Harper Adams University

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Study of *Fusarium langsethiae* infection in UK cereals

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Declaration

This work was composed by the author and is a record of work carried out by him in an original line of research. All other sources of information are shown in the text and written in the references section. None of this work has been presented in any previous application for any degree or qualification.

(Nelson Opoku)

Abstract

Fusarium langsethiae is a relatively newly identified *Fusarium* species. It is responsible for the high levels of the Fusarium mycotoxins HT-2 and T-2 in oats in the UK and other parts of Europe. A field survey was performed to study the infection and development of *F. langsethiae* in the growing season of cereals under commercial production (2009 – 2011). The data showed oats to contain the highest levels of both *F. langsethiae* biomass and HT-2+T-2 mycotoxins in harvested heads of the cereals studied. Head infection if it occurs, was at emergence but before flowering, a deviation from other *Fusarium* species. Seemingly symptomless heads had high levels of *F. langsethiae* DNA and HT-2+T-2, confirming previous suggestions that *F. langsethiae* is a symptomless pathogen of oats. Four field experiments where winter and spring varieties of wheat, barley and oats were cultivated under identical field and agronomic conditions at two sites again showed oats to have the highest *F. langsethiae* DNA and HT-2+T-2 concentration among the cereals studied. Interestingly, there was a significantly higher quantity of HT-2+T-2 per unit of *F. langsethiae* DNA for oats compared to wheat and barley.

An *in-vitro* detached leaf assay was used to screen UK varieties from the HGCA Recommended Lists in 2010 of wheat, barley and oats for resistance against *F. langsethiae* infection. Results from the experiment showed that none of the cereal varieties screened had total resistance to *F. langsethiae* infection, however, in oats, varieties with low HT-2+T-2 in heads under field conditions also had shorter lesion lengths *in-vitro* suggesting that the detached *in-vitro* leaf assay could be a good predictor of HT-2+T-2 concentration in harvested grain.

Data from four different artificial inoculation methods (seed assay, stem base infection, boot-inoculation and a spray inoculation) established that although *F. langsethiae* is a seed borne pathogen it was not systemically transmitted from the seed to the other plant parts. The stem base infection study showed that *F. langsethiae* did not cause any stem base infection even when in close contact with the stem. The spray inoculation resulted in cereal heads having *F. langsethiae* DNA concentrations and subsequent HT-2+T-2 levels comparable to what has been observed under natural infections in commercial fields, suggesting that the infection route for *F. langsethiae* may not be that different from the other Fusarium head blight pathogens.

Based on all the experiments carried out in this thesis, a generalized life-cycle was hypothesized for *F. langsethiae* which deviates from that of the other *Fusarium* species on small grain cereals due to its early head infection and its inability to cause stem base infection.

Outline of thesis

Chapter one presents a brief introduction to the origin of the cereals studied. It also presents a review of the relevant literature on wheat, barley, oat and triticale. Literature on type A trichothecenes as well as the *Fusarium* species implicated in their production is also reviewed. The second chapter outlines the general methodologies used in experiments detailed in the thesis. Where necessary, additions to the methodologies are presented in the appropriate chapters.

Chapter three presents results of a three year field survey of wheat, barley, oats and triticale under commercial cultivation within the counties of Shropshire and Staffordshire in the UK. It presents novel information on the development of *F. langsethiae* in these cereals over the cropping seasons. Chapter four presents results of four field experiments carried out to confirm or otherwise, one of the important outcomes of the field survey.

In chapter five, experiments for two *in-vitro* detached leaf assays are presented. The first experiment examined the aggressiveness within a collection of *F. langsethiae* isolates towards oats. In the second experiment, the most aggressive *F. langsethiae* isolate identified from the first experiment was used to screen UK varieties under testing for the HGCA Recommended list in 2010 of wheat, barley and oats.

Chapter six presents results for four different artificial inoculation methods which were used to study the relationship between *F. langsethiae* and wheat, barley and oats. Chapter seven, the final chapter gives an inclusive discussion of the findings from all experiments detailed in the thesis, conclusions and recommendations for future work.

Statement of advanced studies

During the period of this study, the author completed the following in addition to performing and reporting the experiments detailed in this thesis;

- Obtained a Postgraduate Certificate in Research offered by Harper Adams University College
- Published the following paper in Plant Breeding and Seed Science;

  Opoku, N., Back, M. & Edwards, S.G. 2011. Aggressiveness of *Fusarium langsethiae* isolates towards wheat, barley and oats in an *in-vitro* leaf assay. Plant Breeding and Seed Science, 64, pp. 55-63
- Submitted the following manuscript in May 2012 to European Journal of Plant Pathology;

  Opoku N., Back M. and Edwards S.G. Development of *Fusarium langsethiae* in commercial cereal production
- Gave oral presentations at the following conference and symposia
  1. HGCA-funded Ph.D. Symposium. Harper Adams University College: 7 April 2011
  2. The 33rd Mycotoxin-Workshop, Freising, Germany. 30th May – 1st June 2011
  3. HGCA-funded Ph.D. Symposium. Rothamsted Research Harpenden, Hertfordshire
- Presented posters at the following conferences and symposium
  1. Cropworld Global, Excel, London, 31st October – 2nd November 2011
  2. HGCA-funded Ph.D. Symposium, University of Nottingham. 23rd April 2010
  3. Fusarium seminar; Fusarium – mycotoxins, taxonomy, pathogenicity and host resistance. Radzikow, Poland 20th – 23rd September 2010

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A big thank you to all my Ph.D. colleagues especially Dr. Wiseborn Danquah, for their suggestions, friendly help and extensive discussions around my work which have been very helpful to this study.

