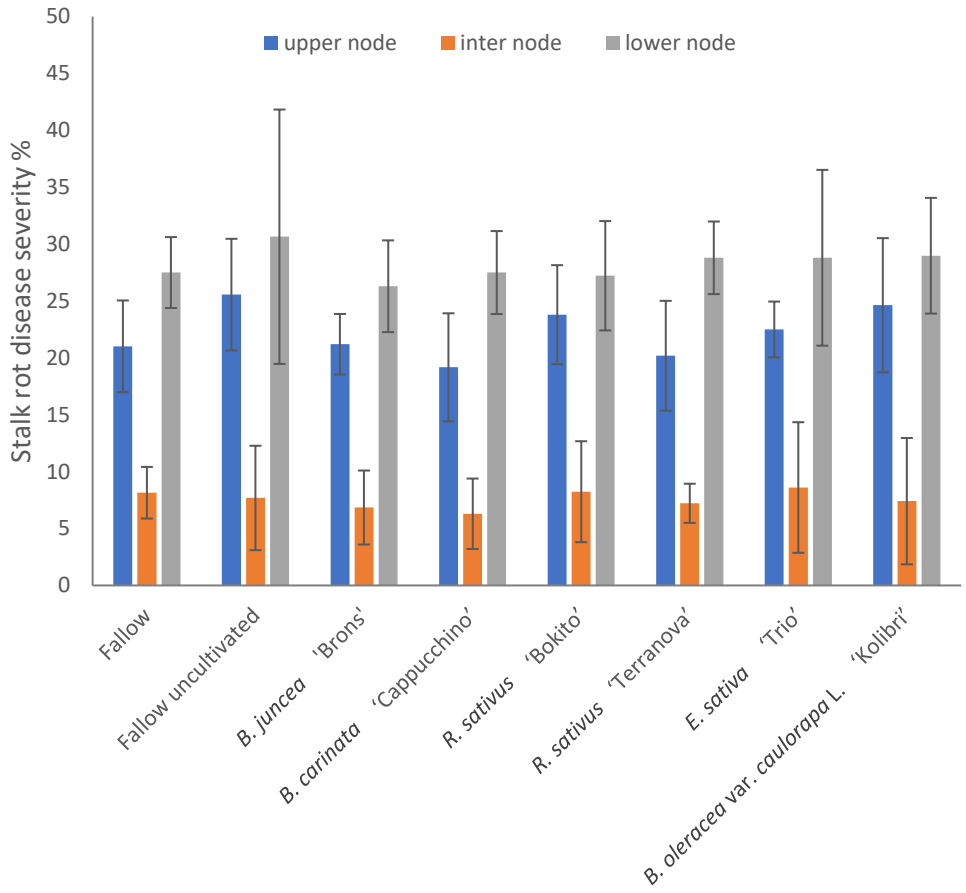


Figure 5.5 Stalk rot severity in maize grown in brassica-incorporated plots preceded by *Fusarium graminearum*-inoculated wheat. Upper node, lower node and internode of stalks were visually assessed according to a 7-class disease severity rating scale of 0 (no infection) to 6 (infection symptoms close to 100% of plant surface) and disease severity % was calculated by McKinney Index. Analysis of variance showed no significant difference between treatments (upper node: p=0.093, CV%=17.8, SED=2.287; inter node: p=0.971, CV%=50.6, SED=2.21; lower node: p=0.942, CV%=21, SED=3.418)

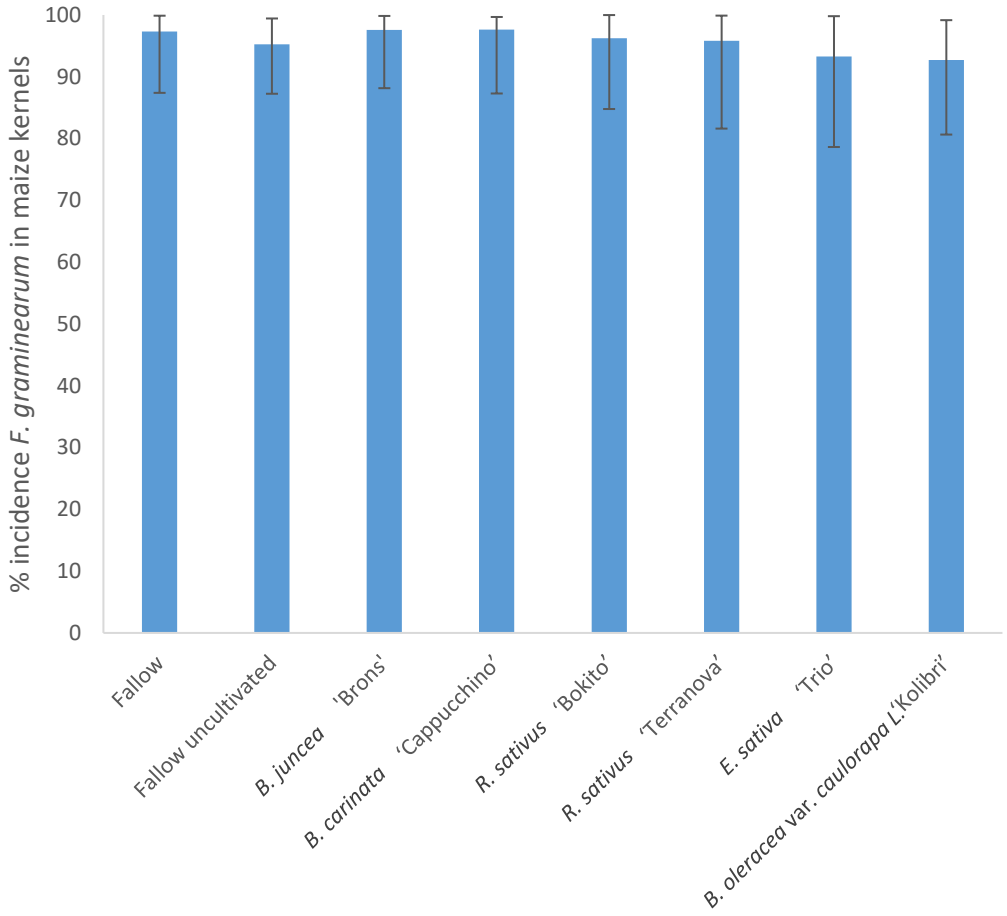


Figure 5.6 Percentage incidence of *Fusarium graminearum* in kernels of maize grown in brassica-incorporated plots preceded by *Fusarium graminearum*-inoculated wheat. Representative kernels were plated out on media and assessed for *F. graminearum* growth. Analysis of variance showed no significant difference between treatments (p=0.411, CV%=8.1, SED=3.642)

5.4.4.2 Assessment of deoxynivalenol and *Fusarium graminearum* DNA in maize

Deoxynivalenol concentrations in maize kernels are shown in Figure 5.7. All samples were found to be contaminated with DON, with 67% samples exceeding the legal limit of 1.75 mg kg^{-1} DON in maize for human consumption. The effect of brassicas on DON contamination was found to be non-significant (p=0.635). The concentrations in the 48 samples ranged from 0.51 to 14.33 mg kg^{-1}. Deoxynivalenol concentrations in maize was 50-60% lower after incorporation of *B. oleracea* var. *caulorapa* L. compared to after the two fallow treatments. *Brassica juncea*-treated plots were found to have the highest variation of DON concentrations in the maize flour with values ranging from 0.55 to 14.33 mg kg^{-1}. Amount of *F. graminearum* DNA in maize is shown in Figure 5.8. Incorporation of *R. sativus* 'Terranova' significantly decreased (p<0.05) the amount of *F. graminearum* DNA by 58% compared with the fallow cultivated treatment, however the level was not significantly different to fallow uncultivated. Maize grown after *B. oleracea* var. *caulorapa* L. treatment also had *F. graminearum* DNA 55% lower compared to maize grown after the cultivated fallow treatment, but the difference was not significant.

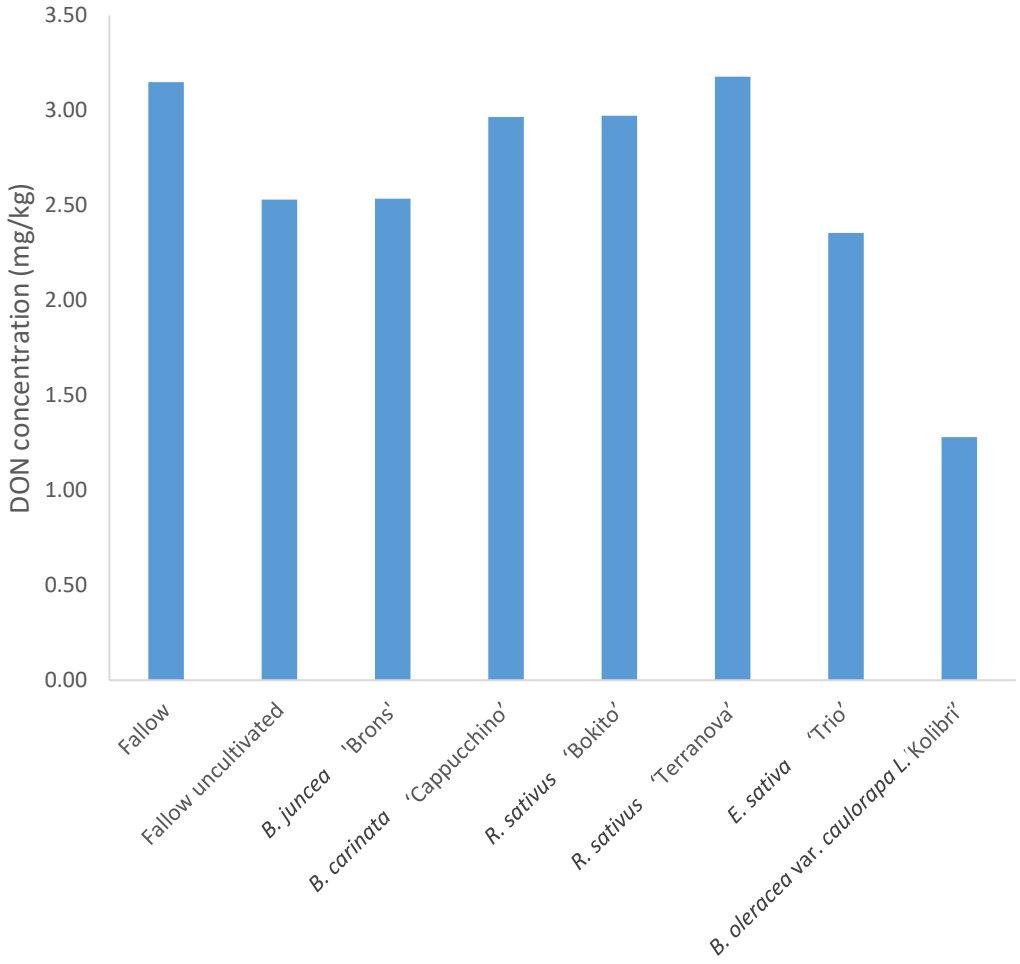


Figure 5.7 Deoxynivalenol (DON, mg kg^{-1}) concentration in maize grown in brassica-incorporated plots preceded by *Fusarium graminearum*-inoculated wheat. Analysis of variance showed no significant differences between treatments (p=0.635, CV%=90.8, SED=0.211)

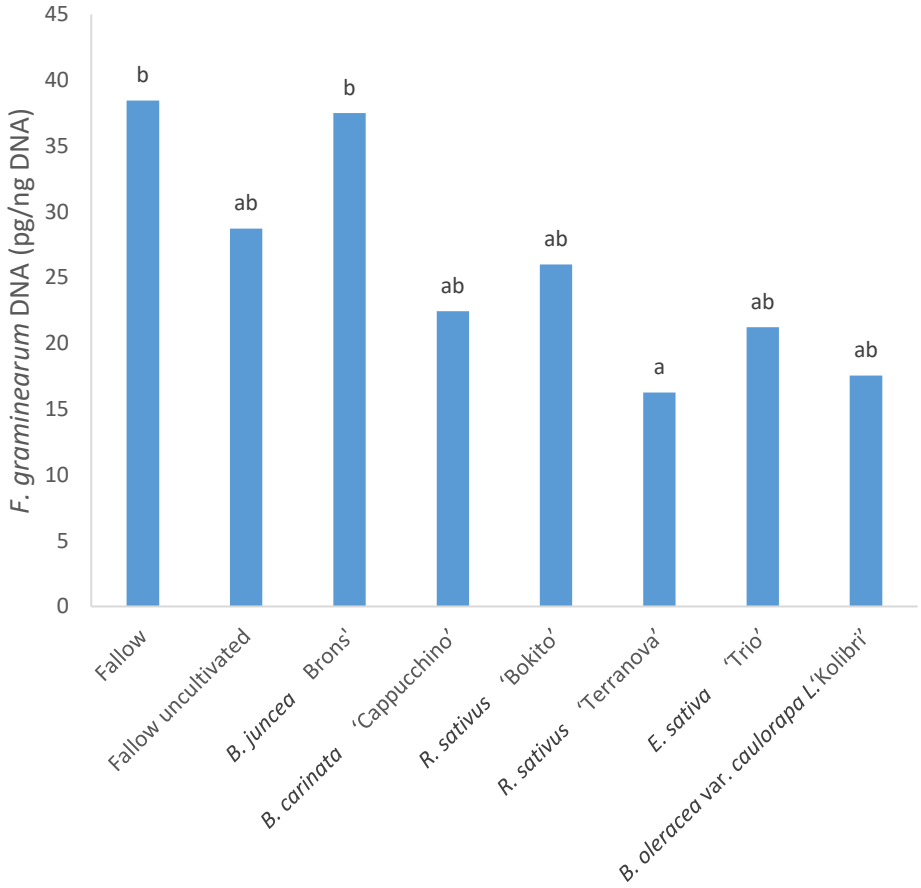


Figure 5.8 *Fusarium graminearum* DNA content in maize grown in brassica-incorporated plots preceded by *Fusarium graminearum*-inoculated wheat. Different letters indicate significant differences according to post hoc Tukey's test at 5% significance level (CV%=13.8, SED=0.111)

5.5 Discussion

In the present two-year study, biofumigation was not effective in suppressing *F. graminearum* in the soil and in the following maize crop. Following incorporation of brassicas, *F. graminearum* infection in maize was not significantly lower compared to control (fallow) plots. This is in contrast to results from previous work in the laboratory (Chapters 3 and 4) indicating brassicas could have a suppressive effect on *F. graminearum* under field conditions.

Biofumigant brassicas have been effective in suppressing soil-borne pathogens in studies conducted elsewhere. For example, Subbarao *et al*. (1999) reported suppression of Verticillium wilt in cauliflower by broccoli residue incorporation in comparison to metam sodium, chloropicrin and other control treatments. The soil population of *Verticillium dahliae* microsclerotia was reduced by 50-75% following incorporation of broccoli, compared to pre-treatment levels. Drakopoulos *et al*. (2020) reported 40-50% DON reduction in wheat flour by using *B. juncea* and *S. alba* mulch in field experiments. While these studies have demonstrated the effectiveness of biofumigation in suppressing disease, lack of disease control has also been reported. In a field experiment (Blok *et al*., 2000), where *F. oxysporum*, *Rhizoctonia solani* and *V. dahliae* inoculum were buried in soil previously amended with broccoli at 34-38 t ha^{-1} (fresh weight), no suppression was recorded. Similarly, in another study (Njoroge *et al*., 2008), incorporation of *B. juncea* and *B. napus* in field was not effective in suppressing *F. oxysporum* or *Pythium* spp. populations in soil.

There could be a number of factors that might have impacted the efficacy of biofumigation in the present study. Soil temperature has a significant impact on ITC production (Price *et al*., 2005). Previously, soil amended with cabbage residues was analysed for volatile production (Gamliel and Stapleton, 1993). It was reported that the concentration of volatiles in the headspace were higher in heated, amended soils than in non-heated amended soils. In the present study, when the plants were incorporated in November, mean soil temperatures at ~10 cm deep were 7.7°C which might not have been high enough to favour effective ITC production. Moreover, due to dry weather conditions, ITC production might not have been sufficient. The average rainfall for November 2018 was 36 mm which was half the amount compared to the average rainfall for November (72 mm) for the five-year

period (2013-2017) according to the data from nearest weather station at Shawbury, UK (Met Office, 2021). Presence of sufficient moisture is important to enable myrosinase activity for production of GSL hydrolysis products (Mora and Kirkegaard, 2002). In a field study conducted by Matthiessen *et al*. (2004), addition of 42 mm of water to *B. juncea* plant material resulted in a 7- to 10-fold increase in ITC concentrations in soil compared to where no water was added. Wang and Mazzola (2019) amended soil with *B. juncea* and *S. alba* seed meal in jars and evaluated AITC emission in the headspace. They reported that AITC production elevated (~0.05-0.265 µg g^{-1} soil) with an increase in soil temperature from 10°C to 30°C and increase in moisture level from -1000 kPa to -40 kPa. Another factor that might have affected the activity of ITC could be the organic matter content in the soil. Gimsing *et al*. (2009) demonstrated that organic matter content is the main sorbent of ITC. Organic matter in arable soils is usually at 2-4% (Weil and Magdoff, 2004) compared to which the organic matter content in soil at the field experiment site was higher at 6%. Sorption of ITC to the organic matter in soil might have resulted in reduced activity of ITC.

In a study investigating the ITC-release potential of brassicas under field conditions, *R. sativus*, at a seed rate of 20 kg ha^{-1} produced a biomass of 71-74 t ha^{-1} and an estimated 31-45 mmol m^{-2} GSL (Doheny-Adams *et al*., 2018). Conversely in the present study, at a similar seed rate, *R. sativus* produced similar biomass (74-79 t ha^{-1}) but the expected GSL concentration in the field was much lower (9-10 mmol m^{-2}). The difference in GSL concentrations could be due to difference in soil conditions such as temperature and moisture. The predominant GSL in the tissue of *B. juncea* was sinigrin as reported previously (Doheny-Adams *et al*., 2018; Ngala *et al*., 2015b). Sinigrin concentration estimated for *B. juncea* (above-ground) was 11 mmol m^{-2}. In comparison, Doheny-Adams *et al*. (2018) found a higher concentration of 16-24 mmol m^{-2} under field conditions. Isothiocyanate-release potential is dependent on GSL concentrations, which were sub-optimal in the present study. In contrast, total GSL concentrations as high as 93, 69 and 61 µmol g^{-1} biomass have been detected previously in *B. juncea*, *R. sativus* and *E. sativa* respectively under field conditions (Ngala *et al*., 2015a).

Biofumigants grown during summer conditions are exposed to higher UV intensity, longer daylight hours and higher temperatures. These factors are known to increase the production of GSL in brassica tissues (Bjorkman *et al*., 2011). Ngala *et al*. (2015a) showed how summer grown brassica crops (*B. juncea*, *E. sativa* and *R.*

sativus) produced higher concentrations of GSL in the summer when compared to being overwintered. The high biomass biofumigants, when chopped and incorporated, are then likely to produce ITC at sufficiently effective concentrations. The average maximum temperature for September and October 2018 was recorded as 17.6°C and 14.5°C whereas in the previous five-year period (2013-2017), the average maximum temperature in September and October were 18.2°C and 15.1°C respectively. Moreover, the average minimum temperature in September 2018 was 8.8°C and in October 2018 was 6.1°C which were 0.6 and 1.6 degrees respectively lower than the average for the previous five years (Shawbury, UK weather station data from Met Office, 2021). Overall, these two months, when the brassica crops were growing, were slightly cooler compared to the past years. Although the average maximum temperature for August 2018 (21.3°C) was a degree higher than the average for the 2013-2017 period, the late sowing (13 August 2018) meant that most of the higher temperatures and longer daylight hours were missed. It would have been better if the cover crops were sown in the first week of August which would have probably resulted in higher biomass and higher GSL concentration in the brassica tissues. Moreover, the sun hours in November 2018 (50 hours) were lower than the average sun hours for November (61 hours) for 2013-2017 period (Shawbury, UK weather station data from Met Office, 2021). The lower sun hours in the month, when brassica crops were to be incorporated, might have caused low GSL concentrations.

Estimation of ITC-release potential based on GSL concentration and biomass (Morra and Kirkegaard, 2002) indicates that sinigrin in *B. juncea* shoots in the present study would produce AITC concentrations at 40 nmol g^{-1} soil, assuming a soil bulk density of 1.4 g cm^{-3} and incorporation to 20 cm. However, this concentration is estimated assuming complete conversion of sinigrin in above-ground tissue to AITC, whereas practically 1% ITC release-efficiency has been reported (Morra and Kirkegaard, 2002). Hence true AITC concentrations were likely to be even lower. In previous *in vitro* assays, AITC ED_{50} for *F. graminearum* was found to be 99 mg l^{-1} (Chapter 3), which is equivalent to 998 nmol ml^{-1}. This suggests that the potential AITC in *B. juncea* plots (≤40 nmol g^{-1} soil) were very low compared to effective AITC concentrations. Requirement of higher ITC concentrations has also been reported for other ITC such as, methyl ITC concentrations of 517 to 1294 nmol g^{-1} soil are estimated to be required for soil sterilisation (Brown *et al*., 1991).

Incorporation of *B. oleracea* var. *caulorapa* L. resulted in a mean *F. graminearum* DNA and DON in maize more than 50% lower compared to fallow. Although the effect was not significant, it does suggest a weak biofumigation effect may have occurred. Growth of *B. oleracea* var. *caulorapa* L. was patchy and lower (fresh wt. 20 t ha^{-1}) than the other brassicas. A greater biomass of this brassica might have suppressed *F. graminearum* growth more efficiently and subsequently decreased DON concentrations in maize significantly. Fan *et al*. (2008) demonstrated inhibitory effect of *B. oleracea* var. *caulorapa* L. against mycelial growth of *F. graminearum* under *in vitro* conditions. They reported that 10 g of powdered frozen tissue per Petri dish inhibited the mycelial growth by 70% on day 4 declining to 51% on day 7. In addition to its suppressive effect against fungi (Fan *et al*., 2008), *B. oleracea* var. *caulorapa* L. has been found effective in controlling nematodes too. Mashed leaves of *B. oleracea* var. *caulorapa* L. reduced population density of the nematode, *Meloidogyne incognita* (infecting cowpea plant) by 78-80%, whereas reduction by metam sodium was 43-65% in pot experiments (El-Nagdi and Youssef, 2019). *Brassica oleracea* var. *caulorapa* L. appears to be a potential biofumigant for suppressing *F. graminearum* in field, hence further investigation on the biofumigation effect of this brassica using different cultivars is recommended. *Fusarium graminearum* DNA content in maize was significantly reduced by *R. sativus*. This is in agreement with findings of a study where in a pot experiment, macerated tissue of *R. sativus* at a rate of 10 g kg^{-1} soil significantly reduced *F. graminearum* DNA content in soil (Vandicke *et al*., 2020). In the same study, macerated tissue of *R. sativus* (5 g per Petri dish) also reduced mycelial growth of *F. graminearum* by 17-19%. The reduction in *F. graminearum* DNA content in maize obtained with *R. sativus* may also be attributed to “partial biofumigation”. The thick roots of *R. sativus*, giving a high under-ground biomass, might have released high concentrations of GSL that were probably hydrolysed by myrosinase or myrosinase-like activity of soil microorganisms (Cheng *et al*., 2004; Rakariyatham *et al*., 2005) or myrosinase residues from chopped brassica tissue, thus producing biofumigation effect. Ngala *et al*. (2015a, 2015c) observed partial biofumigation under growing *R. sativus* crop, causing suppression of the potato cyst nematode *Globodera pallida* in glasshouse and under field conditions.

The present study was not effective in suppressing *F. graminearum* inoculum in soil, and disease and DON contamination in maize, however it provides sound basis for further research. Selection of brassica with the most suitable GSL profile and

concentrations, and good biomass are important factors affecting the outcome of biofumigation. Therefore, to achieve a successful biofumigation effect, the approach needs to be optimised considering environmental factors, such as, temperature and moisture content of soil, as well as establishment and growth of the biofumigant crop.

Chapter 6

Chapter 6

6 General Discussion

With the ongoing phase out of pesticides, where some fumigants are already banned and others are likely to be banned in the near future, finding a safer alternative crop protection strategy is the need of the hour. *Fusarium graminearum*, an important mycotoxin producer in wheat and maize, is currently managed through the use of triazole fungicides. There is evidence that these active substances exhibit endocrine-disrupting properties, which has raised concerns over their use (Anonymous, 2019). Moreover, there is a serious concern about their limited effectiveness and the high selection pressure for fungicide resistance (Becher *et al*., 2010; Yerkovich *et al*., 2020). Prior to the present studies, there has been little research on the biofumigation system to manage *F. graminearum*. Success stories have been documented in *in vitro* studies with other fungal pathogens such as *Rhizoctonia solani*, *F. oxysporum* and *Verticillium dahliae* (Handiseni *et al*., 2016; Neubauer *et al*., 2014; Smolinska *et al*., 2003). The existing literature suggested that there is a need for a comprehensive study to understand the potential of the biofumigation system for management of *F. graminearum*.

This project was initiated with isothiocyanate (ITC) screening to identify the brassica species with known ITC profile which could be used as potential biofumigants against *F. graminearum* in further experiments. Findings from these initial experiments revealed the dose response of different ITC on the mycelial growth and conidial germination of *F. graminearum*. Leaf disc and microcosm experiments were conducted to understand the effectiveness of brassica tissue on *F. graminearum* suppression. Glucosinolate (GSL) content in the brassicas was also analysed to see if efficacy could be linked to specific GSL. Defrosted leaves of *Brassica juncea* collected at different growth stages inhibited *F. graminearum* mycelium growth.

Analysis of GSL using HPLC revealed leaf tissues of *B. juncea* to be rich in sinigrin which is known to release allyl ITC (AITC) upon enzymatic hydrolysis. The level of suppression corresponded to the concentrations of sinigrin. Hence, effective inhibition of *F. graminearum* mycelium by *B. juncea* leaf discs could be attributed to high levels of sinigrin. These findings link to those from ITC assays where AITC was found to be effective against *F. graminearum* mycelial growth and conidial germination. Based on these promising results, a field experiment was conducted to investigate brassicas as potential biofumigants to control *F. graminearum* and mycotoxin production in a wheat-maize rotation. However, the brassicas were found to be not effective in suppressing *F. graminearum* inoculum in soil, disease incidence in maize and subsequent mycotoxin contamination in maize. As discussed in Chapter 5, GSL concentrations were sub-optimal. Firstly, sowing brassica crops in the second week of August 2018 was not ideal, as this meant most of the higher temperatures and longer daylight hours were missed. If sown early August, the brassicas would have probably generated higher biomass and higher GSL concentration in their tissues. Secondly, the environmental conditions were not ideal at the time of the field experiment. Average temperatures in September-October 2018 at the experiment site (data from nearest weather station, Met Office), were slightly cooler (0.6-1.6°C) compared to the previous years. Higher temperatures, longer daylight hours and higher UV intensity are factors that are known to increase the production of GSL in brassica tissues (Bjorkman *et al*., 2011). Again, higher temperatures in the months when the brassica crops were growing would probably have been better in terms of biomass and GSL accumulation in brassica tissues. Moreover, the weather conditions were drier compared to previous years. Due to lower rainfall near the time of brassica incorporation (36 mm in November 2018; 72 mm in November 2013-2017), moisture levels might not have been sufficient for myrosinase activity to produce ITC at effective concentrations.

Brassica juncea ‘Caliente Rojo’ was not used in the field experiment as it was not available at that time. Results from the leaf disc and microcosm experiments suggest that this brassica could be a promising biofumigant against *F. graminearum*. Glucosinolate analysis of this cultivar showed that concentrations of the sinigrin, AITC-precursor, were highest at stem-extension stage compared to flowering stage, and this corresponded to the inhibition levels seen in the leaf disc experiment. Under field conditions, a balance between biomass and GSL concentration could be achieved by identifying an appropriate growth stage between stem-extension and

flowering so that sufficient biomass is attained whilst GSL concentrations remain high during incorporation. This brassica could be sown following harvest of wheat in summer, and incorporated in autumn followed by subsequent cereal production. This could give an advantage of comparatively shorter time period of growing brassica in the field during summer and incorporation could take place while the soil temperature would be comparatively higher favouring ITC production.

In the field experiment, *F. graminearum* DNA and DON in maize grown after *B. oleracea* var. *caulorapa* L. treatment were consistently lower by 50-60% compared to maize grown after the cultivated fallow treatment, but the difference was not significant. Surprisingly, this effect was achieved despite a very patchy growth and hence low biomass compared to the other brassicas. Although the suppressive effect was not significant, this brassica could be a potential biofumigant to reduce mycotoxin production by *F. graminearum*. The thick stems of this brassica are not easy to shred, hence in addition to high biomass, a better shredding technique could result in sufficient production of ITC and other hydrolysis products from *B. oleracea* var. *caulorapa* L. which may prove effective in managing *F. graminearum*. From the GSL analysis of the brassica species used in the field experiments (Chapter 5, Table 5.2), the only GSL that stood out in *B. oleracea* var. *caulorapa* L. 'Kolibri' compared to the other species is neoglucobrassicin. Concentration of neoglucobrassicin in the root tissue of *B. oleracea* var. *caulorapa* L. 'Kolibri' was 4.35 µmol g^{-1} (freeze-dried tissue) compared to the other species where the concentration was <1 µmol g^{-1}. Break down products of neoglucobrassicin in the root tissue of *B. oleracea* var. *caulorapa* L., that were probably produced by myrosinase activity of soil microorganisms (partial biofumigation), or myrosinase residues from chopped brassica tissue might have resulted in the lower DON and *F. graminearum* DNA content in maize. *Brassica oleracea* var. *caulorapa* L. appears to be a promising biofumigant (El-Nagdi and Youssef, 2019; Fan *et al*., 2008) however, it is not a well investigated biofumigant and the literature on its suppressive effect on pests and pathogens is scarce. The fact that the total concentration of GSL of *B. oleracea* var. *caulorapa* L. in field per area was the lowest (2.4 mmol m^{-2}) of all the species, yet seeing *F. graminearum* DNA and DON content in maize grown after this brassica, lower by ~50%, suggests it may have suppression potential against *F. graminearum*. It would be worth further investigating this effect of this brassica on pests and pathogens. Additionally, breeding of GSL-rich cultivars of *B. oleracea* var. *caulorapa* L. might be another avenue to explore. Additionally, as seen in Chapter 5,

incorporation of *Raphanus sativus* 'Terranova' significantly decreased the amount of *F. graminearum* DNA in maize by 58% compared with the fallow cultivated treatment. *Raphanus sativus* also significantly reduced *F. graminearum* incidence by 55% in inoculum recovered in the microcosm experiment. These findings suggest that *R. sativus* holds good biofumigation potential against *F. graminearum*. However as discussed above, the conditions in the field were probably not favourable enough for this brassica to produce greater biofumigation effect on *F. graminearum*. It would be worth exploring and maximising the biofumigation potential of this brassica against *F. graminearum*.

As discussed in Chapter 5, the expected AITC concentrations in the field experiment (~40 nmol l^{-1}) were only a fraction compared to the ED_{50} of AITC determined in the ITC assays (Chapter 3). Such high ITC concentrations might not be very easy to achieve under field conditions, however, biofumigation in integrated pest management (IPM) may be a good approach to manage *F. graminearum* in wheat and maize. According to one report, in response to European Commission's emphasis on implementation of IPM, the Member States drew attention to the lack of sufficient non-chemical alternatives and low risk plant protection products to be an obstacle in the development of IPM (EC, 2017). The approach in the present study if further investigated and optimised could contribute in overcoming this obstacle for the management of *F. graminearum*. It is known that in addition to ITC, other compounds such as thiocyanates, nitriles, dimethyl disulphide and methyl sulphide are also produced as a result of decomposition of brassica tissues. These products are known to have biocidal properties against pests and pathogens (Curto *et al*., 2014; Tsao *et al*., 2002; Wang *et al*., 2009).Therefore the production of ITC alone cannot explain the biofumigation phenomenon, future studies are needed to bridge the gap between *in vitro* work and field work.

The results of this study generally support the use of biofumigation method in cereal dominated crop rotations, however, more field based work is required to demonstrate efficacy. Until now, research on biofumigation, as an alternative for chemical fumigation to control soil-borne diseases, focused on horticultural crops, where soil fumigation was economically feasible. By including biofumigation in cereal dominated crop rotations, the important threat of *F. graminearum* i.e., the generation of mycotoxins, could be reduced.

Recommendations for further studies

- Investigation of synergistic effect of different ITC on *F. graminearum in vitro*
- Investigation of biofumigation effect of *R. sativus* on mycotoxin production by *F. graminearum* in *in vitro* and glasshouse studies
- Investigation of biofumigation effect of *B. oleracea* var. *caulorapa* L. on *F. graminearum* inoculum and mycotoxin production in *in vitro* and glasshouse studies
- Biofumigation effect of neoglucobrassicin hydrolysis products on *F. graminearum in vitro*
- Effect of *B. juncea* 'Caliente Rojo' on *F. graminearum* under field conditions
- Optimisation of biomass and GSL concentrations in brassica cover crops

References

7 References

Abid, M. (2012) *Ecological role of mycotoxins produced by Fusarium graminearum: consequences of the presence of deoxynivalenol (DON) in crop residues on the soil microflora and soil fauna*. PhD thesis, Dijon, Université de Bourgogne.

Abildgren, M. P., Lund, F., Thrane, U., & Elmholt, S. (1987). Czapek-Dox agar containing iprodione and dicloran as a selective medium for the isolation of *Fusarium* species. *Letters in Applied Microbiology*, 5(4), 83-86.

AHDB, (2019a). Stem (black) rust life cycle and risk to UK wheat. Available at: https://ahdb.org.uk/knowledge-library/stem-black-rust-life-cycle-and-risk-to-uk-wheat [Accessed 1 June 2019]

AHDB, (2019b). Integrated pest management (IPM) of cereal diseases. Available at: https://ahdb.org.uk/cereal-dmg [Accessed 1 June 2019]

AHDB, (2021a). Wheat growth guide, pp 1-42. Available at: https://ahdb.org.uk/knowledge-library/wheat-growth-guide

AHDB, (2021b). Recommended Lists variety comments for cereals and oilseed rape. Available at: https://ahdb.org.uk/rlcomments [Accessed 18 November 2021].

AHDB, Cereals and oilseeds, 2016. Wheat disease management guide. Available at: www.cereals.ahdb.org.uk/publications [Accessed 1 June 2019]

Aires, A., Rosa, E., & Carvalho, R. (2006). Effect of nitrogen and sulfur fertilization on glucosinolates in the leaves and roots of broccoli sprouts (*Brassica oleracea* var. italica). *Journal of the Science of Food and Agriculture*, 86(10), 1512-1516.

Alborch, L., Bragulat, M. R., & Cabañes, F. J. (2010). Comparison of two selective culture media for the detection of *Fusarium* infection in conventional and transgenic maize kernels. *Letters in Applied Microbiology*, 50(3), 270-275.

Al-Turki, A. I., & Dick, W. A. (2003). Myrosinase activity in soil. *Soil Science Society of America Journal*, 67(1), 139-145.

Amiri-Jami, A., Sadeghi-Namaghi, H., Gilbert, F., Moravvej, G., & Asoodeh, A. (2016). On the role of sinigrin (mustard oil) in a tritrophic context: plant–aphid–aphidophagous hoverfly. *Ecological Entomology*, 41(2), 138-146.

Andréasson, E., Jørgensen, L. B., Höglund, A., Rask, L., & Meijer, J. (2001). Different myrosinase and idioblast distribution in *Arabidopsis* and *Brassica napus. Plant Physiology*, 127(4), 1750-1763.

Andrews, S., & Pitt, J. I. (1986). Selective medium for isolation of *Fusarium* species and dematiaceous hyphomycetes from cereals. *Applied and Environmental Microbiology*, 51(6), 1235-1238.

Anonymous (2019) While France bans a common endocrine disrupting pesticide, EPA goes silent: Pesticides and You. 39(2): 9-14.

Anonymous, (2014). Growing and feeding forage maize – a review. Research Partnership: Grasslands, Forage and Soil. Work Package 3b: Alternative forages. Report prepared by University of Readingfor AHDB Dairy. February, 2014, pp 1-44.

Anonymous, (2018). Agriculture in the United Kingdom. Produced by: Department for Environment, Food and Rural Affairs; Department of Agriculture, Environment and Rural Affairs (Northern Ireland); Welsh Assembly Government, The Department for Rural Affairs and Heritage; The Scottish Government, Rural and Environment Science and Analytical Services. pp50-62

Audenaert, K., Callewaert, E., Höfte, M., De Saeger, S., & Haesaert, G. (2010). Hydrogen peroxide induced by the fungicide prothioconazole triggers deoxynivalenol (DON) production by *Fusarium graminearum*. *BMC Microbiology*, 10(1), 1-14.

Awad, W. A., Ghareeb, K., Dadak, A., Hess, M., & Böhm, J. (2014). Single and combined effects of deoxynivalenol mycotoxin and a microbial feed additive on lymphocyte DNA damage and oxidative stress in broiler chickens. *PloS one*, 9(1), e88028.

Baffoni, L., Gaggia, F., Dalanaj, N., Prodi, A., Nipoti, P., Pisi, A., et al. (2015). Microbial inoculants for the biocontrol of Fusarium spp. in durum wheat. *BMC Microbiology*, 15(1), 1-10.

Bai, G., Desjardins, A. E., & Plattner, R. D. (2002). Deoxynivalenol-nonproducing *Fusarium graminearum* causes initial infection, but does not cause disease spread in wheat spikes. *Mycopathologia*, 153(2), 91-98.

Bai, G., Su, Z., & Cai, J. (2018). Wheat resistance to Fusarium head blight. *Canadian Journal of Plant Pathology*, 40(3), 336-346.

Baldi, E., Toselli, M., Malaguti, L., & Lazzeri, L. (2015). Evaluation of the biocidal effects of Brassica seed meal on *Armillaria mellea. Annals of Applied Biology*, 167(3), 364-372.

Barba, F. J., Nikmaram, N., Roohinejad, S., Khelfa, A., Zhu, Z., & Koubaa, M. (2016). Bioavailability of glucosinolates and their breakdown products: Impact of processing. *Frontiers in Nutrition*, 3, 24.

Becher, R., Hettwer, U., Karlovsky, P., Deising, H. B., & Wirsel, S. G. (2010). Adaptation of *Fusarium graminearum* to tebuconazole yielded descendants diverging for levels of fitness, fungicide resistance, virulence, and mycotoxin production. *Phytopathology*, 100(5), 444-453.