Finally, to my family especially my dear wife Gifty Opoku and my son Kwaku Nyarko Opoku I say thank you. Your love has been a great pillar of support.

Dedication

To my dear wife Gifty, A. Opoku.

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CHAPTER ONE

1. Literature review

1.1. Origin and domestication of wheat, barley and oats

Evolution of modern day agriculture started with the cultivation and domestication of cereals (Salamini et al., 2002). The window of time that cereals were domesticated is not very certain, but it is believed that cultivation started in the Fertile Crescent in Near East between 10500 and 9250 years ago (Lev-Yadun, 2000; Tanno and Willcox, 2006). The shift from hunting and gathering to cultivating plants is believed to have started with the creation of a set of domesticated cereal varieties some of which are still cultivated today (Salamini et al*.,* 2002). Wheat, (*Triticum*) species were among the first plant species to be cultivated. Einkorn (*Triticum monococcum*), a diploid species, was the first to be cultivated but its cultivation declined in the Bronze Age and is rarely cultivated in modern agriculture (Nesbitt and Samuel, 1995). It has a wild progenitor, *T. boeoticum,* which it can interbreed, with to generate fertile progenies. The domestication of emmer wheat (*T. dicoccum),* a tetraploid and the most important crop in the Fertile Crescent until the early part of the Bronze Age, from its wild progenitor *T. dicoccoides* was the next step in the evolution of modern day wheat (Bar-Yosef, 1998). Emmer is believed to be the wheat that was in use in ancient Egypt and its cultivation continues in Ethiopia today (Salamini et al., 2002). Tetraploid hard wheat (*T. durum*) and hexaploid bread wheat (*T. vulgare*) marked the end of *Triticum* domestication. Bread wheat (*T. aestivum*) is the most cultivated wheat. It has no wild progenitor in nature since it is a hybrid between species with tetraploid genomes and a wild diploid species (*Aegilops tauschii*) (McFadden and Sears, 1946).

The domestication of barley (*Hordeum vulgare*) dates back to about 8000 B.C. (Badr et al., 2000). Wild *H. spontaneum*, the progenitor of domesticated barley colonizes fertile habitats in Israel, Jordan, Southern Turkey, Iraq, Kurdistan and Jordan. In Iran and central Asia, it is a weed that colonizes secondary habitats (Bekele, 1983). Domesticated barley is a selfing diploid that still bares close resemblance to its progenitor. The domesticated barley is, however, differentiated from its wild relative by acquisition of non-brittle rachis, increased seed weight and the presence of some naked seed varieties (Salamini et al., 2002). According to Badr et al. (2000), domestication of barley took place about 10000 years ago in the Western Fertile Crescent, before moving eastwards and diversifying in the Himalayas.

Cultivated common oat (*Avena sativa*), has close genetic affinity and morphological resemblance to two wild and weedy oats; *A. fatua* and *A. sterilis*. Cultivation and/or domestication of oats unlike wheat and barley occurred rather late, around 1000 – 2000 BC in Bronze Age Europe (Zhou et al., 1999). It has been argued that oats were not initially cultivated as a crop but rather as a weed that infested wheat and barley fields and was later collected and planted intentionally (Zohary and Hopf, 2000).

1.2. Wheat

Wheat is the most widely grown cereal in the world covering about a sixth of the total arable land (Slafer and Satorre, 1999). Based on biological classification, three species are commonly grown; *Triticum aestivum*, *T. compactum* and *T. durum* (Atwell, 2001).

Modern wheat types are described using three general terms. The first term describes kernel texture; hard and soft wheat. Hard wheat is known to have higher

protein content than soft wheat and requires more energy to mill (Vaclavik and Christian, 2008). Hard spring wheat contains about 12-18% protein as compared with soft wheat which contains 8-11%. Flour from hard wheat is more desirable for bread making whilst flour from soft wheat is used in making cakes and pastries (Vaclavik and Christian, 2008). The second set of terms describes the pigmentation in the outer layers of the kernel; red and white indicating the presence or absence of red pigmentation. The next set of terms describes the period of cultivation; winter and spring wheat. Winter wheat is planted in autumn and harvested in summer. Winter wheat varieties require a period of low temperatures for heads to form (vernalization). Spring wheat is planted in spring and harvested in summer. Spring wheat varieties do not require vernalization before head formation (Atwell, 2001).

1.2.1. Wheat; optimal conditions and areas of production

Wheat is grown over a wide geographic area. It is grown in almost all temperate countries and some sub-tropical countries in the world. However, production is mostly in the temperate countries (Leonard and Martin, 1963). It is well adapted to areas between latitude 30^{o} and 50^{o} North and 25^{o} and 40^{o} South (Leonard and Martin, 1963). It grows well on well drained soils from sea level to over 3000 m above sea level. Wheat has an optimum growth temperature of about 25^{o}C, but can withstand temperature of about 3 to 4^{o}C and a maximum of 30 to 32^{o}C. For optimum yield, wheat requires an annual rainfall of 25 to 100 cm. High rainfall accompanied with high temperature does not favour wheat production. This is because such conditions encourage undesirable seed sprouting, create a favourable environment for fungal pathogens and insect attack, and reduce protein content and bread making quality (Stoskopf, 1985).

The growing area and production of wheat worldwide has increased steadily since the 1900s (Slater and Satore, 1999). Recent FAO reports indicate that global

wheat production for 2011 stands at 676 million tonnes, a growth of 3.4% from 2010 (FAOSTAT, 2010). China (Non-EU 27) is the world's largest producer of wheat (Table 1.1).

In the EU, wheat production for 2011 is estimated at 95 million tonnes, 1% below that of 2010 (COCERAL, 2012). Available data from COCERAL (2012) showed France as the leading producer of wheat for 2009, 2010 and 2011 production seasons followed by Germany and UK respectively (Figure 1.1).

Table 1.1. World leading producers of wheat in 2011

Country	Production (Million tonnes)
EU 27	131
China	120
India	91
USA	61
Russia	56
Canada	27
Australia	26
Pakistan	23
Turkey	17
Kazakhstan	15
Iran	14

Source: USDA, 2011.

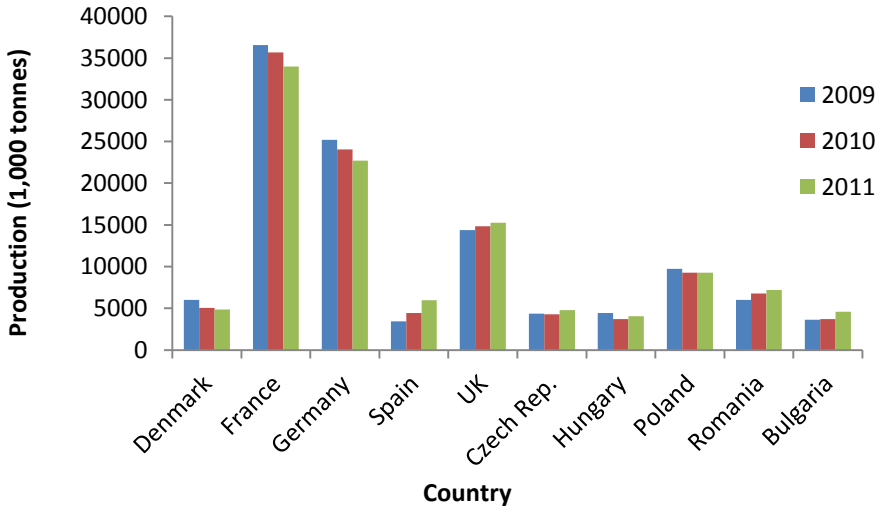

Figure 1.1.Top ten producers of wheat (soft) in EU (27) in 2009, 2010 and 2011. Source: COCERAL (2012)

In 2011, UK wheat production totalled 15.2 million tonnes, an increase of 5.3% above that of 2010

1.2.2. Wheat; nutritional value and uses

It is estimated that wheat supplies about 20% of the food calories of the world's growing population (Gibson and Benson, 2002). Typically a wheat grain contains about 70% carbohydrates, 12% protein, 12% water, 2% crude fibre, 2% vitamins and minerals and 2% lipids (Stone and Savin, 1999). Wheat, apart from its ability to do well in a diverse range of environments has become a popular cereal crop mainly because of its gluten, a protein with unique physical and chemical properties. Gluten is known to exhibit three main physical properties; plasticity, strength and elasticity. This means that gluten is capable of deformation when under pressure but can also resist this deformation, and has the ability to regain its original form when this pressure is released. The interaction of these properties allow wheat flour to form dough that can expand and yet resist stretching to bursting point making it an excellent choice for making bread (Stone and Savin, 1999).

Generally, wheat proteins are put into four classes based on their sequential extraction in different solutions: albumins (water soluble), globulins (water insoluble but soluble in dilute salt solution), gliadins (insoluble in both water and salt solution but soluble in alcohol) and glutenins (soluble in dilute acids and alkalis) (Stone and Savin, 1999). Albumin and globulin are known as the metabolic proteins and are essential for the growth and development of the wheat seedling and contribute very little to dough strength and bread making quality (Stone and Savin, 1999). Gliadin and glutenin are known as the storage or gluten proteins. Primarily, they serve as a store of energy and nutrients for the germination and growth of the seedling and contribute more to the physical properties of dough, thus bread making qualities (Gupta et al., 1995). Gliadins are the smaller of the two, non-aggregating and do not contribute much to gluten strength but are largely responsible for extensibility (ability to stretch without breaking) (Gupta et al., 1993). Glutenins are aggregating proteins and have the ability to form macromolecules which are central to the formation of cohesive gluten network required for dough strength which is needed for bread making.

Wheat is used mainly as human food although it is an important source of animal feed. Its ability to be processed into various types of food combined with its nutritional value has made it a household energy source. It is estimated that wheat provides about 20% of the world total food calories and in the USA, per capita consumption of wheat exceeds any other single food staple (Gibson and Benson, 2002).