Beddow, J. M., Pardey, P. G., Chai, Y., Hurley, T. M., Kriticos, D. J., Braun, H., et al. (2015). Research investment implications of shifts in the global geography of wheat stripe rust. *Nature Plants*, 1(10), 1-5.

Belhassen, H., Jiménez-Díaz, I., Arrebola, J., Ghali, R., Ghorbel, H., Olea, N., & Hedili, A. (2015). Zearalenone and its metabolites in urine and breast cancer risk: A case-control study in Tunisia. *Chemosphere,* 128, 1-6.

Bellostas, N., Kachlicki, P., Sørensen, J. C., & Sørensen, H. (2007). Glucosinolate profiling of seeds and sprouts of *B. oleracea* varieties used for food. *Scientia Horticulturae*, 114(4), 234-242.

Bellostas, N., Sørensen, J. C., & Sørensen, H. (2004). Qualitative and quantitative evaluation of glucosinolates in cruciferous plants during their life cycles. *Agroindustria*, 3(3), 5-10.

Bending, G. D., & Lincoln, S. D. (1999). Characterisation of volatile sulphur-containing compounds produced during decomposition of *Brassica juncea* tissues in soil. *Soil Biology and Biochemistry*, 31(5), 695-703.

Bennett, J., & Klich, M. (2003). Mycotoxins. *Clinical Microbiology Reviews*, 16(3), 497-516.

Bensassi, F., Zaied, C., Abid, S., Hajlaoui, M.R., & Bacha, H. (2010) Occurrence of deoxynivalenol in durum wheat in Tunisia. *Food Control*, 21(3), 281-285.

Bhandari, S. R., Jo, J. S., & Lee, J. G. (2015). Comparison of glucosinolate profiles in different tissues of nine Brassica crops. *Molecules*, 20(9), 15827-15841.

Bhat, R., Ramakrishna, Y., Beedu, S., & Munshi, K. (1989). Outbreak of trichothecene mycotoxicosis associated with consumption of mould-damaged wheat products in Kashmir valley, India. *The Lancet,* 333(8628), 35-37.

Björkman, M., Klingen, I., Birch, A. N., Bones, A. M., Bruce, T. J., Johansen, T. J., et al. (2011). Phytochemicals of Brassicaceae in plant protection and human health–Influences of climate, environment and agronomic practice. *Phytochemistry*, 72(7), 538-556.

Blandino, M., Pilati, A., Reyneri, A., & Scudellari, D. (2010). Effect of maize crop residue density on Fusarium head blight and on deoxynivalenol contamination of common wheat grains. *Cereal Research Communications*, 38(4), 550-559.

Blok, W. J., Lamers, J. G., Termorshuizen, A. J., & Bollen, G. J. (2000). Control of soilborne plant pathogens by incorporating fresh organic amendments followed by tarping. *Phytopathology*, 90(3), 253-259.

Bo, P., Lien, J., Chen, Y., Yu, F., Lu, H., Yu, C., et al. (2016). Allyl isothiocyanate induces cell toxicity by multiple pathways in human breast cancer cells. *The American Journal of Chinese Medicine*, 44(02), 415-437.

Boenisch, M. J., & Schäfer, W. (2011). *Fusarium graminearum* forms mycotoxin producing infection structures on wheat. *BMC Plant Biology*, 11(1), 1-14.

Bones, A. M., & Rossiter, J. T. (1996). The myrosinase-glucosinolate system, its organisation and biochemistry. *Physiologia Plantarum*, 97(1), 194-208.

Borek, V., Morra, M. J., & McCaffrey, J. P. (1996). Myrosinase activity in soil extracts. *Soil Science Society of America Journal*, 60(6), 1792-1797.

Bottalico, A., Visconti, A., Logrieco, A., Solfrizzo, M., & Mirocha, C. J. (1985). Occurrence of zearalenols (diastereomeric mixture) in corn stalk rot and their production by associated *Fusarium* species. *Applied and Environmental Microbiology,* 49(3), 547-551.

Bragulat, M. R., Martinez, E., Castella, G., & Cabanes, F. J. (2004). Selective efficacy of culture media recommended for isolation and enumeration of *Fusarium* spp. *Journal of Food Protection*, 67(1), 207-211.

Brar, G. S., Brûlé-Babel, A. L., Ruan, Y., Henriquez, M. A., Pozniak, C. J., Kutcher, H. R., et al. (2019). Genetic factors affecting Fusarium head blight resistance improvement from introgression of exotic Sumai 3 alleles (including Fhb1, Fhb2, and Fhb5) in hard red spring wheat. *BMC Plant Biology*, 19(1), 1-19.

Brown, N. A., Evans, J., Mead, A., & Hammond-Kosack, K. E. (2017). A spatial temporal analysis of the *Fusarium graminearum* transcriptome during symptomless and symptomatic wheat infection. *Molecular Plant Pathology*, 18(9), 1295-1312.

Brown, P. D., & Morra, M. J. (1997). Control of soil-borne plant pests using glucosinolate-containing plants. *Advances in Agronomy,* 61,167-231.

Brown, P. D., Morra, M. J., McCaffrey, J. P., Auld, D. L., & Williams, L. (1991). Allelochemicals produced during glucosinolate degradation in soil. *Journal of Chemical Ecology*, 17(10), 2021-2034.

Brown, P. D., Tokuhisa, J. G., Reichelt, M., & Gershenzon, J. (2003). Variation of glucosinolate accumulation among different organs and developmental stages of Arabidopsis thaliana. *Phytochemistry*, 62(3), 471-481.

Buxdorf, K., Yaffe, H., Barda, O., & Levy, M. (2013). The effects of glucosinolates and their breakdown products on necrotrophic fungi. *PloS one*, 8(8), e70771.

Calmes, B., N'Guyen, G., Dumur, J., Brisach, C. A., Campion, C., Iacomi, B. et al. (2015). Glucosinolate-derived isothiocyanates impact mitochondrial function in fungal cells and elicit an oxidative stress response necessary for growth recovery. *Frontiers in Plant Science*, 6, 414.

Canessa, E. F., & Morrell, J. J. (1995). Effect of mixtures of carbon disulfide and methylisothiocyanate on survival of wood-colonizing fungi. *Wood and Fiber Science*, 27(3), 207-224.

CAST (2003) *Mycotoxins: Risks in plant, animal, and human systems*. Task force report no. 139, Council for Agricultural Science and Technology Ames, Iowa, USA.

Castellá, G., Bragulat, M. R., Rubiales, M. V., & Cabañes, F. J. (1997). Malachite green agar, a new selective medium for *Fusarium* spp. *Mycopathologia*, 137(3), 173-178.

Cataldi, T. R., Rubino, A., Lelario, F., & Bufo, S. A. (2007). Naturally occurring glucosinolates in plant extracts of rocket salad (Eruca sativa L.) identified by liquid chromatography coupled with negative ion electrospray ionization and quadrupole

ion-trap mass spectrometry. *Rapid Communications in Mass Spectrometry*, 21(14), 2374-2388.

CCFAC, 2000. Codex Committee on Food Additives and Contaminants. Posting date. Joint FAO/WHO Expert Committee on Food Additives: Position paper on zearalenone. Publication CCFAC 00/19. Codex Alimentarius Commission, Rome, Italy.

Champeil, A., Fourbet, J., Doré, T., & Rossignol, L. (2004). Influence of cropping system on Fusarium head blight and mycotoxin levels in winter wheat. *Crop protection*, 23(6), 531-537.

Chan, M., & Close, R. C. (1987). Aphanomyces root rot of peas 3. Control by the use of cruciferous amendments. New Zealand Journal of Agricultural Research, 30(2), 225-233.

Charron, C. S., & Sams, C. E. (1999). Inhibition of *Pythium ultimum* and *Rhizoctonia solani* by shredded leaves of *Brassica* species. *Journal of the American Society for Horticultural Science*, 124(5), 462-467.

Chen, J., Griffey, C. A., Saghai Maroof, M. A., et al., (2006). Validation of two major quantitative trait loci for Fusarium head blight resistance in Chinese wheat line W14. *Plant Breeding* 125, 99–101.

Chen, L., Heng, J., Qin, S., & Bian, K. (2018). A comprehensive understanding of the biocontrol potential of *Bacillus velezensis* LM2303 against Fusarium head blight. *PLoS One*, 13(6), e0198560.

Chen, L., Tian, X., & Yang, B. (1990). A study on the inhibition of rat myocardium glutathione peroxidase and glutathione reductase by moniliformin. *Mycopathologia,* 110(2), 119-124.

Cheng, D., Hashimoto, K., & Uda, Y. (2004). *In vitro* digestion of sinigrin and glucotropaeolin by single strains of *Bifidobacterium* and identification of the digestive products. *Food and Chemical Toxicology*, 42(3), 351-357.

Chin, H. W., & Lindsay, R.C. (1993). Volatile sulfur compounds formed in disrupted tissues of different cabbage cultivars. *Journal of Food Science* 58, 835-841.

Clarke, D. B. (2010). Glucosinolates, structures and analysis in food. *Analytical Methods*, 2(4), 310-325.

Cohen, M. F., Yamasaki, H., & Mazzola, M. (2005). *Brassica napus* seed meal soil amendment modifies microbial community structure, nitric oxide production and incidence of Rhizoctonia root rot. *Soil Biology and Biochemistry*, 37(7), 1215-1227.

Commission Delegated Regulation (EU) 2017/2100 of 4 September 2017 setting out scientific criteria for the determination of endocrine-disrupting properties pursuant to Regulation (EU) No 528/2012 of the European Parliament and Council. *Official Journal of the European Union* L 301/1-5.

Commission Implementing Regulation (EU) 2017/2324 of 12 December 2017 renewing the approval of the active substance glyphosate in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market, and amending the Annex to Commission Implementing Regulation (EU) No 540/2011. *Official Journal of the European Union* L 333/10-16.

Commission Implementing Regulation (EU) 2020/2087 of 14 December 2020 concerning the non-renewal of the approval of the active substance mancozeb, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market, and amending the Annex to Commission Implementing Regulation (EU) No 540/2011. *Official Journal of the European Union* L 423/50-52.

Commission Regulation (EU) 2018/605 of 19 April 2018 amending Annex II to Regulation (EC) No 1107/2009 by setting out scientific criteria for the determination of endocrine disrupting properties. *Official Journal of the European Union* L 101/33-36.

Cook, R. J. (2003). Take-all of wheat. *Physiological and Molecular Plant Pathology*, 62(2), 73-86.

Culp, S. J., Mellick, P. W., Trotter, R. W., Greenlees, K. J., Kodell, R. L., & Beland, F. A. (2006). Carcinogenicity of malachite green chloride and leucomalachite green in B6C3F1 mice and F344 rats. *Food and Chemical Toxicology*, 44(8), 1204-1212.

Curtis, B.C. (2002) Wheat in the World. In: Curtis, B.C., Rajaram, S. and Macpherson, H.G. (Eds.) *Bread Wheat Improvement and Production*, Plant Production and Protection Series 30, FAO, Rome, 1-18.

Curto, G., Dongiovanni, C., Sasanelli, N., Santori, A., & Myrta, A. (2014). Efficacy of dimethyl disulfide (DMDS) in the control of the root-knot nematode *Meloidogyne*

incognita and the cyst nematode *Heterodera carotae* on carrot in field condition in Italy, 405-410.

D'Mello, J., Placinta, C., & Macdonald, A. (1999). Fusarium mycotoxins: A review of global implications for animal health, welfare and productivity. *Animal Feed Science and Technology,* 80(3), 183-205.

Dahlin, P., & Hallmann, J. (2020). New insights on the role of allyl isothiocyanate in controlling the root knot nematode Meloidogyne hapla. *Plants*, 9(5), 603.

Dai, Y., Duan, Y., Liu, H. et al., (2017). Molecular cytogenetic characterization of two Triticum–Secale–Thinopyrum trigeneric hybrids exhibiting superior resistance to Fusarium head blight, leaf rust, and stem rust race Ug99. *Frontiers in Plant Science*, 8, 797.

Dandurand, L., Morra, M. J., Zasada, I. A., Phillips, W. S., Popova, I., & Harder, C. (2017). Control of Globodera spp. using Brassica juncea seed meal and seed meal extract. *Journal of Nematology*, 49(4), 437.

D'Angelo, D. L., Bradley, C. A., Ames, K. A., Willyerd, K. T., Madden, L. V., & Paul, P. A. (2014). Efficacy of fungicide applications during and after anthesis against Fusarium head blight and deoxynivalenol in soft red winter wheat. *Plant Disease*, 98(10), 1387-1397.

David Miller, J., Culley, J., Fraser, K., Hubbard, S., Meloche, F., Ouellet, T., *et al*. (1998). Effect of tillage practice on Fusarium head blight of wheat. *Canadian Journal of Plant Pathology*, 20(1), 95-103.

De los Santos, B., Chamorro, M., Medina-Mínguez, J. J., Capote, N., Aguado, A., & Romero, F. (2016) Emerging diseases in strawberry crop: Charcoal rot and Fusarium wilt. In: Husaini, A. M., & Neri, D. (Eds.) Strawberry: Growth, development and diseases. CAB International, pp. 212-250.

De Nicola, G. R., D'Avino, L., Curto, G., Malaguti, L., Ugolini, L., Cinti, S., *et al*. (2013). A new biobased liquid formulation with biofumigant and fertilising properties for drip irrigation distribution. *Industrial Crops and Products*, 42, 113-118.

DEFRA, Department for Environment, Food & Rural Affairs (2018). National statistics Farming Statistics – 2018, UK wheat and barley production first estimate. Available at: https://www.gov.uk/government/statistics/farming-statistics-2018-uk-wheat-and-barley-production-first-estimate [Accessed 26 November 2018]

Delaquis, P. J., & Sholberg, P. L. (1997). Antimicrobial activity of gaseous allyl isothiocyanate. *Journal of Food Protection*, 60(8), 943-947.

Desjardins, A. E., Proctor, R. H., Bai, G., McCormick, S. P., Shaner, G., Buechley, G., *et al*. (1996). Reduced virulence of trichothecene-nonproducing mutants of *Gibberella zeae* in wheat field tests. *Molecular Plant-Microbe Interactions,*9(9), 775-781.

Diab, H. G., Hu, S., & Benson, D. M. (2003). Suppression of Rhizoctonia solani on impatiens by enhanced microbial activity in composted swine waste-amended potting mixes. *Phytopathology*, 93(9), 1115-1123.

Dill-Macky, R., & Jones, R. K. (2000). The effect of previous crop residues and tillage on Fusarium head blight of wheat. *Plant Disease*, 84(1), 71-76.

D'Mello, J.P.F., & Macdonald, A.M.C. (1997). Mycotoxins. *Animal Feed Science Technol*ogy, 69, 155-166.

Doheny-Adams, T., Lilley, C. J., Barker, A., Ellis, S., Wade, R., Atkinson, H. J., *et al*. (2018). Constant isothiocyanate-release potentials across biofumigant seeding rates. *Journal of Agricultural and Food Chemistry*, 66(20), 5108-5116.

Donkin, S. G., Eiteman, M. A., & Williams, P. L. (1995). Toxicity of glucosinolates and their enzymatic decomposition products to Caenorhabditis elegans. *Journal of Nematology*, 27(3), 258.

Drakopoulos, D., Kägi, A., Gimeno, A., Six, J., Jenny, E., Forrer, H., *et al*. (2020). Prevention of Fusarium head blight infection and mycotoxins in wheat with cut-and-carry biofumigation and botanicals. *Field Crops Research*, 246, 107681.

Dubin, H. J., & Duveiller, E. (2011) Fungal, bacterial and nematode diseases of wheat: breeding for resistance and other control measures. In: Bonjean, A. P., Angus, W. J. & Van Ginkel M. (Eds.) *The World Wheat Book: A History of Wheat Breeding*, Paris: Lavoisier Vol. 2, pp. 1131-1181.

EC, European Commission. (2017). Report from the Commission to the European Parliament and the Council on Member State national action plans and on progress in the implementation of Directive 2009/128/EC on the sustainable use of pesticides. European Commission Brussels, 10.10.2017 COM(2017) 587 final pp. 1-19. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=COM:2017:0587:FIN [Accessed 18 April 2018].

EC, European Commission (2021). Neonicotinoids. Available at: https://ec.europa.eu/food/plants/pesticides/approval-active-substances/renewal-approval/neonicotinoids_en [Accessed 19 March 2021].

EC, European Commission (2006) Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of European Union*, L364, 5-24.

EC, European Commission (2019a). Cereals, oilseeds, protein crops and rice. Available at: https://ec.europa.eu/info/food-farming-fisheries/plants-and-plant-products/plant-products/cereals_en [Accessed 23 July 2019]

EC, European Commission (2019b). RustWatch: A European early-warning system for wheat rust diseases, Factsheet. Available at: https://cordis.europa.eu/project/id/773311 [Accessed 23 July 2019]

Edwards, S. G., & Jennings, P. (2018). Impact of agronomic factors on Fusarium mycotoxins in harvested wheat. *Food Additives & Contaminants: Part A*, 35(12), 2443-2454.

Edwards, S. G., & Seddon, B. (2001). Selective media for the specific isolation and enumeration of *Botrytis cinerea* conidia. *Letters in Applied Microbiology*, 32(2), 63-66.

Edwards, S. G., Pirgozliev, S. R., Hare, M. C., & Jenkinson, P. (2001). Quantification of trichothecene-producing *Fusarium* species in harvested grain by competitive PCR to determine efficacies of fungicides against Fusarium head blight of winter wheat. *Applied and Environmental Microbiology*, 67(4), 1575-1580.

Edwards, S. G., Pirgozliev, S. R., Hare, M. C., & Jenkinson, P. (2001). Quantification of trichothecene-producing *Fusarium* species in harvested grain by competitive PCR to determine efficacies of fungicides against Fusarium head blight of winter wheat. *Applied and Environmental Microbiology*, 67(4), 1575-1580.

EFSA, (2011). Scientific opinion on the risks for animal and public health related to the presence of T-2 and HT-2 toxin in food and feed. EFSA J 9(12):2481.

Einhellig, F. A. (1995) Allelopathy-current status and future goals. In: Inderjit, A., Dakshini, K. M. M., & Einhellig F. A. (Eds.) *Allelopathy: Organisms, Processes, and Applications*. Washington, DC: American Chemical Society Press, pp. 1–24.

El Nagdi, W. M. & Youssef, M. M. (2019). Brassica vegetable leaf residues as promising biofumigants for the control of root knot nematode, *Meloidogyne incognita* infecting cowpea. *Agricultural Engineering International: CIGR Journal*, 21(1), 134-139.

El-Sharouny, E. E. (2015). Effect of different soil amendments on the microbial count correlated with resistance of apple plants towards pathogenic Rhizoctonia solani AG-5. *Biotechnology & Biotechnological Equipment*, 29(3), 463-469.

Enebak S. (2007) Methyl bromide and the Montreal protocol: An update on the critical use exemption and quarantine pre-shipment process. In: Riley, L. E., Dumroese, R. K. & Landis, T. D. Tech. coords*. National proceedings: Forest and Conservation Nursery Associations-2006*, December 2007, Proc. RMRS-P-50. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain.

Erickson, B. E. (2016). EU bans endocrine disrupting herbicides. Available at: https://cen.acs.org/articles/94/i17/EU-bans-endocrine-disrupting-herbicides.html [Accessed 12 April 2017].

Escrivá, L., Font, G., & Manyes, L. 2015. In vivo toxicity studies of Fusarium mycotoxins in the last decade: A review. *Food and Chemical Toxicology,* 78, 185-206.

Eskola, M., Kos, G., Elliott, C. T., Hajšlová, J., Mayar, S., & Krska, R. (2020). Worldwide contamination of food-crops with mycotoxins: Validity of the widely cited 'FAO estimate'of 25%. Critical reviews in food science and nutrition, 60(16), 2773-2789.

Eurostat, Wheat and spelt by area, production and humidity. Available at: https://ec.europa.eu/eurostat/web/products-datasets/-/tag00047 [Accessed: 27 June 2019]

Fahey, J. W., Zalcmann, A. T., & Talalay, P. (2001). The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry,* 56 (1), 5-51.

Fan, C. M., Xiong, G. R., Qi, P., Ji, G. H., & He, Y. Q. (2008). Potential biofumigation effects of *Brassica oleracea* var. *caulorapa* on growth of fungi. *Journal of Phytopathology*, 156(6), 321-325.

FAOSTAT, Food and Agriculture Organization of the United Nations. Available at: http://www.fao.org/ faostat/en/.

Figueroa, M., Hammond-Kosack, K. E., & Solomon, P. S. (2018). A review of wheat diseases—a field perspective. *Molecular Plant Pathology*, 19(6), 1523-1536.

Flannigan, B. (1991) Mycotoxins. In: D'Mello, J.P.F., Duffus, C.M., & Duffus, J.H. (Eds.), Toxic substances in crop plants. The Royal Society of Chemistry, Cambridge, pp. 226-257.

Fones, H., & Gurr, S. (2015). The impact of Septoria tritici Blotch disease on wheat: An EU perspective. *Fungal Genetics and Biology*, 79, 3-7.

Frąc, M., Gryta, A., Oszust, K., & Kotowicz, N. (2016). Fast and accurate microplate method (Biolog MT2) for detection of *Fusarium* fungicides resistance/sensitivity. *Frontiers in Microbiology*, 7, 489.

Frizzell, C., Ndossi, D., Verhaegen, S., Dahl, E., Eriksen, G., Sørlie, M., Ropstad, E., Muller, M., Elliott, C., & Connolly, L. (2011). Endocrine disrupting effects of zearalenone, alpha-and beta-zearalenol at the level of nuclear receptor binding and steroidogenesis. *Toxicology Letters,* 206(2), 210-217.

Furlan, L., Bonetto, C., Finotto, A., Lazzeri, L., Malaguti, L., Patalano, G., *et al*. (2010). The efficacy of biofumigant meals and plants to control wireworm populations. *Industrial Crops and Products*, 31(2), 245-254.

Galletti, S., Sala, E., Leoni, O., Burzi, P. L., & Cerato, C. (2008). *Trichoderma* spp. tolerance to *Brassica carinata* seed meal for a combined use in biofumigation. *Biological Control*, 45(3), 319-327.

Gamliel, A., & Stapleton, J. J. (1997). Improvement of soil solarization with volatile compounds generated from organic amendments. *Phytoparasitica*, 25(1), S31-S38.

Gao, F., Jiang, L., Chen, M., Geng, C., Yang, G., Ji, F., *et al*. (2013). Genotoxic effects induced by zearalenone in a human embryonic kidney cell line. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 755(1), 6-10.

García-Álvarez, A., Bello, A., Sanz, R., Piedra Buena, A., Monserrat, A. & Díez-Rojo, M.A. (2004). Biofumigation as an alternative to methyl bromide for the production of tomatoes and other vegetables. In: T. Batchelor and F. Alfarroba. *eds. Alternatives to methyl bromide: proceedings of International conference 27-30 September 2004, Lisbon, Portugal*. Brussels: European Commission. pp. 171-175.

Gardiner, D. M., Osborne, S., Kazan, K., & Manners, J. M. (2009). Low pH regulates the production of deoxynivalenol by *Fusarium graminearum*. *Microbiology,* 155 (9), 3149-3156.

Gathercole, P. S., Thiel, P. G., & Hofmeyr, J. H. (1986). Inhibition of pyruvate dehydrogenase complex by moniliformin. *The Biochemical Journal,* 233(3), 719-723.

Georgiou, C. D., Patsoukis, N., Papapostolou, I., & Zervoudakis, G. (2006). Sclerotial metamorphosis in filamentous fungi is induced by oxidative stress. *Integrative and Comparative Biology*, 46(6), 691-712.

Gervais, L., Dedryver, F., Morlais, J., Bodusseau, V., Negre, S., Bilous, M., *et al*. (2003). Mapping of quantitative trait loci for field resistance to Fusarium head blight in an European winter wheat. *Theoretical and Applied Genetics*, 106(6), 961-970.

Ghoname, A. A., Riad, G. S., El-Bassiony, A. M. M., & Tantawy, A. S. (2017). Biofumigation with fresh manure or Brassicaceae residuals could be a promising methyl bromide alternative in head lettuce production. *Gesunde Pflanzen,* 69 (1), pp. 29-37.

Giamoustaris, A., & Mithen, R. (1995). The effect of modifying the glucosinolate content of leaves of oilseed rape (*Brassica napus* ssp. oleifera) on its interaction with specialist and generalist pests. *Annals of Applied Biology,* 126(2), 347-363.

Gilchrist, L., & Dubin, H. J. (2002) Fusarium head blight, in Bread Wheat Improvement and Production, Plant Production and Protection. Series 30, FAO, Rome.

Gilsinger, J., Kong, L., Shen, X., & Ohm, H. (2005). DNA markers associated with low Fusarium head blight incidence and narrow flower opening in wheat. *Theoretical and Applied Genetics*, 110(7), 1218-1225.

Gimsing, A. L., & Kirkegaard, J. A. (2006). Glucosinolate and isothiocyanate concentration in soil following incorporation of brassica biofumigants. *Soil Biology and Biochemistry*, 38(8), 2255-2264.

Gimsing, A. L., & Kirkegaard, J. A. (2009). Glucosinolates and biofumigation: Fate of glucosinolates and their hydrolysis products in soil. *Phytochemistry Reviews,* 8(1), 299-310.

Gimsing, A. L., Poulsen, J. L., Pedersen, H. L., & Hansen, H. C. B. (2007). Formation and degradation kinetics of the biofumigant benzyl isothiocyanate in soil. *Environmental Science & Technology*, 41(12), 4271-4276.

Gimsing, A. L., Sørensen, J. C., Tovgaard, L., Jørgensen, A. M. F., & Hansen, H. C. B. (2006). Degradation kinetics of glucosinolates in soil. *Environmental Toxicology and Chemistry,* 25(8), 2038-2044.

Gimsing, A. L., Strobel, B. W., & Hansen, H. C. (2009). Degradation and sorption of 2-propenyl and benzyl isothiocyanate in soil. *Environmental Toxicology and Chemistry: An International Journal*, 28(6), 1178-1184.

Halkier, B. A., & Gershenzon, J. (2006). Biology and biochemistry of glucosinolates. *Annual Review of Plant Biology,* 57, 303-333.

Hall, R. (1981). Correction: Benomyl increases the selectivity of the Nash-Snyder medium for *Fusarium solani* f. sp. *phaseoli*. *Canadian Journal of Plant Pathology*, 3(2), 97-102.

Handiseni, M., Jo, Y. K., Lee, K. M., & Zhou, X. G. (2016). Screening brassicaceous plants as biofumigants for management of *Rhizoctonia solani* AG1-IA. *Plant Disease* 100, 758-763.

Hanschen, F. S., & Schreiner, M. (2017). Isothiocyanates, nitriles, and epithionitriles from glucosinolates are affected by genotype and developmental stage in *Brassica oleracea* varieties. *Frontiers in Plant Science,* 8, 1095.

Hanschen, F. S., Yim, B., Winkelmann, T., Smalla, K., & Schreiner, M. (2015). Degradation of biofumigant isothiocyanates and allyl glucosinolate in soil and their effects on the microbial community composition. *Plos One,* 10(7), e0132931.

Hofgaard, I. S., Seehusen, T., Aamot, H. U., Riley, H., Razzaghian, J., Le, V. H., *et al*. (2016). Inoculum potential of *Fusarium* spp. relates to tillage and straw management in Norwegian fields of spring oats. *Frontiers in Microbiology*, 7, 556.

Holbert, J. R., Trost, J. F., & Hoffer, G. N. (1919). Wheat scab as affected by system of rotation. *Phytopathology* 9, 45-47.

Hollins, T. W., Ruckenbauer, P., & De Jong, H. (2003). Progress towards wheat varieties with resistance to Fusarium head blight. *Food Control*, 14(4), 239-244.

Hooda, K. S., Khokhar, M. K., Shekhar, M., Karjagi, C. G., Kumar, B., Mallikarjuna, N., *et al*. (2017). Turcicum leaf blight—sustainable management of a re-emerging maize disease. *Journal of Plant Diseases and Protection*, 124(2), 101-113.

Hopkins, R. J., Ekbom, B., & Henkow, L. (1998). Glucosinolate content and susceptibility for insect attack of three populations of *Sinapis alba*. *Journal of Chemical Ecology,* 24 (7), 1203-1216.

Hossain, S., Bergkvist, G., Berglund, K., Glinwood, R., Kabouw, P., Mårtensson, A., *et al*. (2014). Concentration-and time-dependent effects of isothiocyanates produced from Brassicaceae shoot tissues on the pea root rot pathogen Aphanomyces euteiches. *Journal of Agricultural and Food Chemistry*, 62(20), 4584-4591.

Hu, P., Hollister, E. B., Somenahally, A. C., Hons, F. M., & Gentry, T. J. (2015). Soil bacterial and fungal communities respond differently to various isothiocyanates added for biofumigation. *Frontiers in Microbiology,* 5, 729.

Hueza, I. M., Raspantini, P. C. F., Raspantini, L. E. R., Latorre, A. O., & Górniak, S. L. (2014). Zearalenone, an estrogenic mycotoxin, is an immunotoxic compound. *Toxins,* 6(3), 1080-1095.

Huisman, O. C., & Ashworth Jr, L. J. (1974). Verticillium albo-atrum: Quantitative Isolation of Microsclerotia. *Phytopathology*, 64, 1159-1163.

Hunter, M. C., Smith, R. G., Schipanski, M. E., Atwood, L. W., & Mortensen, D. A. (2017). Agriculture in 2050: recalibrating targets for sustainable intensification. *Bioscienc*e, 67(4), 386-391.

Hussein, H. S., & Brasel, J. M. (2001). Toxicity, metabolism, and impact of mycotoxins on humans and animals. *Toxicology*, 167(2), 101-134.

IARC (International Agency for Research on Cancer) (1987). IARC monographs on the evaluation of the carcinogenic risks to humans. Overall Evaluations of Carcinogenicity: An Updating of IARC Monographs Volumes 1 to 42. Suppl. 7, p. 71. Lyon: IARC.

IARC (International Agency for Research on Cancer) (2002). Traditional herbal medicines, some mycotoxins, naphthalene and styrene. IARC Monographs on the evaluation of carcinogenic risks to humans. Lyon, France: International Agency for Research on Cancer 82:1–556.

IARC (International Agency for Research on Cancer) (2021). IARC Monographs on the Identification of Carcinogenic Hazards to Humans. Gentian violet, leucogentian violet, malachite green, leucomalachite green, and CI Direct Blue 218. IARC Working Group. Lyon, France; Feb 25–March 5, 2021. Volume 129: (in press).

IARC (International Agency for Research on Cancer) (1993). Monographs on the Evaluation of Carcinogenic Risks to Humans; Some Naturally Occurring Substances, Food Items and Constituents. Heterocyclic Aromatic Amines and Mycotoxins, vol. 56. International Agency for Research on Cancer, World Health Organization, Lyon, France, 1993, pp. 397–444.

Imathiu, S. M., Ray, R. V., Back, M. I., Hare, M. C., & Edwards, S. G. (2013). A survey investigating the infection of *Fusarium langsethiae* and production of HT-2 and T-2 mycotoxins in UK oat fields. *Journal of Phytopathology*, 161(7-8), 553-561.

Inch, S. A., & Gilbert, J. (2003). Survival of Gibberella zeae in Fusarium-damaged wheat kernels. *Plant Disease*, 87(3), 282-287.

Ishida, M., Hara, M., Fukino, N., Kakizaki, T., & Morimitsu, Y. (2014). Glucosinolate metabolism, functionality and breeding for the improvement of Brassicaceae vegetables. *Breeding Science,* 64 (1), pp. 48-59.

ISO 9167 Rapeseed and rapeseed meals – Determination of glucosinolates content – Method using HPLC. Available at: https://www.iso.org/standard/72207.html [Accessed 21 March 2022]

Jahangir, M., Kim, H. K., Choi, Y. H., & Verpoorte, R. (2009). Health-affecting compounds in Brassicaceae. *Comprehensive Reviews in Food Science and Food Safety,* 8(2), 31-43.

Jakšić, S., Abramović, B., Jajić, I., Baloš, M. Ž, Mihaljev, Ž, Despotović, V., *et al*. (2012). Co-occurrence of fumonisins and deoxynivalenol in wheat and maize harvested in Serbia. *Bulletin of Environmental Contamination and Toxicology*, 89(3), 615-619.

JECFA. (2017). *Evaluation of certain food additives and contaminants*. Eighty-third report of the Joint FAO/WHO Expert Committee on Food Additives (WHO Technical Report Series, No. 1002). Geneva, Switzerland: World Health Organization.

Jeon, J., Bong, S. J., Park, J. S., Park, Y., Arasu, M. V., Al-Dhabi, N. A., & Park, S. U. (2017). De novo transcriptome analysis and glucosinolate profiling in watercress (*Nasturtium officinale* R. br.). *BMC Genomics,* 18(1), 401.

Jochum, C. C., Osborne, L. E., & Yuen, G. Y. (2006). Fusarium head blight biological control with Lysobacter enzymogenes strain C3. *Biological Control*, 39(3), 336-344.

Jørgensen, M. E., Nour-Eldin, H. H., & Halkier, B. A. (2015). Transport of defense compounds from source to sink: Lessons learned from glucosinolates. *Trends in Plant Science,* 20(8), 508-514.

Jung, B., Lee, S., Ha, J., Park, J., Han, S., Hwang, I., *et al*. (2013). Development of a selective medium for the fungal pathogen *Fusarium graminearum* using toxoflavin produced by the bacterial pathogen *Burkholderia glumae*. *The Plant Pathology Journal*, 29(4), 446.

Justes, E., Beaudoin, N., Bertuzzi, P., Charles, R., Constantin, J., Dürr, C., Joannon, A., Le Bas, C., Mary, B., Montfort, F., Ruiz, L., Sarthou, J., Souchère, V., & Tournebize, J. (2017). Main lessons drawn from the analysis of literature. In: Cover crops for sustainable farming. Justes, E. (Ed.) Springer, Dordrecht. pp. 33-35.

Kaiser, S. J., Mutters, N. T., Blessing, B., & Günther, F. (2017). Natural isothiocyanates express antimicrobial activity against developing and mature biofilms of *Pseudomonas aeruginosa*. *Fitoterapia,* 119, 57-63.

Kano, R., Ishida, R., Nakane, S., Sekiguchi, M., Hasegawa, A., & Kamata, H. (2012). The first reported case of canine subcutaneous Cryptococcus flavescens infection. *Mycopathologia*, 173(2), 179-182.

Karlovsky, P. (2011). Biological detoxification of the mycotoxin deoxynivalenol and its use in genetically engineered crops and feed additives. *Applied Microbiology and Biotechnology,* 91 (3), 491-504.

Kazan, K., Gardiner, D. M., & Manners, J. M. (2012). On the trail of a cereal killer: recent advances in *Fusarium graminearum* pathogenomics and host resistance. *Molecular Plant Pathology*, 13(4), 399-413.

Keller, M. D., Bergstrom, G. C., & Shields, E. J. (2014). The aerobiology of *Fusarium graminearum*. *Aerobiologia*, 30(2), 123-136.

Khosrokhavar, R., Rahimifard, N., Shoeibi, S., Hamedani, M. P., & Hosseini, M. (2009). Effects of zearalenone and α-zearalenol in comparison with raloxifene on T47D cells. *Toxicology Mechanisms and Methods,* 19(3), 246-250.

Kim, D., Shin, Y., Lee, S., Wimonmuang, K., Kang, K. B., Lee, Y., *et al*. (2021). FgPKS7 is an essential player in mating-type-mediated regulatory pathway required for completing sexual cycle in *Fusarium graminearum*. *Environmental Microbiology*, 23(4), 1972-1990.

Kim, M. S., Zhang, H., Yan, H., Yoon, B., & Shim, W. B. (2018). Characterizing co-expression networks underpinning maize stalk rot virulence in Fusarium verticillioides through computational subnetwork module analyses. *Scientific Reports*, 8(1), 1-13.

Kim, S., & Ishii, G. (2006). Glucosinolate profiles in the seeds, leaves and roots of rocket salad (*Eruca sativa* mill.) and anti-oxidative activities of intact plant powder and purified 4-methoxyglucobrassicin. *Soil Science and Plant Nutrition,* 52 (3), 394-400.

Kirkegaard J. A., Gardner P. A., Desmarchelier J. M. & Angus J. F. (1993) Biofumigation: using *Brassica* species to control pests and diseases in horticulture and agriculture. In: Wratten, N. & Mailer, R. J. (Eds.) *9th Australian Research Assembly on brassicas*, 5-7 October 1993, Wagga Wagga, NSW, Australia, pp. 77–82.

Kirkegaard, J. A., & Sarwar, M. (1998). Biofumigation potential of brassicas. *Plant and Soil*, 201(1), 71-89.

Kirkegaard, J. A., Gardner, P. A., Angus, J. F., & Koetz, E. (1994). Effect of Brassica break crops on the growth and yield of wheat. *Australian Journal of Agricultural Research*, 45(3), 529-545.

Kirkegaard, J. A., Sarwar, M., Wong, P., Mead, A., Howe, G., & Newell, M. (2000). Field studies on the *biofumigation of take-all by Brassica break crops. Australian Journal of Agricultural Research*, 51(4), 445-456.

Kirkegaard, J. A., Simpfendorfer, S., Holland, J., Bambach, R., Moore, K. J., & Rebetzke, G. J. (2004). Effect of previous crops on crown rot and yield of durum and bread wheat in northern NSW. *Australian Journal of Agricultural Research*, 55(3), 321-334.

Kirkegaard, J. A., Wong, P., & Desmarchelier, J. M. (1996). *In vitro* suppression of fungal root pathogens of cereals by Brassica tissues. *Plant Pathology*, 45(3), 593-603.

Kirkegaard, J., & Matthiessen, J., (2004). Developing and refining the biofumigation concept. *Agroindustria*, 3, 233-239.

Kirkegaard, J., Christen, O., Krupinsky, J., & Layzell, D. (2008). Break crop benefits in temperate wheat production. *Field Crops Research*, 107(3), 185-195.

Kissane, Z., & Shephard, J. M. (2017). The rise of glyphosate and new opportunities for biosentinel early-warning studies. *Conservation Biology*, 31(6), 1293-1300.

Klix, M. B., Verreet, J., & Beyer, M. (2007). Comparison of the declining triazole sensitivity of Gibberella zeae and increased sensitivity achieved by advances in triazole fungicide development. *Crop Protection*, 26(4), 683-690.

Komada, H. (1975). Development of a selective medium for quantitative isolation of *Fusarium oxysporum* from natural soil. *Review of Plant Protection Research*, 8, 114-124.