The value of wheat as a food crop cannot be overemphasized. Wheat flour is used intensively in the milling industry and other food products such as macaroni, spaghetti and breakfast foods amongst others (Gibson and Benson, 2002). However, wheat has other industrial uses. Wheat is used as a raw material for the

manufacture of starch. Wheat starch is used as a food thickener and in the textile industry as a size (a kind of glue that protects the warp yarns against abrasion during the weaving operation) (Cornell and Hoveling, 1998). Wheat derived products are used as substitutes for milk and meat, although in small quantities, wheat flour and gluten for example are used in making sausage and meat loaf. Wheat is also used as a substitute for rice in the form of 'bulgur' in Asia (Cornell and Hoveling, 1998).

1.3. Barley

Barley ranks second to wheat as the most important cereal crop cultivated in temperate countries and the fourth most important in the world (Briggs, 1978). It is an important crop in Asia, Argentina, Australia, Europe and North Africa where it serves as human food and feed for livestock (Leonard and Martin, 1963).

There are three main cultivated species; *Hordeum vulgare*, a six-row barley rachis, *H. distichum*, a two rowed and *H. irregulare,* known as irregular barley (Briggs, 1978). All three species have a tough rachis. A number of terms have been used to describe different barley types: Barley end uses puts barley into two main classes, feed and malting types. Feed barley is grown for animal feed whilst malting barley is grown for the brewing industry. Using lemma and palea characteristics, barley is classified into hulled and hulless types. Hulled barley has the lemma and palea tightly attached to the seed at maturity and remains in place during harvest. With hulless types, the lemma and palea are loosely attached and the seed threshes free from the lemma and palea during harvesting (Briggs, 1978). Hulled varieties are the most common types grown in most parts of the world. Hulless varieties are important in the Andes and Himalayan regions as well as in Ethiopia. Based on seed colour, barley is also classified into colourless, white and yellow types (Kling,

2004). When barley is processed by removing the hull and polished, it is termed as pearl barley and is used for food.

1.3.1. Barley; optimal conditions and areas of production

Cultivation of barley occurs across almost all the temperate zones, sub-tropical areas and high altitude sections of the tropics. It is cultivated from the equator in Ethiopia to the Arctic circle in Norway (Leonard and Martin, 1963). Barley can be grown on virtually all arable soils; however, it is usually grown on coarse textured soils with a pH range of 6.0 to 7.5 (Briggs, 1978). Barley is more drought tolerant than wheat and oats. Growth is favoured by moderate rather than high rainfall (Leonard and Martin, 1963). For optimum production, barley requires an annual rainfall of 500-1000 mm and a temperature of about 15–30^{o}C (Leonard and Martin, 1963)

Barley is grown in over 100 countries with Russia (Non-EU 27) the current world leading producer (Table 1.2). Global barley production rose from 124 million tonnes in 2010 to 135 million tonnes in 2011.

Table 1.2. World leading producers of barley in 2011

Country	Production (Million metric tonnes)
EU 27	53.6
Russia	16.5
Canada	9.0
Australia	8.0
Ukraine	7.5
Turkey	6.2
Argentina	5.4
USA	4.3
Iran	3.4
China	2.6
Belarus	2.0

Source: USDA 2011.

France, Germany and Spain are the leading producers in the EU (27) (Figure 1.3). Total winter barley production in the EU (27) was 51.0, 43.2 and 41.7 million tonnes in 2009, 2010 and 2011 respectively. Spring barley production was 24.5 and 20.1 and 22.2 million tonnes for the same periods.

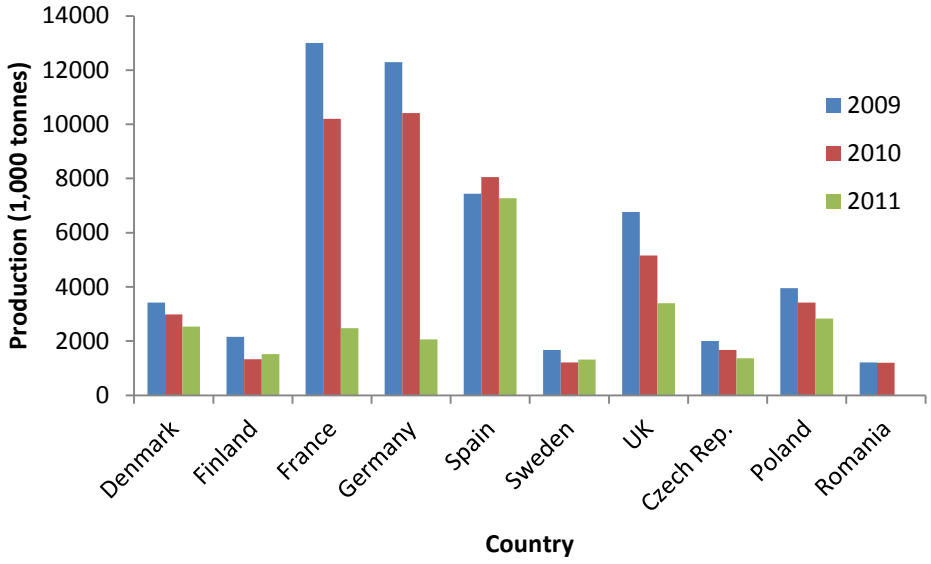

Figure 1.2. Top ten producers of winter barley in the EU (27) in 2009, 2010 and 2011. Source: COCERAL (2012)

1.3.2. Barley; nutritional values and uses

Barley is an important source of energy, especially for animals and a rich source of minerals. It has been estimated that 100 g boiled pearl barley provides (% recommended daily amount) 6% vitamin B6, 12% copper, 10% zinc and 15% phosphorus (HGCA, 2009).

Barley is used as animal feed, malt and human food. About 85% of the world's total barley production is used as animal feed (Fischbeck, 2002). For example, it is used to feed finishing beef cattle in the USA and also pigs and poultry in regions where maize cannot be economically produced (Mahdi et al., 2008). In ancient Rome,

barley was the main source of energy in human diets and gladiators were called ‘hordearii’, meaning barley men (Grando, 2005)

In the highlands of Central Asia, the North Horn of Africa, the Andean countries and the Baltic States, barley is still a major staple food (Grando, 2005). Morocco is one of the world’s largest consumers of barley as food. About 20% of barley grain in Morocco is used as food. Its use as food is more common in the rural areas where annual consumption is estimated at 54 kg per person (Amri et al., 2005). In the highlands of Ethiopia, barley accounts for about 60% total food consumed (Amri et al., 2005). In the Andes of Colombia, Peru and Bolivia, barley is the main staple food for farmers living at high altitudes.

This is mainly because it is the main crop best suited for the prevailing harsh environmental and soil conditions (Capettini, 2005). In the developed world less than 5% of total barley production is used as food (Jadhav et al., 1998). In recent years, however, the interest in the food uses of barley has been renewed as a result of the health benefits of barley. Barley contains β-glucan (a soluble fibre) and tocols (tocophenols and tocotrienols) which have been reported to lower cholesterol levels and postprandial blood glucose (Brennan and Cleary, 2005; Wang et al., 1993).

In developed countries, the main food product of barley is malt. Malt is predominantly used in the brewing industry. However, it is used in small quantities as additives to give flavour and colour to breakfast cereals and baked goods (Mahdi et al., 2008). In the baking industry, barley is used for making fermented bakery product. Soluble sugars, proteins and amylases present in malt extracts promotes yeast activity resulting in good bread texture, bigger loaf volume, good flavour and colour to finished baked products. Malt products are also used in non-

fermented baking products such as crackers, cookies and muffins (Mahdi et al., 2008).

Barley malt is an important constituent of both alcoholic and non-alcoholic beverages. Malt is used widely in the brewery industry for the making of beer and distillation of whisky. Two barley types are used in making malt; *H. distichun* (two-rowed barley) and *H. vulgare* (six-rowed barley) (Pandey, 2004). According to Goldammer (2008), American brewers prefer *H. vulgare* to *H. distichun* because of its higher enzyme content. *Hordeum vulgare* contains high levels of diastatic enzymes (amylase) which increases conversion of adjunct starches during mashing. It also has a higher protein content and thicker husk (Goldammer, 2008). The high protein content, however, results in high amount of protein-polyphenol complexes during beer making increasing the haziness in the finished beer. The husk is rich in polyphenols which not only contribute to haze, but also create astringent taste in the product (Goldammer, 2008). *Hordeum distichun* is the preferred choice of UK brewers due to its better starch/husk ratio and better malty flavour. It has lower enzyme content and fewer proteins (Goldammer, 2008).

1.4. Oats

Oat is the sixth most important cultivated cereal worldwide, contributing about 2.6% of the total world grain production. Generally oats are grown when the land is considered to be less suitable for wheat and barley production (Hoffman, 1995; HGCA, 1989). Oats belongs to the genus *Avena* which has many wild and few cultivated species (Murphy and Hoffman, 1992). There are two main cultivated species *Avena sativa* (white oat) and *A. byzantine* (red oat). These two species are cultivated for both food and feed. *Avena sativa* is more important than *A. byzantine* as it is more commonly grown in many countries (Leggette, 1992). It is estimated that about 75% of all oats produced worldwide is used as animal feed (Hoffman,

1995), out of which a significant portion is used as high-energy feed for race horses (Welch, 1995). Oats are primarily used as a forage crop for cattle in southern hemisphere countries such as Argentina and Uruguay and as feed grain in Australia (Schrickel, 1986).

1.4.1. Oats; optimal conditions and areas of production

Oat cultivation is concentrated between latitudes 35^{o}-50^{o} North and 20^{o}-40^{o} South (Hoffman, 1995). It thrives well in cool moist climate and is very sensitive to hot dry weather throughout its growth cycle and has the ability to tolerate considerable cold during the seedling and tillering stages (Hoffman, 1995). Apart from rice, oats require more moisture than any other cultivated cereal per unit of dry matter produced (Forsberg and Reeves, 1995). Oats are adapted to many different soils and once temperature and moisture conditions are favourable any reasonable well drained arable land is suitable for its cultivation (Alan and Adams, 1979). However, growth and production are favoured by well drained soils with a pH between 5.3 and 5.7, although it can tolerate pH as low as 4.5 (Forsbeg and Reeves, 1995).

Russia (Non-EU 27) is currently the world leading producer of oats followed by Canada and the USA respectively (Table 1.3).

Table 1.3. World leading producers of oats in 2011

Country	Production (million tonnes)
EU-27	7.8
Russia	5.0
Canada	3.2
Australia	1.6
U.S.A.	1.1
Belarus	0.6
China	0.6
Chile	0.6
Ukraine	0.5
Argentina	0.4
Brazil	0.3

Source**:** USDA, 2011

In Europe (EU 27), total oat production in 2009, 2010 and 2011 were 6.0, 5.1 and 5.4 million tonnes respectively with Poland being the leading producer followed by Finland and Spain respectively (Figure.1.4). Oat production in the UK decreased from 694 thousand tonnes in 2010 to 625 thousand tonnes in 2011 (11% decrease) (COCERAL, 2012).