Koroleva, O. A., Gibson, T. M., Cramer, R., & Stain, C. (2010). Glucosinolate-accumulating S-cells in Arabidopsis leaves and flower stalks undergo programmed cell death at early stages of differentiation. *The Plant Journal*, 64(3), 456-469.

Kotowicz, N. K., Frąc, M., & Lipiec, J. (2014). The importance of Fusarium fungi in wheat cultivation–pathogenicity and mycotoxins production: a review. *Journal of Animal & Plant Sciences*, 21(2), 3326-3343.

Krska, R., Welzig, E., & Boudra, H. (2007). Analysis of Fusarium toxins in feed. *Animal Feed Science and Technology*, 137(3-4), 241-264.

Kruger, D., Fourie, J. C., & Malan, A. P. (2013). Cover crops with biofumigation properties for the suppression of plant-parasitic nematodes: A review. *South African Journal of Enology and Viticulture*, 34(2), 287-295.

Kuchernig, J. C., Burow, M., & Wittstock, U. (2012). Evolution of specifier proteins in glucosinolate-containing plants. *BMC Evolutionary Biology*, 12(1), 1-14.

Kuiper-Goodman, T., Scott, P., & Watanabe, H. (1987). Risk assessment of the mycotoxin zearalenone. *Regulatory Toxicology and Pharmacology,* 7(3), 253-306.

Kupke, F., Herz, C., Hanschen, F. S., Platz, S., Odongo, G. A., Helmig, S., *et al*. (2016). Cytotoxic and genotoxic potential of food-borne nitriles in a liver in vitro model. *Scientific reports*, 6(1), 1-11.

Kurt, Ş, Güneş, U., & Soylu, E. M. (2011). *In vitro* and *in vivo* antifungal activity of synthetic pure isothiocyanates against Sclerotinia sclerotiorum. *Pest Management Science*, 67(7), 869-875.

Kushad, M. M., Brown, A. F., Kurilich, A. C., Juvik, J. A., Klein, B. P., Wallig, M. A., *et al*. (1999). Variation of Glucosinolates in Vegetable Crops of Brassica oleracea. *Journal of Agricultural and Food Chemistry*, 47(4), 1541-1548.

Kushiro, M., Thammawong, M., Hossen, S. M., Kozawa, T., Yoshida, M., Nakagawa, H., Nagashima, H., Okadome, H., & Nakajima, T. (2012). Effects of noodle making and cooking on the levels of a mycotoxin deoxynivalenol in Japanese soft wheat varieties. *Japanese Journal of Food Chemistry and Safety*, 19(2), 129-135.

Larkin, R. P., & Griffin, T. S. (2007). Control of soilborne potato diseases using *Brassica* green manures. *Crop protection*, 26(7), 1067-1077.

Lazarovits, G., Hawke, M. A., Tomlin, A. D., Olthof, Th. H. A. & Squre, S. (1991). Soil solarization to control *Verticillium dahliae* and *Pratylenchus penetrons* on potatoes in central Ontario. *Canadian Journal of Plant Pathology*, 13(2), 116-123.

Lazzeri, L., Curto, G., Dallavalle, E., D'avino, L., Malaguti, L., Santi, R., *et al*. (2009). Nematicidal efficacy of biofumigation by defatted Brassicaceae meal for control of Meloidogyne incognita (Kofoid et White) Chitw. on a full field zucchini crop. *Journal of Sustainable Agriculture*, 33(3), 349-358.

Lazzeri, L., Leoni, O., & Manici, L. M. (2004). Biocidal plant dried pellets for biofumigation. *Industrial Crops and Products*, 20(1), 59-65.

Lazzeri, L., Malaguti, L., Cinti, S., Ugolini, L., De Nicola, G. R., Bagatta, M., Casadei, N., D'Avino, L., & Matteo, R. (2012). The *Brassicaceae* biofumigation system for plant cultivation and defence. An Italian twenty-year experience of study and application. Proc. VIth IS on Brassicas and XVIIIth Crucifer Genetics Workshop. Branca, F., & Tribulato, A. (Eds.) Acta Hort. 1005, ISHS 2013 pp. 375-382.

LeCoz, C.J., & Ducombs, G. (2006) Plants and plant products. *In*: Frosch, P.J., Menne, T. and Lepottevin, J.P. (Eds.) *Contact Dermatitis.* 4th ed. Berlin-Heidelberg, Germany: Springer Verlag. pp. 751-800.

Lefebvre, M., Leblanc, M. L., & Watson, A. K. (2018). Seed dormancy and seed morphology related to weed susceptibility to biofumigation – Corrigendum. *Weed Science,* doi: 10.1017/wsc.2018.17

Lembright, H. W. (1990). Soil fumigation: principles and application technology. *Journal of Nematology*, 22(4S), 632.

Lenc, L., Czecholinski, G., Wyczling, D., Turow, T., & Kazmierczak, A. (2015). Fusarium head blight (FHB) and Fusarium spp. on grain of spring wheat cultivars grown in Poland. *Journal of Plant Protection Research,* 55, 266-77.

Leplat, J., Friberg, H., Abid, M., & Steinberg, C. (2013). Survival of *Fusarium graminearum*, the causal agent of Fusarium head blight. A review. *Agronomy for Sustainable Development*, 33(1), 97-111.

Leslie, J. F., & Summerell, B. A. (2006) *The* Fusarium *laboratory manual*. Ames IA, USA: Blackwell Publishing.

Lewis, J. A., & Papavizas, G. C. (1971). Effect of sulfur-containing volatile compounds and vapors from cabbage decomposition *on Aphanomyces euteiches. Phytopathology*, 61, 208-214.

Lewis, J. A., & Papavizas, G. C. (1974). Effect of volatiles from decomposing plant tissues on pigmentation, growth and survival of *Rhizoctonia solani*. *Soil Science*, 118, 156-163.

Li, T., Liu, T., Zheng, C., Kang, C., Yang, Z., Yao, X., *et al*. (2017). Changes in soil bacterial community structure as a result of incorporation of Brassica plants compared with continuous planting eggplant and chemical disinfection in greenhouses. *PLoS One*, 12(3), e0173923.

Li, W., Ping, H. E., & Jin, J. (2010). Effect of potassium on ultrastructure of maize stalk pith and young root and their relation to stalk rot resistance*. Agricultural Sciences in Chin*a, 9(10), 1467-1474.

Li, Y. C., Ledoux, D. R., Bermudez, A. J., Fritsche, K. L., & Rottinghaus, G. E. (2000). The individual and combined effects of fumonisin B1 and moniliformin on

performance and selected immune parameters in turkey poults. *Poultry Science,* 79(6), 871-878.

Lim, L.T. (1999) *Permeation of allyl isothiocyanate, oxygen and water vapor in synthetic and biopolymer films*. PhD thesis, Guelph, The University of Guelph.

Luo, Y. (1988). Fusarium toxins contamination of cereals in China. In: Aibara K, Kumagai S, Ohtsubo K, Yoshizawa T, (Eds.). Proceedings of the 7th International IUPAC Symposium on Mycotoxins and Phycotoxins. Tokyo: Japanese Assn. of Mycotoxicology. p. 97-98.

Lutz, M. P., Feichtinger, G., Défago, G., & Duffy, B. (2003). Mycotoxigenic Fusarium and deoxynivalenol production repress chitinase gene expression in the biocontrol agent Trichoderma atroviride P1. *Applied and Environmental Microbiology*, 69(6), 3077-3084.

Lv, X., Pan, L., Wang, J., Lu, L., Yan, W., Zhu, Y., *et al*. (2017). Effects of triazole fungicides on androgenic disruption and CYP3A4 enzyme activity. *Environmental pollution*, 222, 504-512.

Maaroufi, K., Chekir, L., Creppy, E. E., Ellouz, F., & Bacha, H. (1996). Zearalenone induces modifications of haematological and biochemical parameters in rats. *Toxicon,* 34 (5), 535-540.

Malik, M. S., Riley, M. B., Norsworthy, J. K., & Bridges Jr, W. (2010). Glucosinolate profile variation of growth stages of wild radish (*Raphanus raphanistrum*). *Journal of Agricultural and Food Chemistry*, 58(6), 3309-3315.

Manners, J. G. (1966) Assessment of germination. In: Madelin, M. F. (Ed.) *The fungus spore*. London: Butterworths, pp. 165-173.

Mari, M., Iori, R., Leoni, O., & Marchi, A. (1993). In vitro activity of glucosinolate-derived isothiocyanates against postharvest fruit pathogens. *Annals of Applied Biology*, 123(1), 155-164.

Mari, M., Leoni, O., Bernardi, R., Neri, F., & Palmieri, S. (2008). Control of brown rot on stonefruit by synthetic and glucosinolate-derived isothiocyanates. *Postharvest Biology and Technology*, 47(1), 61-67.

Markell, S. G., & Francl, L. J. (2003). Fusarium head blight inoculum: species prevalence and Gibberella zeae spore type. *Plant Disease*, 87(7), 814-820.

Maršić, N., Može, K. S., Mihelič, R., Nečemer, M., Hudina, M., & Jakopič, J. (2021). Nitrogen and Sulphur Fertilisation for Marketable Yields of Cabbage (Brassica oleracea L. var. Capitata), Leaf Nitrate and Glucosinolates and Nitrogen Losses Studied in a Field Experiment in Central Slovenia. *Plants*, 10(7), 1304.

Masiello, M., Somma, S., Ghionna, V., Logrieco, A. F., & Moretti, A. (2019). *In vitro* and in field response of different fungicides against *Aspergillus flavus* and *Fusarium* species causing ear rot disease of maize. *Toxins*, 11(1), 11.

Matarese, F., Sarrocco, S., Gruber, S., Seidl-Seiboth, V., & Vannacci, G. (2012). Biocontrol of Fusarium head blight: interactions between Trichoderma and mycotoxigenic Fusarium. *Microbiology*, 158(1), 98-106.

Mathur, S., Constable, P. D., Eppley, R. M., Waggoner, A. L., Tumbleson, M. E., & Haschek, W. M. (2001). Fumonisin B1 is hepatotoxic and nephrotoxic in milk-fed calves. *Toxicological Sciences*, 60(2), 385-396.

Matteo, R (2017) *Non-food brassicas for green chemistry purposes through a biorefinery approach*. PhD thesis, Bologna, University of Bologna.

Matthews, J. (2016). Anglia farmer. Available at: http://www.angliafarmer.co.uk/new-maize-varieties-recommended-for-anaerobic-digestion/ [Accessed 27 June 2019]

Matthiessen, J. N., & Shackleton, M. A. (2005). Biofumigation: environmental impacts on the biological activity of diverse pure and plant-derived isothiocyanates. *Pest Management Science*, 61(11), 1043-1051.

Matthiessen, J. N., Warton, B., & Shackleton, M. A. (2004). The importance of plant maceration and water addition in achieving high Brassica-derived isothiocyanate levels in soil. *Agroindustria*, 3(3), 277-280.

Mayton, H. S., Olivier, C., Vaughn, S. F., & Loria, R. (1996). Correlation of fungicidal activity of Brassica species with allyl isothiocyanate production in macerated leaf tissue. *Phytopathology*, 86(3), 267-271.

Mazzola, M., Brown, J., Izzo, A. D., & Cohen, M. F. (2007). Mechanism of action and efficacy of seed meal-induced pathogen suppression differ in a Brassicaceae species and time-dependent manner. *Phytopathology*, 97(4), 454-460.

Mazzola, M., Hewavitharana, S. S., & Strauss, S. L. (2015). Brassica seed meal soil amendments transform the rhizosphere microbiome and improve apple production through resistance to pathogen reinfestation. *Phytopathology*, 105(4), 460-469.

McCormick, S. P., Stanley, A. M., Stover, N. A., & Alexander, N. J. (2011). Trichothecenes: from simple to complex mycotoxins. *Toxins*, 3(7), 802-814.

McKevith, B. (2004). Nutritional aspects of cereals. British Nutrition Foundation, *Nutrition Bulletin*, 29, 111-142.

McKinney, H. H. (1923). Influence of soil temperature and moisture on infection of wheat seedlings by *Helminthosporium sativum*. *Journal of Agricultural Research,* 26(5),195-217.

McMullen, M., Bergstrom, G., De Wolf, E., Dill-Macky, R., Hershman, D., Shaner, G., *et al*. (2012). A unified effort to fight an enemy of wheat and barley: Fusarium head blight. *Plant Disease*, 96(12), 1712-1728.

McMullen, M., Jones, R., & Gallenberg, D. (1997). Scab of wheat and barley: a re-emerging disease of devastating impact. *Plant Disease*, 81(12), 1340-1348.

Mehta, Y. R. (2014) *Wheat Diseases and their Management*. New York: Springer. pp. 256.

Merhej, J., Boutigny, A.L., Pinson-Gadais, L., Richard-Forget, F., & Barreau, C. (2010). Acidic pH as a determinant of TRI gene expression and trichothecene B biosynthesis in *Fusarium graminearum*. *Food Additives & Contaminants: Part A,* 27, 710–717.

Mesterhazy, A. (1995). Types and components of resistance to Fusarium head blight of wheat. *Plant breeding*, 114(5), 377-386.

Met Office (2021) *Historic station data*. Available at: https://www.metoffice.gov.uk/research/climate/maps-and-data/historic-station-data [Accessed 26 October 2021].

Mielniczuk, E., & Skwaryło-Bednarz, B. (2020). Fusarium Head Blight, mycotoxins and strategies for their reduction. *Agronomy*, 10(4), 509.

Miller, J. D., Young, J. C., & Sampson, D. R. (1985). Deoxynivalenol and Fusarium head blight resistance in spring cereals. *Journal of Phytopathology*, 113(4), 359-367.

Mills, K., Salgado, J. D., & Paul, P. A. (2016). Fusarium head blight or head scab of wheat, barley and other small grain crops. PLPATH-CER-06 Agriculture and Natural Resources. Ohioline, Ohio State University Extension. Available at: http://ohioline.osu.edu/factsheet/plpath-cer-06 [Accessed 24 May 2018]

Mirocha, C. J., & Swanson, S. P. (1983). Regulation of perithecia production in *Fusarium roseum* by zearalenone. *Journal of Food Safety*, 5(1), 41-53.

Mittal, J., Szymczak, W. A., Pirofski, L., & Galen, B. T. (2018). Fungemia caused by Aureobasidium pullulans in a patient with advanced AIDS: a case report and review of the medical literature. *JMM case reports*, 5(4).

Mitter, V., Francl, L. J., Ali, S., Simpfendorfer, S., & Chakraborty, S. (2006). Ascosporic and conidial inoculum of Gibberella zeae play different roles in Fusarium head blight and crown rot of wheat in Australia and the USA. *Australasian Plant Pathology*, 35(4), 441-452.

Morales-Rodríguez, C., Vettraino, A. M., & Vannini, A. (2016). Efficacy of biofumigation with Brassica carinata commercial pellets (BioFence) to control vegetative and reproductive structures of Phytophthora cinnamomi. *Plant Disease*, 100(2), 324-330.

Morra, M. J., & Kirkegaard, J. A. (2002). Isothiocyanate release from soil-incorporated *Brassica* tissues. *Soil Biology and Biochemistry*, 34(11), 1683-1690.

Motisi, N., Doré, T., Lucas, P., & Montfort, F. (2010). Dealing with the variability in biofumigation efficacy through an epidemiological framework. *Soil Biology and Biochemistry*, 42(12), 2044-2057.

Mueller, D. S., Wise, K. A., Sisson, A. J., Allen, T. W., Bergstrom, G. C., Bosley, D. B., *et al*. (2016). Corn yield loss estimates due to diseases in the United States and Ontario, Canada from 2012 to 2015. *Plant Health Progress*, 17(3), 211-222.

Münch, S., Lingner, U., Floss, D. S., Ludwig, N., Sauer, N., & Deising, H. B. (2008). The hemibiotrophic lifestyle of *Colletotrichum* species. *Journal of Plant Physiology*, 165(1), 41-51.

Munkvold, G. P. (2003). Epidemiology of *Fusarium* diseases and their mycotoxins in maize ears. *European Journal of Plant Pathology*, 109(7), 705-713.

Nagaraj, R., Wu, W., Will, J., & Vesonder, R. (1996). Acute cardiotoxicity of moniliformin in broiler chickens as measured by electrocardiography. *Avian Diseases,* 223-227.

Nash, S.M., & Snyder, W.C. (1962) Quantitative estimations by plate counts of propagules of the bean root rot *Fusarium* in field soils. *Phytopathology,* 52, 567–572.

Nazareth, T. M., Bordin, K., Manyes, L., Meca, G., Mañes, J., & Luciano, F. B. (2016). Gaseous allyl isothiocyanate to inhibit the production of aflatoxins, beauvericin and enniatins by Aspergillus parasiticus and Fusarium poae in wheat flour. *Food Control*, 62, 317-321.

Nesic, K., Ivanovic, S., & Nesic, V. (2014). Fusarial toxins: secondary metabolites of Fusarium fungi. *Reviews of Environmental Contamination and Toxicology*, 228, 101-120.

Neubauer, C., Heitmann, B., & Müller, C. (2014). Biofumigation potential of Brassicaceae cultivars to *Verticillium dahliae. European Journal of Plant Pathology*, 140(2), 341-352.

Neubauer, C., Hüntemann, K., Heitmann, B., & Müller, C. (2015). Suppression of Verticillium dahliae by glucosinolate-containing seed meal amendments. *European Journal of Plant Pathology*, 142(2), 239-249.

Ngala, B. M., Haydock, P. P., Woods, S., & Back, M. A. (2015a). Biofumigation with *Brassica juncea, Raphanus sativus* and *Eruca sativa* for the management of field populations of the potato cyst nematode *Globodera pallida. Pest Management Science*, 71(5), 759-769.

Ngala, B. M., Woods, S. R., & Back, M. A. (2015b). *In vitro* assessment of the effects of *Brassica juncea* and *Raphanus sativus* leaf and root extracts on the viability of *Globodera pallida* encysted eggs. *Nematology*, 17(5), 543-556.

Ngala, B. M., Woods, S. R., & Back, M. A. (2015c). Sinigrin degradation and *G. pallida* suppression in soil cultivated with brassicas under controlled environmental conditions. *Applied Soil Ecology*, 95, 9-14.

Ngwene, B., Neugart, S., Baldermann, S., Ravi, B., & Schreiner, M. (2017). Intercropping Induces Changes in Specific Secondary Metabolite Concentration in Ethiopian Kale (Brassica carinata) and African Nightshade (Solanum scabrum) under Controlled Conditions. *Frontiers in Plant Science*, 8, 1700.

Njoroge, S. M., Riley, M. B., & Keinath, A. P. (2008). Effect of incorporation of *Brassica* spp. residues on population densities of soilborne microorganisms and on damping-off and Fusarium wilt of watermelon. *Plant Disease*, 92(2), 287-294.

Norred, W., Plattner, R., Vesonder, R., Bacon, C. & Voss, K. (1992). Effects of selected secondary metabolites of *Fusarium moniliforme* on unscheduled synthesis of DNA by rat primary hepatocytes. *Food and Chemical Toxicology,* 30(3), 233-237.

Norsworthy, J. K., & Meehan, J. T. (2005). Use of isothiocyanates for suppression of Palmer amaranth (Amaranthus palmeri), pitted morningglory (Ipomoea lacunosa), and yellow nutsedge (Cyperus esculentus). *Weed Science*, 53(6), 884-890.

Nowicki, D., Rodzik, O., Herman-Antosiewicz, A., & Szalewska-Pałasz, A. (2016). Isothiocyanates as effective agents against enterohemorrhagic Escherichia coli: insight to the mode of action. *Scientific reports*, 6, 22263.

OECD/FAO (2021), *OECD-FAO Agricultural Outlook OECD Agriculture statistics (database)*. Available at: http://dx.doi.org/10.1787/agr-outl-data-en. [Accessed 3 October 2021].

Ojaghian, M. R., Jiang, H., Xie, G., Cui, Z., Zhang, J., & Li, B. (2012). *In vitro* biofumigation of *Brassica* tissues against potato stem rot caused by *Sclerotinia sclerotiorum*. *The Plant Pathology Journal*, 28(2), 185-190.

Omirou, M. D., Papadopoulou, K. K., Papastylianou, I., Constantinou, M., Karpouzas, D. G., Asimakopoulos, I., *et al*. (2009). Impact of nitrogen and sulfur fertilization on the composition of glucosinolates in relation to sulfur assimilation in different plant organs of broccoli. *Journal of Agricultural and Food Chemistry*, 57(20), 9408-9417.

Paillard, S., Schnurbusch, T., Tiwari, R. *et al*., (2004). QTL analysis of resistance to Fusarium head blight in Swiss winter wheat (*Triticum aestivum* L.). *Theoretical and Applied Genetics*, 109, 323-32.

Palazzini, J., Fumero, V., Yerkovich, N., Barros, G., Cuniberti, M., & Chulze, S. (2015). Correlation between *Fusarium graminearum* and deoxynivalenol during the 2012/13 wheat Fusarium head blight outbreak in Argentina. *Cereal Research Communications*, 43(4), 627-637.

Pallez-Barthel, M., Cocco, E., Vogelgsang, S., & Beyer, M. (2021). Frequency of deoxynivalenol concentrations above the maximum limit in raw winter wheat grain during a 12-year multi-site survey. *Agronomy*, 11(5), 960.

Palop, M. L., Smiths, J. P., & ten Brink, B. (1995). Degradation of sinigrin by Lactobacillus agilis strain R16. *International Journal of Food Microbiology*, 26(2), 219-229.

Papavizas, G. C. (1967). Evaluation of various media and anti-microbial agents for isolation of *Fusarium* from soil. *Phytopathology*, 57(8), 848-852.

Parry, D. W., Jenkinson, P., & McLeod, L. (1995). Fusarium ear blight (scab) in small grain cereals—a review. *Plant Pathology*, 44(2), 207-238.

Paul, P. A., Lipps, P. E., Hershman, D. E., McMullen, M. P., Draper, M. A., & Madden, L. V. (2008). Efficacy of triazole-based fungicides for Fusarium head blight and deoxynivalenol control in wheat: A multivariate meta-analysis. *Phytopathology*, 98(9), 999-1011.

Pestka, J. (2010). Toxicological mechanisms and potential health effects of deoxynivalenol and nivalenol. *World Mycotoxin Journal,* 3 (4), 323-347.

Peterson, C. J., Cosse, A., & Coats, J. R. (2000). Insecticidal components in the meal of *Crambe abyssinica. Journal of Agricultural and Urban Entomology*, 17(1).

Peterson, C. J., Tsao, R., & Coats, J. R. (1998). Glucosinolate aglucones and analogues: insecticidal properties and a QSAR. *Pesticide Science*, 54(1), 35-42.

Piccinini, E., Ferrari, V., Campanelli, G., Fusari, F., Righetti, L., Pagnotta, E., *et al*. (2015). Effect of two liquid formulations based on Brassica carinata co-products in containing powdery mildew on melon. *Industrial Crops and Products*, 75, 48-53.

Pirgozliev, S. R., Edwards, S. G., Hare, M. C., & Jenkinson, P. (2002). Effect of dose rate of azoxystrobin and metconazole on the development of Fusarium head blight and the accumulation of deoxynivalenol (DON) in wheat grain. *European Journal of Plant Pathology*, 108, 469-78.

Pirgozliev, S. R., Edwards, S. G., Hare, M. C., & Jenkinson, P. (2003). Strategies for the control of Fusarium head blight in cereals. *European Journal of Plant Pathology*, 109(7), 731-742.

Pitt, J.I. (1996). What are mycotoxins?, *Australian Mycotoxin Newsletter*, 7(4), 1.

Placinta, C. M., D'Mello, J. F., & Macdonald, A. (1999). A review of worldwide contamination of cereal grains and animal feed with Fusarium mycotoxins. *Animal Feed Science and Technology*, 78(1-2), 21-37.

Pleadin, J., Vahčić, N., Perši, N., Ševelj, D., Markov, K., & Frece, J. (2013). Fusarium mycotoxins' occurrence in cereals harvested from Croatian fields. *Food Control*, 32(1), 49-54.

Pokharel, R. R. (2012). Efficacy of bio-fumigation and soil solarization on soil-borne onion pathogens. Agricultural experiment station, Technical report, TR 12-1 January, Colorado State University, pp 1-18.

Ponts, N. (2015). Mycotoxins are a component of *Fusarium graminearum* stress-response system. *Frontiers in Microbiology,* 6, 1234.

Ponts, N., Pinson-Gadais, L., Barreau, C., Richard-Forget, F., & Ouellet, T. (2007). Exogenous H_2O_2 and catalase treatments interfere with tri genes expression in liquid cultures of *Fusarium graminearum*. *FEBS Letters,* 581(3), 443-447.

Popova, I. E., & Morra, M. J. (2014). Simultaneous quantification of sinigrin, sinalbin, and anionic glucosinolate hydrolysis products in Brassica juncea and Sinapis alba seed extracts using ion chromatography. *Journal of Agricultural and Food Chemistry*, 62(44), 10687-10693.

Postic, J., Cosic, J., Vrandecic, K., Jurkovic, D., Saleh, A. A., & Leslie, J. F. (2012). Diversity of Fusarium species isolated from weeds and plant debris in Croatia. *Journal of Phytopathology*, 160(2), 76-81.

Poulsen, R., Luong, X., Hansen, M., Styrishave, B., & Hayes, T. (2015). Tebuconazole disrupts steroidogenesis in Xenopus laevis. *Aquatic Toxicology*, 168, 28-37.

Price, A. J., Charron, C. S., Saxton, A. M., & Sams, C. E. (2005). Allyl isothiocyanate and carbon dioxide produced during degradation of *Brassica juncea* tissue in different soil conditions. *HortScience*, 40(6), 1734-1739.

Pritsch, C., Muehlbauer, G. J., Bushnell, W. R., Somers, D. A., & Vance, C. P. (2000). Fungal development and induction of defense response genes during early infection of wheat spikes by *Fusarium graminearum*. *Molecular Plant-Microbe Interactions*, 13(2), 159-169.

Radojčić Redovniković, I., Glivetić, T., Delonga, K., & Vorkapić-Furač, J. (2008). Glucosinolates and their potential role in plant. *Periodicum Biologorum*, 110(4), 297-309.

Rakariyatham, N., Butrindr, B., Niamsup, H., & Shank, L. (2005). Screening of filamentous fungi for production of myrosinase. *Brazilian Journal of Microbiology*, 36(3), 242-245.

Ramirez, M. L., Chulze, S., & Magan, N. (2006). Temperature and water activity effects on growth and temporal deoxynivalenol production by two Argentinean strains of *Fusarium graminearum* on irradiated wheat grain. *International Journal of Food Microbiology,* 106(3), 291-296.

Ramos García, M., Hernández López, M., Barrera Necha, L. L., Bautista Baños, S., Troncoso Rojas, R., & Bosquez Molina, E. (2012). *In vitro* response of *Fusarium oxysporum* isolates to isothiocyanates application. *Revista mexicana de fitopatología*, 30(1), 1-10.

Rao, K. V., & Fernandes, C. L. (1996). Progressive effects of malachite green at varying concentrations on the development of N-nitrosodiethylamine induced hepatic preneoplastic lesions in rats. *Tumori*, 82(3), 280-286.

Rask, L., Andréasson, E., Ekbom, B., Eriksson, S., Pontoppidan, B., & Meijer, J. (2000). Myrosinase: gene family evolution and herbivore defense in Brassicaceae. *Plant Molecular Biology*, 42(1), 93-114.

Regulation (EC) No 1005/2009 of the European parliament and of the council of 16 September 2009 on substances that deplete the ozone layer. *Official Journal of the European Union* L 286/1-30.

Rheeder, J. P., Marasas, W. F., & Vismer, H. F. (2002). Production of fumonisin analogs by Fusarium species. *Applied and Environmental Microbiology*, 68(5), 2101-2105.

Richardson, K. E., Hagler Jr, W. M., & Mirocha, C. J. (1985). Production of zearalenone,. alpha.-and. beta.-zearalenol, and. alpha.-and. beta.-zearalanol by *Fusarium* spp. in rice culture. *Journal of Agricultural and Food Chemistry,* 33(5), 862-866.

Rocha, O., Ansari, K., & Doohan, F. M. (2005). Effects of trichothecene mycotoxins on eukaryotic cells: a review. *Food Additives and Contaminants*, 22(4), 369-378.

Rodríguez-Molina, M. C., Serrano-Pérez, P., & Palo, C. (2016). Effect of biofumigation with brassica pellets combined with Brassicaceae cover crops and

plastic cover on the survival and infectivity of inoculum of Phytophthora nicotianae Breda de Haan. *Pest Management Science*, 72(7), 1295-1301.

Romero, L. R. (2016) *Occurrence and Importance of Foliar Diseases on Maize (Zea mays L.) in Central Europe*. PhD thesis, Göttingen, Georg-August-University Göttingen.

Ropejko, K., & Twarużek, M. (2021). Zearalenone and its metabolites—General overview, occurrence, and toxicity. *Toxins*, 13(1), 35.

Rosa, E. A., Heaney, R. K., Portas, C. A., & Fenwick, G. R. (1996). Changes in Glucosinolate Concentrations in BrassicaCrops (BoleraceaandBnapus) Throughout Growing Seasons. *Journal of the Science of Food and Agriculture*, 71(2), 237-244.

Rosen, C. J., Fritz, V. A., Gardner, G. M., Hecht, S. S., Carmella, S. G., & Kenney, P. M. (2005). Cabbage yield and glucosinolate concentrations as affected by nitrogen and sulfur fertility. *HortScience*, 40(5), 1493-1498.

Rossetto, M. R. M., Shiga, T. M., Vianello, F., & Lima, G. P. P. (2013). Analysis of total glucosinolates and chromatographically purified benzylglucosinolate in organic and conventional vegetables. *LWT-Food Science and Technology*, 50(1), 247-252.

Rothrock, C. S., & Gottlieb, D. (1981). Importance of antibiotic production in antagonism of selected Streptomyces species to two soil-borne plant pathogens. *The Journal of Antibiotics*, 34(7), 830-835.

Rudelsheim, P. L. J., & Smets, G. (2011). Baseline information on agricultural practices in the EU Maize (*Zea mays* L.). Study performed for EuropaBio aisbl, Avenue de l' Armee 6. B- 1040 Brussels' Belgium' PERSEUS BVBA, p 58.

Rumberger, A., & Marschner, P. (2003). 2-Phenylethylisothiocyanate concentration and microbial community composition in the rhizosphere of canola. *Soil Biology and Biochemistry*, 35(3), 445-452.

Sakorn, P., Rakariyatham, N., Niamsup, H., & Kovitaya, P. (1999). Sinigrin degradation by Aspergillus sp. NR-4201 in liquid culture. *Science Asia*, 25(4), 189-194.

Salgado, J. D., & Paul, P. A. (2016). Leaf blotch diseases of wheat- Septoria tritici blotch, Stagonospora nodorum blotch and tan spot. PLPATH-CER-07 Agriculture and Natural Resources. Ohioline, Ohio State University Extension. Available at: https://ohioline.osu.edu/factsheet/plpath-cer-07 [Accessed 24 May 2018]

Salgado, J. D., Roche, E., & Paul, P. A. (2016). Rust diseases of wheat. PLPATH-CER-12 Agriculture and Natural Resources. Ohioline, Ohio State University Extension. Available at: https://ohioline.osu.edu/factsheet/plpath-cer-12 [Accessed 24 May 2018]

Sarwar, M., Kirkegaard, J. A., Wong, P., & Desmarchelier, J. M. (1998). Biofumigation potential of brassicas. 3. In vitro toxicity of isothiocyanates to soil-borne fungal pathogens. *Plant and Soil*, 201, 103-112.

Scarpino, V., Reyneri, A., Sulyok, M., Krska, R., & Blandino, M. (2015). Effect of fungicide application to control Fusarium head blight and 20 Fusarium and Alternaria mycotoxins in winter wheat (Triticum aestivum L.). *World Mycotoxin Journal*, 8(4), 499-510.

Schisler, D. A., Khan, N. I., Boehm, M. J., & Slininger, P. J. (2002). Greenhouse and field evaluation of biological control of Fusarium head blight on durum wheat. *Plant Disease*, 86(12), 1350-1356.

Schisler, D. A., Slininger, P. J., Boehm, M. J., & Paul, P. A. (2011). Co-culture of yeast antagonists of Fusarium head blight and their effect on disease development in wheat. *Plant Pathology Journal*, 10(4), 128-137.

Schlaeppi, K., Abou-Mansour, E., Buchala, A., & Mauch, F. (2010). Disease resistance of Arabidopsis to Phytophthora brassicae is established by the sequential action of indole glucosinolates and camalexin. *The Plant Journal*, 62(5), 840-851.

Schollenberger, M., Müller, H., Rüfle, M., Suchy, S., Plank, S., & Drochner, W. (2006). Natural occurrence of 16 Fusarium toxins in grains and feedstuffs of plant origin from Germany. *Mycopathologia,* 161(1), 43-52.

Schoneberg, A., Musa, T., Voegele, R. T., & Vogelgsang, S. (2015). The potential of antagonistic fungi for control of *Fusarium graminearum* and *Fusarium crookwellense* varies depending on the experimental approach. *Journal of Applied Microbiology*, 118(5), 1165-1179.

Schonhof, I., Blankenburg, D., Müller, S., & Krumbein, A. (2007). Sulfur and nitrogen supply influence growth, product appearance, and glucosinolate concentration of broccoli. *Journal of Plant Nutrition and Soil Science*, 170(1), 65-72.

Schroeder, H. W., & Christensen, J. J. (1963). Factors affecting the resistance of wheat to scab caused by *Gibberella zeae*. *Phytopathology* 53, 831-838.

Segalin, M., & Reis, E. M. (2010). Semi-selective medium for *Fusarium graminearum* detection in seed samples. *Summa Phytopathologica*, 36, 338-341.

Shah, F. A., Ansari, M. A., Watkins, J., Phelps, Z., Cross, J., & Butt, T. M. (2009). Influence of commercial fungicides on the germination, growth and virulence of four species of entomopathogenic fungi. *Biocontrol Science and Technology*, 19(7), 743-753.

Shah, L., Ali, A., Yahya, M., Zhu, Y., Wang, S., Si, H., *et al*. (2018). Integrated control of Fusarium head blight and deoxynivalenol mycotoxin in wheat. *Plant Pathology*, 67(3), 532-548.

Shah, L., Ali, A., Zhu, Y., Wang, S., Si, H., & Ma, C. (2017). Wheat resistance to Fusarium head blight and possibilities of its improvement using molecular marker-assisted selection. *Czech Journal of Genetics and Plant Breeding*, 53(2), 47-54.

Shephard, G. S. (2008). Impact of mycotoxins on human health in developing countries. *Food Additives & Contaminants*, 25(2), 146-151.

Shewry, P. R., & Hey, S. J. (2015). The contribution of wheat to human diet and health. *Food and Energy Security*, 4(3), 178-202.

Shiferaw, B., Smale, M., Braun, H., Duveiller, E., Reynolds, M., & Muricho, G. (2013). Crops that feed the world 10. Past successes and future challenges to the role played by wheat in global food security. *Food Security*, 5(3), 291-317.

Singh, R. S., & Nene, Y. L. (1965). Malachite green in synthetic medium for the isolation of *Fusarium* spp. from plant tissues. *Naturwissenschaften*, 52(4), 94.

Smith, B. J., & Kirkegaard, J. A. (2002). In vitro inhibition of soil microorganisms by 2-phenylethyl isothiocyanate. *Plant Pathology*, 51(5), 585-593.

Smolinska, U., Morra, M. J., Knudsen, G. R., & James, R. L. (2003). Isothiocyanates produced by Brassicaceae species as inhibitors of *Fusarium oxysporum. Plant Disease*, 87(4), 407-412.

Sobrova, P., Adam, V., Vasatkova, A., Beklova, M., Zeman, L., & Kizek, R. (2010). Deoxynivalenol and its toxicity. *Interdisciplinary Toxicology*, 3(3), 94-99.

Sotelo, T., Lema, M., Soengas, P., Cartea, M. E., & Velasco, P. (2015). *In vitro* activity of glucosinolates and their degradation products against brassica-pathogenic bacteria and fungi. *Applied and Environmental Microbiology*, 81(1), 432-440.

Srivastava, S., Sinha, R., & Roy, D. (2004). Toxicological effects of malachite green. *Aquatic toxicology*, 66(3), 319-329.

Stadnik, A., & Borzecki, A. (2009). Influence of the zearalenone on the activity of chosen liver enzymes in a rat. *Annals of Agricultural and Environmental Medicine,* 16(1), 31-35.

Stanciu, O., Banc, R., Cozma, A., Filip, L., Miere, D., Mañes, J., *et al*. (2015). Occurence of Fusarium mycotoxins in wheat from Europe—A review. *Acta Univ. Cibiniensis Series E Food Technology*, 19, 35-60.

Stockmann-Juvala, H., Alenius, H., & Savolainen, K. (2008). Effects of fumonisin B1 on the expression of cytokines and chemokines in human dendritic cells*. Food and Chemical Toxicology*, 46(5), 1444-1451.

Subbarao, K. V., Hubbard, J. C., & Koike, S. T. (1999). Evaluation of broccoli residue incorporation into field soil for Verticillium wilt control in cauliflower. *Plant Disease*, 83(2), 124-129.

Subedi, S. (2015). A review on important maize diseases and their management in Nepal. *Journal of Maize Research and Development*, 1(1), 28-52.

Suproniene, S., Kadziene, G., Irzykowski, W., Sneideris, D., Ivanauskas, A., Sakalauskas, S., *et al*. (2019). Weed species within cereal crop rotations can serve as alternative hosts for *Fusarium graminearum* causing Fusarium head blight of wheat. *Fungal Ecology*, 37, 30-37.

Sutton, J. C. (1982). Epidemiology of wheat head blight and maize ear rot caused by *Fusarium graminearum*. *Canadian Journal of Plant Pathology*, 4(2), 195-209.

Szczech, M. M. (1999). Suppressiveness of vermicompost against Fusarium wilt of tomato. *Journal of Phytopathology*, 147(3), 155-161.

Szuets, P., Mesterhazy, A., Falkay, G. Y., & Bartok, T. (1997). Early telarche symptoms in children and their relations to zearalenone contamination in foodstuffs. *Cereal Research Communications*, 429-436.

Tagele, S. B., Kim, R., & Shin, J. (2021). Interactions between Brassica Biofumigants and Soil Microbiota: Causes and Impacts. *Journal of Agricultural and Food Chemistry*, 69(39), 11538-11553.

Talgre, L., Lauringson, E., Roostalu, H., Astover, A., & Makke, A. (2012). Green manure as a nutrient source for succeeding crops. *Plant, Soil and Environment*, 58(6), 275-281.