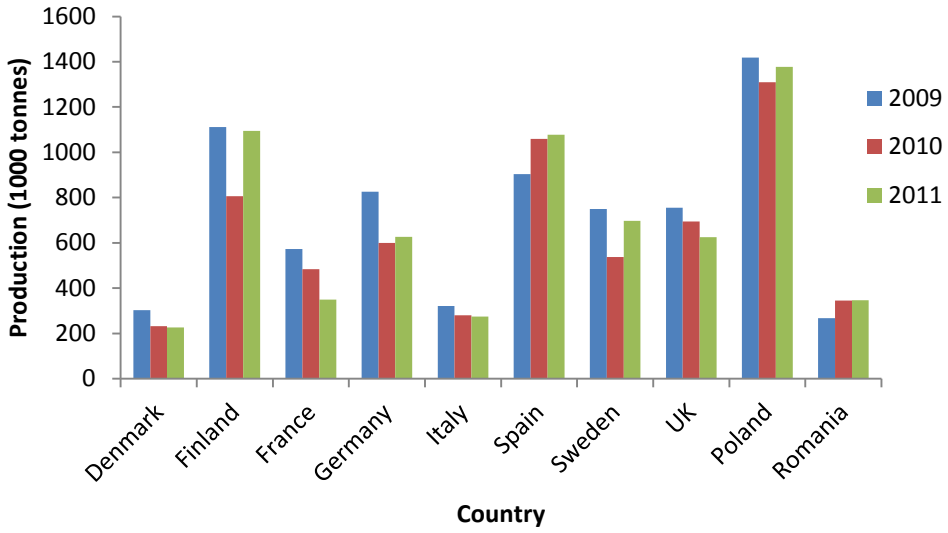

Figure 1.3. Top ten producers of oats in the EU (27) in 2009, 2010 and 2011. Source; COCERAL (2012)

1.4.2. Oats; nutritional values and uses

The use of oats as human food dates back several thousands of years but since the second century BC, its use has declined and has become a more valued animal feed (Ranhotra and Gelroth, 1995). Oats are a source of dietary fibre, vitamins, antioxidants and contain a relatively high levels of proteins (Paterson, 2001).

Oats are an important animal feed. Traditionally, oats have been used as feed for horses since the sixteenth century in the UK (Welch, 1995). In the animal feed industry, naked oat varieties are preferred to the husk varieties (Brand and van de Merwe, 1995). This is because the husk which constitute about 25-26% of the

harvested grain is mainly made of fibrous material which lowers the energy value of the grain (Peltonen-Saino et al., 2004). Naked oats have an energy value equivalent to maize as well as a high protein and fat content and a low fibre content (Christison and Bell 1980).

1.5. Triticale

Triticale is an amphiploid cross from wheat and rye. It is either the descendant of common wheat (*T. aestivum*) or durum wheat (*T. durum*) as the seed parent and cultivated diploid rye (*Secale cereal* L) as the pollen parent (Ammar et al., 2004). Records of its evolution dates back to 1888 but it was not until 1968 that commercial triticale varieties were made available to farmers (Zillinsky, 1974).

1.5.1. Triticale; optimal conditions and areas of production.

Triticale performs well under rain fed and irrigated conditions when soil fertility is good. Like wheat, it can be grown under a wide range of conditions but on record it performs better than wheat under stress conditions (Salmon, 2004). It has been shown to be competitive under abiotic stress conditions such as drought, extreme temperatures, high pH and salinity and trace element deficiency or toxicity (Belaid, 1994).

The production of triticale as a commercial crop was very low until the mid-1980s and has been increasing at an average rate of 150,000 tonnes per year in the UK (Salmon, 2004) and according to the FAO (2003) the steady increase in production was attributed to an increase in planting area. Production in 2008 was around 14 million tonnes and 90% of this production was in Europe. During this period Poland was the highest producer with a total production of 4.5 million metric tonnes followed by Germany and France (Figure 1.4)