Taylor, F. I. (2013) *Control of soil borne potato pathogens using Brassica spp. mediated biofumigation*. PhD thesis, Glasgow, University of Glasgow.

Teich, A. H., & Nelson, K. (1984). Survey of Fusarium head blight and possible effects of cultural practices in wheat fields in Lambton County in 1983. *Canadian Plant Disease Survey*, 64(1), 11-13.

Thangstad, O. P., Iversen, T., Slupphaug, G., & Bones, A. (1990). Immunocytochemical localization of myrosinase in Brassica napus L. *Planta*, 180(2), 245-248.

The World Bank, Cereal production (metric tons). Available at: https://data.worldbank.org/indicator/AG.PRD.CREL.MT [Accessed: 27 June 2019]

Thompson, M. E., & Raizada, M. N. (2018). Fungal pathogens of maize gaining free passage along the silk road. *Pathogens*, 7(4), 81.

Thompson, R. S., Aveling, T., & Prieto, R. B. (2013). A new semi-selective medium for *Fusarium graminearum, F. proliferatum, F. subglutinans* and *F. verticillioides* in maize seed. *South African Journal of Botany*, 84, 94-101.

Thrane, U. (1989) *Fusarium* species and their specific profiles of secondary metabolites. In: Chelkowski, J. (Ed.) Fusarium - *Mycotoxins, taxonomy and pathogenicity*. New York: Elsevier, pp. 199-225.

Thrane, U. (1996). Comparison of three selective media for detecting *Fusarium* species in foods: A collaborative study. *International Journal of Food Microbiology*, 29(2-3), 149-156.

Thrane, U., Filtenborg, O., Frisvad, J.C., & Lund, F. (1992) Improved methods for detection and identification of toxigenic *Fusarium* species. In: Samson, R.A., Hocking, A.D., Pitt, J.I. & King, A.D. (Eds.) *Modern Methods in Food Mycology.* Amsterdam: Elsevier, pp. 285–291.

Torriani, S. F., Melichar, J. P., Mills, C., Pain, N., Sierotzki, H., & Courbot, M. (2015). Zymoseptoria tritici: a major threat to wheat production, integrated approaches to control. *Fungal Genetics and Biology*, 79, 8-12.

Trail, F. (2009). For blighted waves of grain: *Fusarium graminearum* in the postgenomics era. *Plant Physiology*, 149(1), 103-110.

Tsao, R., Peterson, C. J., & Coats, J. R. (2002) Glucosinolate breakdown products as insect fumigants and their effect on carbon dioxide emission of insects. *BMC Ecology* 2, 5.

Tsao, R., Reuber, M., Johnson, L., & Coats, J. R. (1996). Insecticidal Toxicities of Glucosinolate· containing Extracts from Crambe Seeds. *Journal of Agricultural and Urban Entomology*, 13(2), 109.

Tschanz, A. T., Horst, R. K., & Nelson, P. E. (1976). The effect of environment on sexual reproduction of *Gibberella zeae. Mycologia*, 68(2), 327-340.

Tsror, L., Shlevin, E., & Peretz-Alon, I. (2005). Efficacy of metam sodium for controlling *Verticillium dahliae* prior to potato production in sandy soils. *American Journal of Potato Research*, 82(5), 419.

Utermark, J., & Karlovsky, P. (2007). Role of zearalenone lactonase in protection of Gliocladium roseum from fungitoxic effects of the mycotoxin zearalenone. *Applied and Environmental Microbiology*, 73(2), 637-642.

US EPA-OPP United States Environmental Protection Agency. Office of Pesticide Programs (2018). Chemicals evaluated for carcinogenic potential annual cancer report 2018, pp. 1-40.

Vandicke, J., De Visschere, K., Deconinck, S., Leenknecht, D., Vermeir, P., Audenaert, K., *et al*. (2020). Uncovering the biofumigant capacity of allyl isothiocyanate from several Brassicaceae crops against *Fusarium* pathogens in maize. *Journal of the Science of Food and Agriculture*, 100(15), 5476-5486.

Vanwyk, P. S., Scholtz, D. J., & Los, O. (1986). A selective medium for the isolation of *Fusarium* spp. from soil debris. *Phytophylactica*, 18(2), 67-70.

Vaughn, S. F., Palmquist, D. E., Duval, S. M., & Berhow, M. A. (2006). Herbicidal activity of glucosinolate-containing seedmeals. *Weed Science*, 54(4), 743-748.

Veeken, A., Blok, W. J., Curci, F., Coenen, G., Termorshuizen, A. J., & Hamelers, H. (2005). Improving quality of composted biowaste to enhance disease suppressiveness of compost-amended, peat-based potting mixes. *Soil Biology and Biochemistry*, 37(11), 2131-2140.

Velasco, P., Soengas, P., Vilar, M., Cartea, M. E., & del Rio, M. (2008). Comparison of glucosinolate profiles in leaf and seed tissues of different Brassica napus crops. *Journal of the American Society for Horticultural Science*, 133(4), 551-558.

Visconti, A., Haidukowski, E. M., Pascale, M., & Silvestri, M. (2004). Reduction of deoxynivalenol during durum wheat processing and spaghetti cooking. *Toxicology Letters,* 153(1), 181-189.

Vogelgsang, S., Beyer, M., Pasquali, M., Jenny, E., Musa, T., Bucheli, T. D., *et al*. (2019). An eight-year survey of wheat shows distinctive effects of cropping factors on different Fusarium species and associated mycotoxins. *European Journal of Agronomy*, 105, 62-77.

Voutsina, N., Payne, A. C., Hancock, R. D., Clarkson, G. J., Rothwell, S. D., Chapman, M. A., *et al*. (2016). Characterization of the watercress (Nasturtium officinale R. Br.; Brassicaceae) transcriptome using RNASeq and identification of candidate genes for important phytonutrient traits linked to human health. *BMC Genomics*, 17(1), 378.

Vujanovic, V., Hamel, C., Jabaji-Hare, S., & St-Arnaud, M. (2002). Development of a selective myclobutanil agar (MBA) medium for the isolation of *Fusarium* species from asparagus fields. *Canadian Journal of Microbiology*, 48(9), 841-847.

Waalwijk, C., van der Heide, R., de Vries, I., van der Lee, T., Schoen, C., Costrel-de Corainville, G. *et al*. (2004). Quantitative detection of *Fusarium* species in wheat using TaqMan. *European Journal of Plant Pathology*, 110(5-6), 481-494.

Wachowska, U., & Głowacka, K. (2014). Antagonistic interactions between *Aureobasidium pullulans* and *Fusarium culmorum*, a fungal pathogen of winter wheat. *Biocontrol*, 59(5), 635-645.

Walker, J. C., Morell, S., & Foster, H. H. (1937). Toxicity of mustard oils and related sulfur compounds to certain fungi. *American Journal of Botany*, 536-541.

Wang, D., Rosen, C., Kinkel, L., Cao, A., Tharayil, N., & Gerik, J. (2009). Production of methyl sulfide and dimethyl disulfide from soil-incorporated plant materials and implications for controlling soilborne pathogens. *Plant and Soil*, 324(1), 185-197.

Wang, J., Gu, H., Yu, H., Zhao, Z., Sheng, X., & Zhang, X. (2012). Genotypic variation of glucosinolates in broccoli (*Brassica oleracea* var. italica) florets from China. *Food Chemistry*, 133(3), 735-741.

Wang, J., Qiu, Y., Wang, X., Yue, Z., Yang, X., Chen, X., *et al*. (2017). Insights into the species-specific metabolic engineering of glucosinolates in radish (Raphanus sativus L.) based on comparative genomic analysis. *Scientific reports*, 7(1), 16040.

Wang, L., & Mazzola, M. (2019). Effect of soil physical conditions on emission of allyl isothiocyanate and subsequent microbial inhibition in response to Brassicaceae seed meal amendment. *Plant Disease*, 103(5), 846-852.

Wang, Q., Ma, Y., Wang, G., Gu, Z., Sun, D., An, X., *et al*. (2014). Integration of biofumigation with antagonistic microorganism can control Phytophthora blight of pepper plants by regulating soil bacterial community structure. *European Journal of Soil Biology*, 61, 58-67.

Wang, T., Li, Y., Bi, Y., Zhang, M., Zhang, T., Zheng, X., *et al*. (2020). Benzyl isothiocyanate fumigation inhibits growth, membrane integrity and mycotoxin production in *Alternaria alternata. RSC Advances*, 10(3), 1829-1837.

Warton, B., Matthiessen, J. N., & Shackleton, M. A. (2001). Glucosinolate content and isothiocyanate evolution− Two measures of the biofumigation potential of plants. *Journal of Agricultural and Food Chemistry*, 49(11), 5244-5250.

Warton, B., Matthiessen, J. N., & Shackleton, M. A. (2003). Cross-enhancement: enhanced biodegradation of isothiocyanates in soils previously treated with metham sodium. *Soil Biology and Biochemistry*, 35(8), 1123-1127.

Wathelet, J., Iori, R., Leoni, O., Quinsac, A., & Palmieri, S. (2004). Guidelines for glucosinolate analysis in green tissues used for biofumigation. *Agroindustria*, 3(3), 257-266.

Wei, F., Passey, T., & Xu, X. (2016). Effects of individual and combined use of bio-fumigation-derived products on the viability of *Verticillium dahliae* microsclerotia in soil. *Crop Protection*, 79, 170-176.

Weil, R. R., & Magdoff, F. (2004) Significance of soil organic matter to soil quality and health. In: Magdoff, F., & Weil, R. R. (Eds.) *Soil Organic Matter in Sustainable Agriculture*. CRC Press, p.10.

White, T. J., Bruns, T., Lee, S., & Taylor, J. W. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M. A., Gelfand, D. H., Sninsky, J. J., & White, T.J. (Eds.) *PCR Protocols: A Guide to Methods and Applications*. New York, USA: Academic Press, pp. 315–322.

Wilson, W., Dahl, B., & Nganje, W. (2018). Economic costs of Fusarium Head Blight, scab and deoxynivalenol. *World Mycotoxin Journal*, 11(2), 291-302.

Wittstock, U., & Burow, M. (2010). Glucosinolate breakdown in Arabidopsis: mechanism, regulation and biological significance. *The Arabidopsis book/American Society of Plant Biologists*, 8, e0134.

Wittstock, U., & Halkier, B. A. (2002). Glucosinolate research in the *Arabidopsis* era. *Trends in Plant Science*, 7(6), 263-270.

Wittstock, U., Meier, K., Dörr, F., & Ravindran, B. M. (2016). NSP-dependent simple nitrile formation dominates upon breakdown of major aliphatic glucosinolates in roots, seeds, and seedlings of Arabidopsis thaliana Columbia-0. *Frontiers in Plant Science*, 7, 1821.

Wood, C., Kenyon, D. M., & Cooper, J. M. (2017). Allyl isothiocyanate shows promise as a naturally produced suppressant of the potato cyst nematode, *Globodera pallida*, in biofumigation systems. *Nematology*, 19(4), 389-402.

Wu, F. (2004). Mycotoxin risk assessment for the purpose of setting international regulatory standards. *Environmental Science & Technology*, 38 (15), 4049-4055

Wu, F. (2007). Measuring the economic impacts of Fusarium toxins in animal feeds. *Animal Feed Science and Technology*, 137(3-4), 363-374.

Xiao, D., Powolny, A. A., & Singh, S. V. (2008). Benzyl isothiocyanate targets mitochondrial respiratory chain to trigger reactive oxygen species-dependent apoptosis in human breast cancer cells. *Journal of Biological Chemistry*, 283(44), 30151-30163.

Xue, A. G., Chen, Y., Voldeng, H. D., Fedak, G., Savard, M. E., Längle, T., *et al*. (2014). Concentration and cultivar effects on efficacy of CLO-1 biofungicide in controlling Fusarium head blight of wheat. *Biological Control*, 73, 2-7.

Yang, J., Zhang, Y., Wang, Y., &Cui, S. (2007). Toxic effects of zearalenone and α-zearalenol on the regulation of steroidogenesis and testosterone production in mouse Leydig cells. *Toxicology in vitro,* 21(4), 558-565.

Yaws, C. L. & Satyro, M. A. (2015) Vapor Pressure - Organic Compounds. In: Yaws, C. L. (Ed.) *The Yaws Handbook of Vapor Pressure*. Elsevier, Gulf Professional Publishing, pp. 1-314.

Yazar, S., & Omurtag, G. Z. (2008). Fumonisins, trichothecenes and zearalenone in cereals. *International Journal of Molecular Sciences,* 9(11), 2062-2090.

Ye, L., & Zhang, Y. (2001). Total intracellular accumulation levels of dietary isothiocyanates determine their activity in elevation of cellular glutathione and induction of Phase 2 detoxification enzymes. *Carcinogenesis*, 22(12), 1987-1992.

Yerkovich, N., Cantoro, R., Palazzini, J. M., Torres, A., & Chulze, S. N. (2020). Fusarium head blight in Argentina: Pathogen aggressiveness, triazole tolerance and biocontrol-cultivar combined strategy to reduce disease and deoxynivalenol in wheat. *Crop Protection*, 137, 105300.

Yi, G., Lim, S., Chae, W. B., Park, J. E., Park, H. R., Lee, E. J., *et al*. (2015). Root Glucosinolate profiles for screening of radish (Raphanus sativus L.) genetic resources. *Journal of Agricultural and Food Chemistry*, 64(1), 61-70.

Yim, B., Hanschen, F. S., Wrede, A., Nitt, H., Schreiner, M., Smalla, K., *et al*. (2016). Effects of biofumigation using Brassica juncea and Raphanus sativus in comparison to disinfection using Basamid on apple plant growth and soil microbial communities at three field sites with replant disease. *Plant and Soil*, 406(1), 389-408.

Yuen, G. Y., & Schoneweis, S. D. (2007). Strategies for managing Fusarium head blight and deoxynivalenol accumulation in wheat. *International Journal of Food Microbiology*, 119(1-2), 126-130.

Zadoks, J. C., Chang, T. T., & Konzak, C. F. (1974). A decimal code for the growth stages of cereals. *Weed Research*, 14(6), 415-421.

Zatecka, E., Ded, L., Elzeinova, F., Kubatova, A., Dorosh, A., Margaryan, H., Dostalova, P., Korenkova, V., Hoskova, K., & Peknicova, J. (2014). Effect of zearalenone on reproductive parameters and expression of selected testicular genes in mice. *Reproductive Toxicology,* 45, 20-30

Zhang, M., Li, Y., Bi, Y., Wang, T., Dong, Y., Yang, Q., *et al*. (2020). 2-Phenylethyl isothiocyanate exerts antifungal activity against *Alternaria alternata* by affecting membrane integrity and mycotoxin production. *Toxins*, 12(2), 124.

Zhang, Y., & Talalay, P. (1998). Mechanism of differential potencies of isothiocyanates as inducers of anticarcinogenic Phase 2 enzymes. *Cancer research*, 58(20), 4632-4639.

Zhao, F., Li, R., Xiao, S., Diao, H., Viveiros, M. M., Song, X. *et al*. (2013). Postweaning exposure to dietary zearalenone, a mycotoxin, promotes premature onset of puberty and disrupts early pregnancy events in female mice. *Toxicological Sciences*, 132(2), 431-442.

Zhao, P., Quan, C., Wang, Y., Wang, J., & Fan, S. (2014). *Bacillus amyloliquefaciens* Q-426 as a potential biocontrol agent against *Fusarium oxysporum* f. sp. *spinaciae. Journal of Basic Microbiology*, 54(5), 448-456.

Zhao, Y., Zhu, X., Wu, H., Zhuang, D., Yu, G., Li, X., Li, F., & Yu, A. (2012). Evaluation of fetal skeletal malformations in deoxynivalenol-treated mice using microarray analysis. *Archives of Environmental Contamination and Toxicology,* 63 (3), 445-452.

Zhen-xin, G. U., Qiang-hui G. U. O., & Ying-juan G. U. (2012). Factors influencing glucoraphanin and sulforaphane formation in brassica plants: A review. *Journal of Integrative Agriculture,* 11(11), 1804-1816.

Zhu, B., Yang, J., & Zhu, Z. (2013). Variation in glucosinolates in pak choi cultivars and various organs at different stages of vegetative growth during the harvest period. *Journal of Zhejiang University SCIENCE B*, 14(4), 309-317.

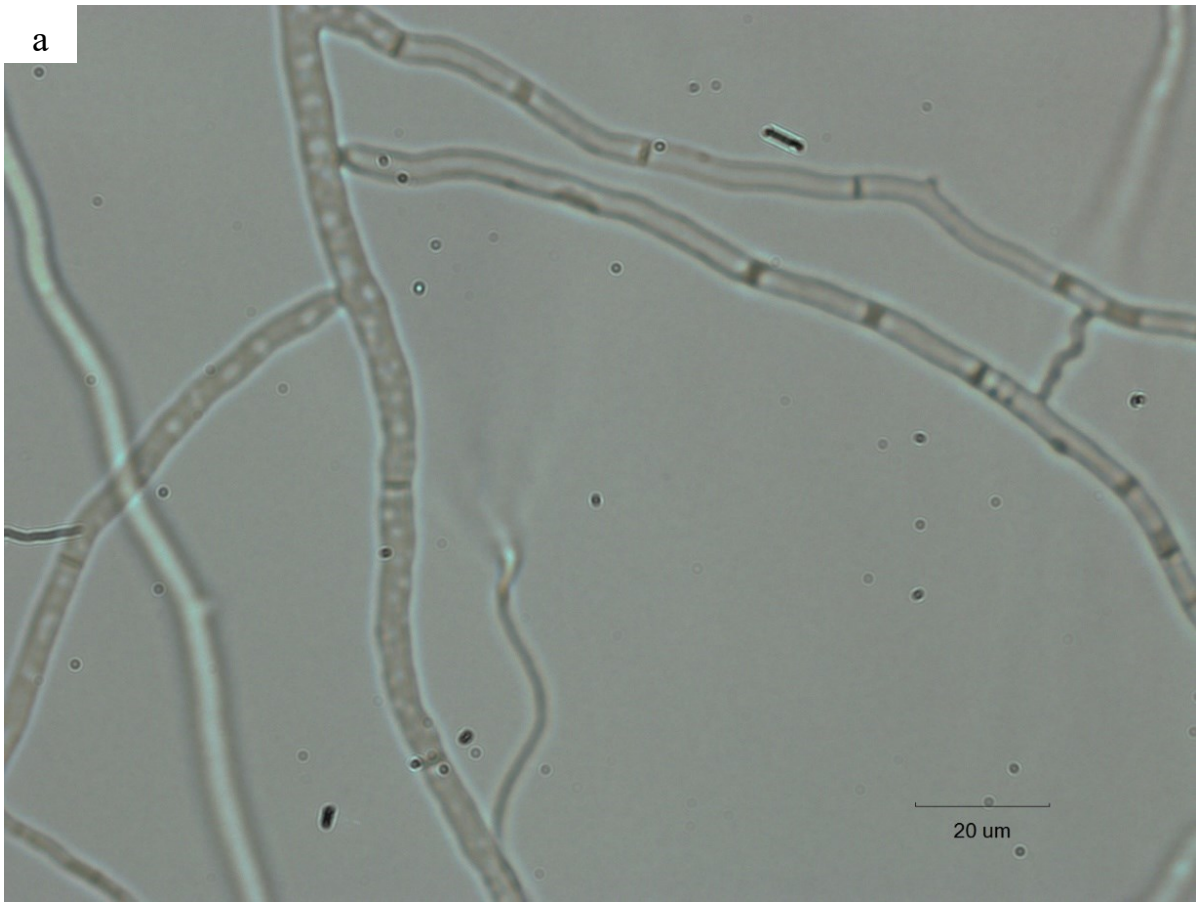
Zhu, Z., Hao, Y., Mergoum, M., Bai, G., Humphreys, G., Cloutier, S., *et al*. (2019). Breeding wheat for resistance to Fusarium head blight in the Global North: China, USA, and Canada. *The Crop Journal*, 7(6), 730-738.