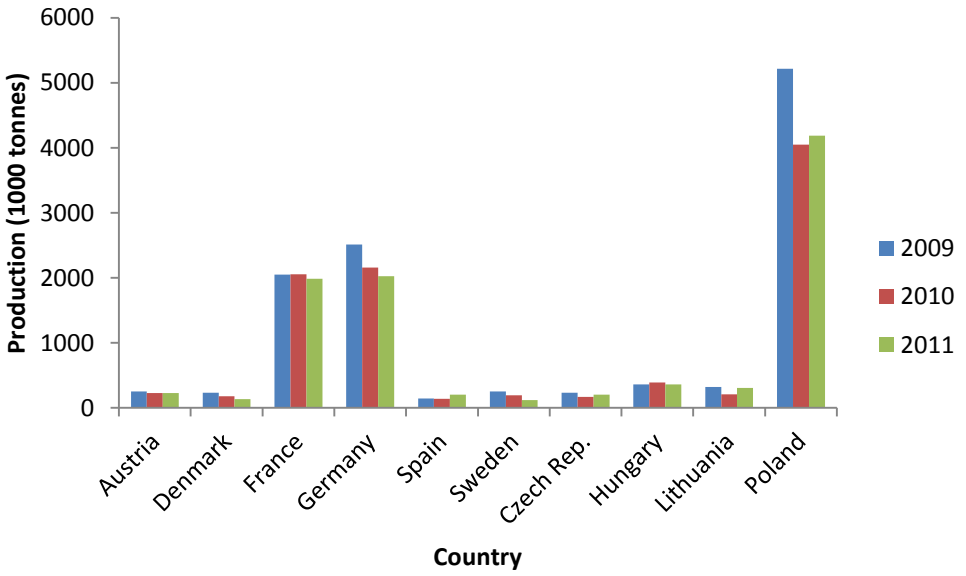

Figure 1.4. Top ten producers of triticale in the EU (27) in 2009, 2010 and 2011. Source: COCERAL (2012).

1.5.2. Triticale, nutritional values and uses.

Triticale is commonly used as animal feed although the initial intension was to be used as food for humans (Salmon, 2004). It has however been evaluated for a number of food uses such as bread making, oriental noodles, malting and brewing among others with a promising outcome. On dry basis, triticale contains about 10.2 – 15.6% protein, 53 - 65% starch and 2.3 – 4.5% crude fibre (Pena, 2004).

1.6. *Fusarium* infection in cereals

1.6.1. Fusarium

The *Fusarium* genus belongs to Kingdom Fungi; Phylum Ascomycota; Class Sordariomycetes; Order Hypocreales; Family Nectriaceae; (Michielse and Martin, 2009). Although a number of *Fusarium* species occupy plant aerial parts and can disperse through the atmosphere (actively or passively), *Fusarium* are considered to be soil-borne fungi because of their abundance in the soil and close association with plant roots (Nelson et al., 1994). The ability of members of this genus to cause

both plant and animal disease has made them very important and over the past 20 years a number of species have been studied extensively (Desjardins, 2006). In man and animals, their potential as disease causing organisms is associated with their mycotoxin production. *Fusarium* species cause disease to a wide range of crops. Of the 101 economically important plants on the plant disease list (maintained by the American Phytopathological society), Leslie and Summerell (2006) indicated that 81 had at least one *Fusarium*–associated disease.

Fusarium diseases range from crown rot, head blight, root rots and cankers among others (Nelson et al., 1994). The degree of damage and economic losses caused by various *Fusarium* species cannot be overemphasised. In the 1960s the commercial banana industry was nearly brought to a total collapse as a result of the disease Panama wilt caused by *F. oxysporum* f. sp. *cubense* (Ploetz and Pegg, 1999).

1.6.2. Fusarium Head Blight

Fusarium head blight (FHB) also known as fusarium ear blight and scab or fusarium panicle blight (FPB) specifically in oats is a disease of all small grain cereals. It is caused by a number of *Fusarium* species such as *F. avenaceum*, *F. culmorum*, *F. graminearum*, *F. poae* and *Microdochium nivale* (Parry et al., 1995; Xu et al., 2005). Among these species, *F. culmorum* and *F. graminearum* are more pathogenic, thus most important (Edwards, 2004). Although the disease was first reported in the 1880s (Stack, 2003), it still causes significant damage to cereals in Europe and is reported to be a limiting factor in wheat and barley production (Stack, 2000). Disease symptoms are similar in wheat, barley and oats. Blighted areas turn brown and senesce prematurely. Advanced stages of the disease become visible as purple to black perithecia (sexual fruiting bodies) and/or pink

sporodochia (asexual fruiting body) on infected heads, panicles or glumes (Osborne and Stein, 2007).

The growth, spore dispersal and infection of *Fusarium* species responsible for FHB is weather dependant, thus, occurrence and severity of FHB vary from year to year and from one geographical region to another (Parry et al., 1995). The disease is associated with warm, humid summers (Sutton, 1982; Jelinek et al., 1989). Cereals are most susceptible to FHB from head emergence through to flowering and may extend to the soft dough stage of grain development (Xu, 2003; Edwards, 2004).

The disease cycle of *Fusarium* species causing FHB (Figure 1.5) is complex. This is because species occur as part of a group that has the ability to cause Fusarium seedling blight (FSB) and brown foot rot worldwide and the importance of different inocula is not clearly understood (Xu, 2003). All three diseases can influence each other, each serving as an inoculum reservoir for the other (Cockerell, 1997).