Zinedine, A., Soriano, J. M., Molto, J. C., & Manes, J. (2007.) Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: An oestrogenic mycotoxin. *Food and Chemical Toxicology,* 45(1), 1-18.

Appendix

8 Appendix

a



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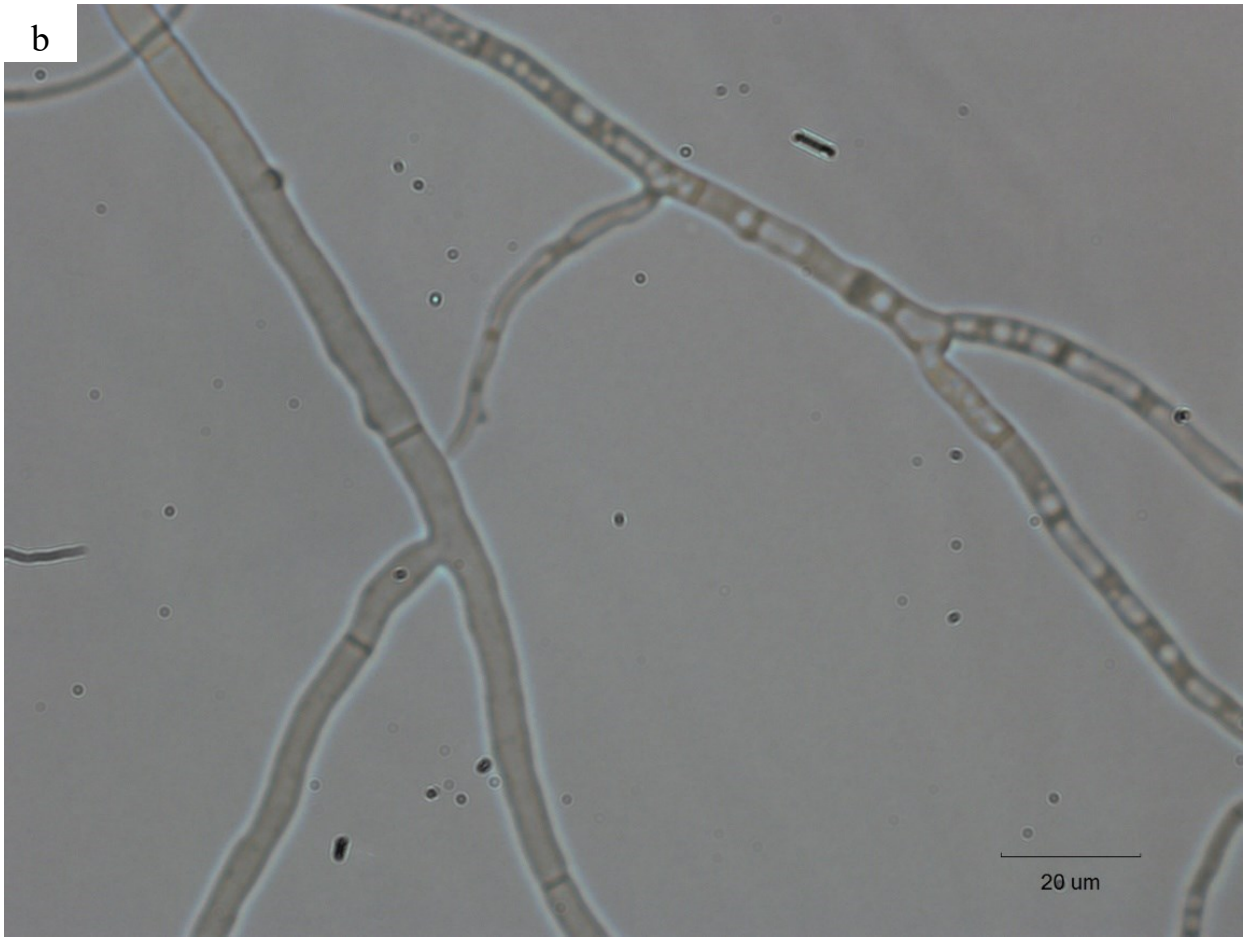

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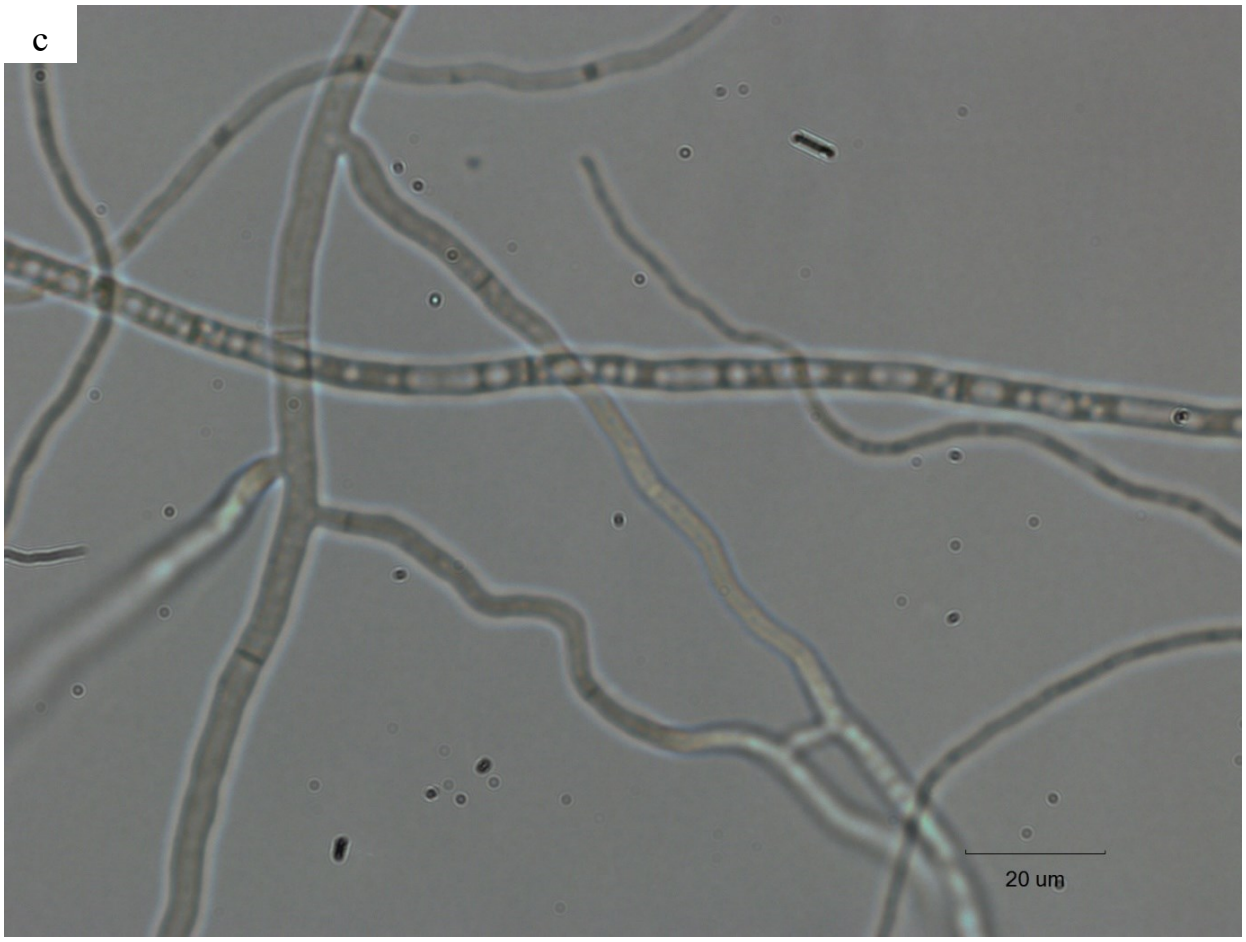

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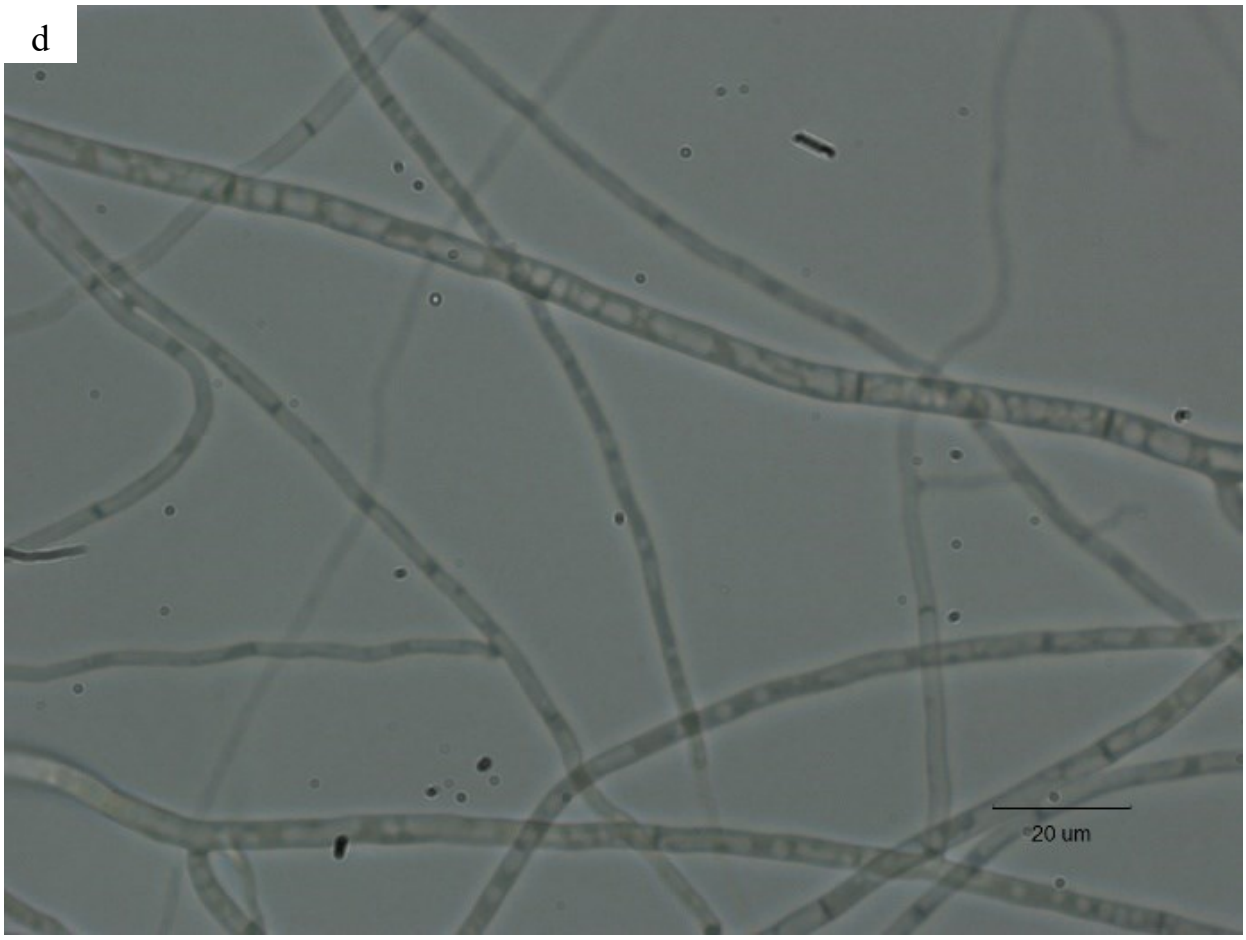

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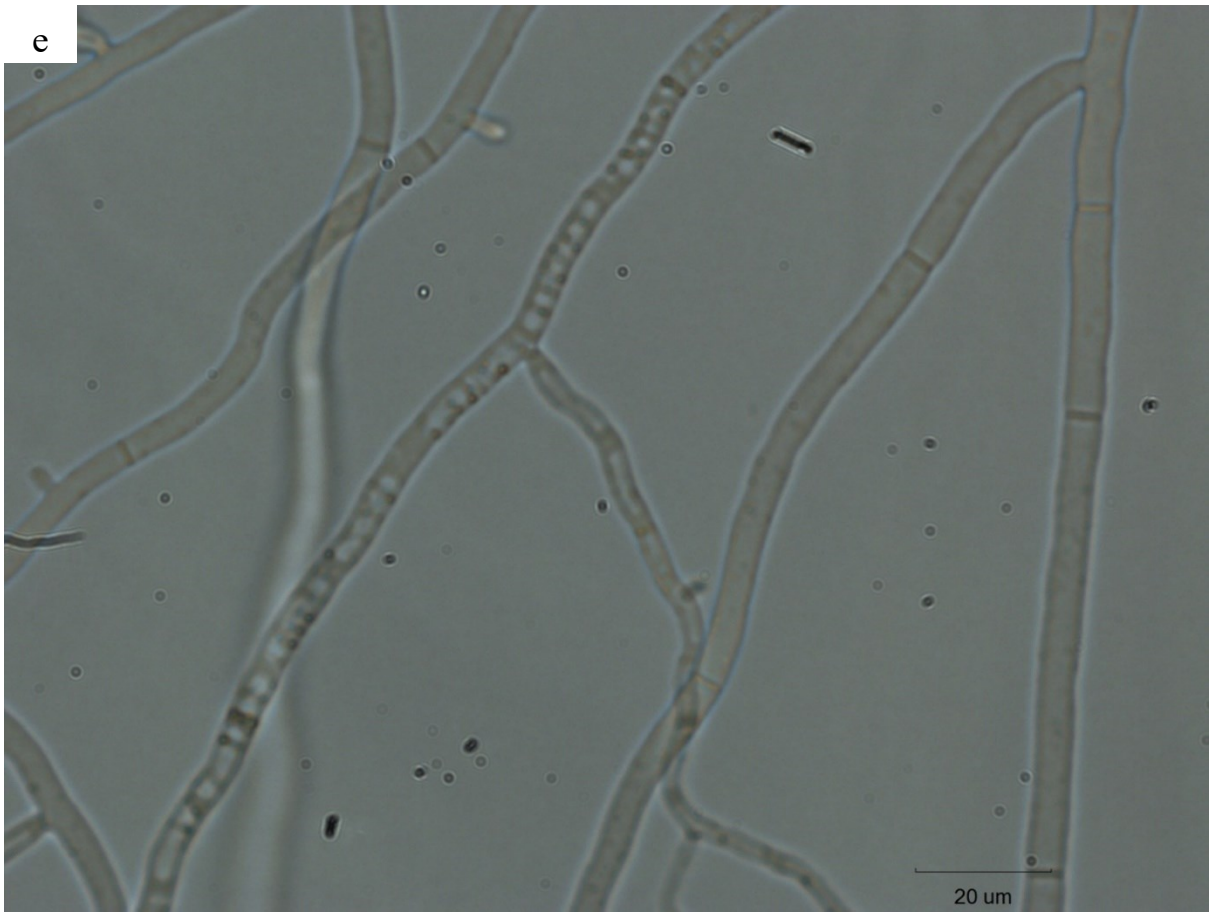

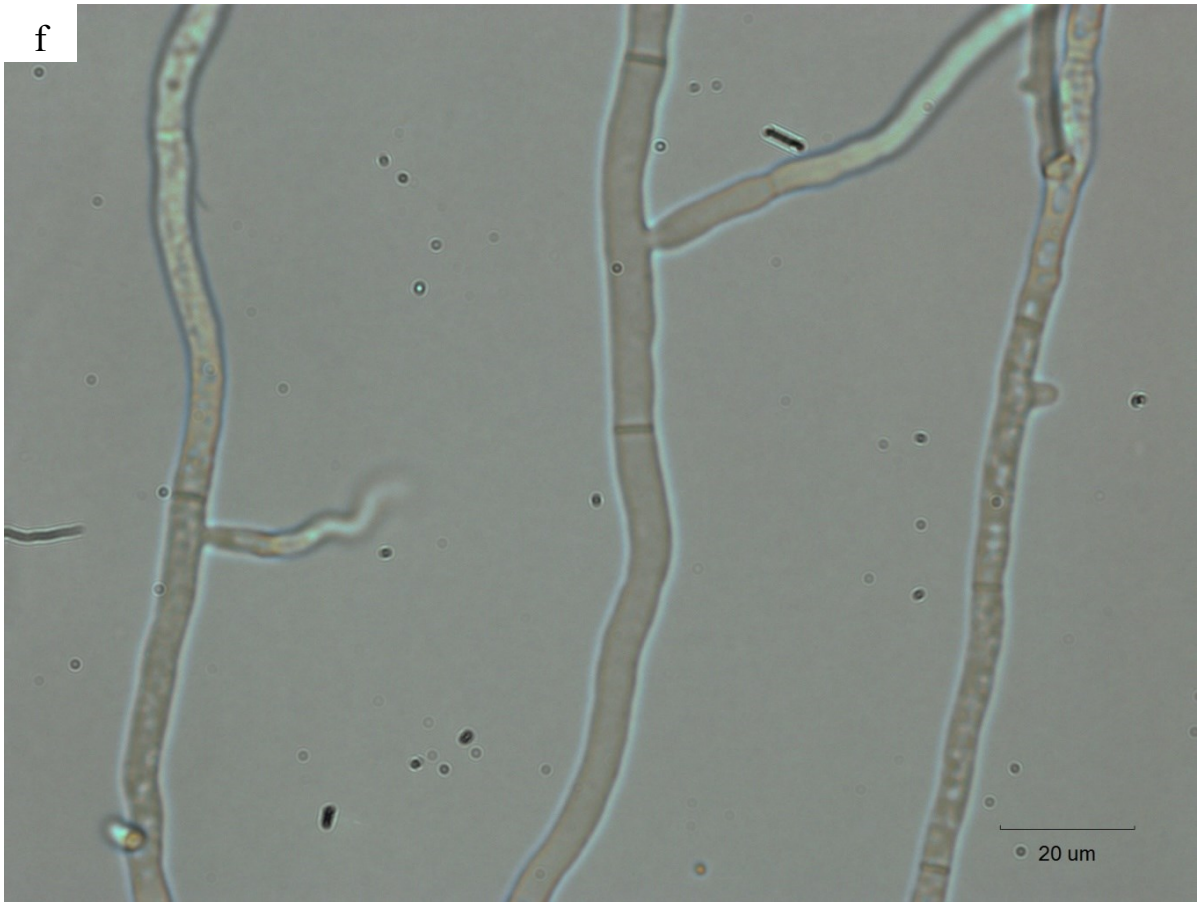

Appendix Figure 1. *Fusarium graminerum* mycelia (a) untreated, and exposed to (b) 69 mg l^{-1} allyl isothiocyanate, (c) 11 mg l^{-1} 2-phenylethyl isothiocyanate, (d) 2 mg l^{-1} benzyl isothiocyanate, (e) 35 mg l^{-1} methyl isothiocyanate and (f) 977 mg l^{-1} ethyl isothiocyanate after 9 days at 15°C (x400 magnification)