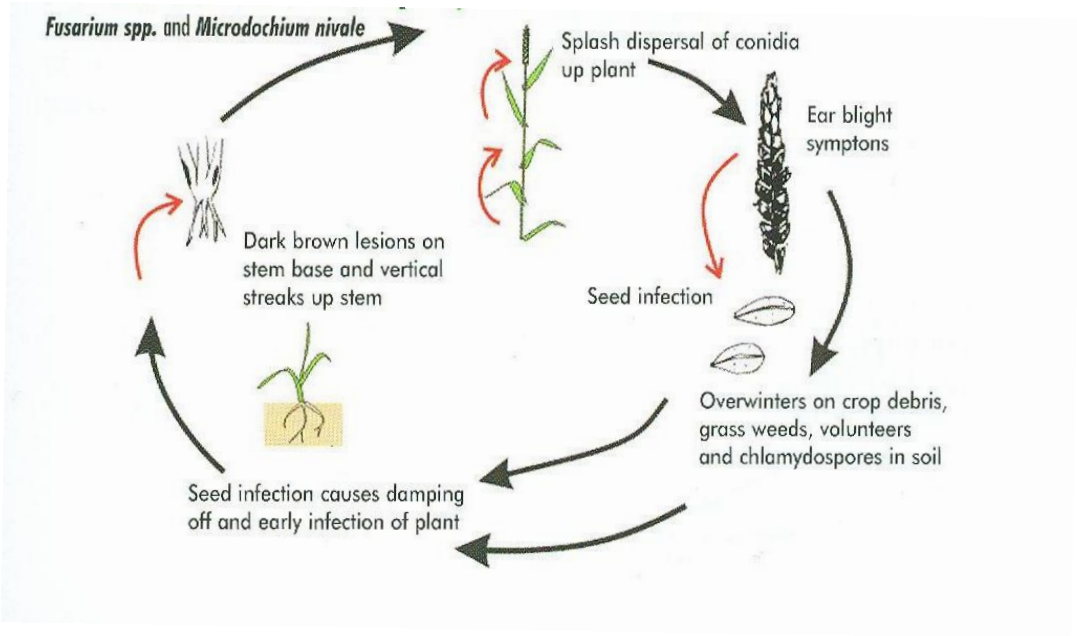

Figure 1.5. Generalized disease cycle of *Fusarium* species. Source: Encyclopedia of Cereal diseases (HGCA; BASF, not dated)

Early infection of susceptible cereals by a virulent *Fusarium* species can result in FSB. This can cause pre- and post-emergence death of seedlings and just as in the case of FHB, symptoms and disease severity is weather dependent (Holmes and Channon, 1975). After the seedling stage and on more mature plants, infection can result in brown foot rot. Brown foot rot is characterized by browning of the stem base, rotting, lodging and white heads, a situation less common in the UK (Parry, 1990). In the UK foot rot infection is commonly seen as browning on or around one of the basal nodes (Parry, 1990).

Cereals are most susceptible to FHB infection from head emergence through to end of flowering (Xu 2003; Edwards 2004). According to Shaner (2003), crop residues seem to be the major source of inoculum for the initiation of infection. However, infected seeds and soil could also serve as inoculum sources (Maiorano et al., 2008).

1.6.3. Economic importance of fusarium head blight

Fusarium head blight is an economically important disease of cereals. This is because it has the ability to influence every aspect of the grain industry (Gilbert and Tekauz, 2000). It has negative effects on cereal yield, grain quality and the ability to contaminate grains with mycotoxins. Fusarium head blight infection reduces yield directly by reducing plant growth and grain production (Osborne and Stein, 2007). Infected grains are shrivelled with reduced weight and as such, some are expelled from the combine with the chaff during harvest (Goswami and Kistler, 2004).

Economic losses due to FHB are well documented. In Europe, outbreaks in Ireland in 1954 resulted in 50% yield reduction in oats and wheat (McKay, 1957). In 1980 and 1981, German farmers suffered a similar fate, but with a lesser loss; 30%

reduction in yield (Charmley et al., 1994). In 1993 in the USA, FHB infection resulted in about a 50% drop in wheat yield compared to the previous year in the north eastern part of North Dakota and a 40% drop in north western Minnesota (NASS, 1999). The FHB epidemics in the US in the 1990s led to an estimated loss of $2.5 billion and $400 million to wheat and barley farmers respectively (Windels, 2000). In Canada, epidemics in the 1990s led to wheat farmers in Quebec and Ontario incurring economic losses estimated at US $220 million and another $300 million in Manitoba between 1993 and 1998 (Windels, 2000). Windels (2000) cited Wood (1999) reporting that the United State Department of Agricultural (USDA) considered FHB as the most devastating disease ever to occur in the United States after the 1950s epidemic of stem rust. Fusarium head blight infection results in grains with destroyed starch granules, cell walls and endosperm proteins, reducing the quality of grains used for human food (Bethel et al., 1985; Snijders, 2004).

Mycotoxin production in wheat, barley and oats is of great concern. This is because their presence seems to be unavoidable in processed feeds and foods from these grains and more importantly some of these mycotoxins have been associated with chronic and acute mycotoxicoses in animals and humans (Desjardins, 2006; Bottalico and Perrone, 2002). Among the mycotoxins produced by *Fusarium* species, deoxynivalenol (DON) seems to be the most important in terms of abundance and frequency of contamination worldwide (Edwards et al., 2009). Other mycotoxins of importance associated with FHB are nivalenol (NIV), zearelenone (ZEA), HT-2 and T-2

Seeds from FHB infected crops serve as inoculum for primary infection. Such seeds have reduced germination as well as reduced vigour and can result in seedling blight (Gilbert and Tekauz, 1995; Winson et. al., 2001). Bechtel et al. (1995) indicated that *Fusarium* contamination in cereal seeds could result in a

significant reduction in germination and seedling vigour, thus contaminated grains should not be used as seed stock.

1.7. Fusarium head blight and mycotoxin production in cereals

Mycotoxin is derived from the Greek word 'mukos' (fungus) and a Latin word 'toxicum' (poison) (Turner et al., 2009). Mycotoxins are produced by fungi from several genera including *Aspergillus, Fusarium* and *Penicillium* (Smith et al., 1994). Mycotoxins have been defined differently by different authors, for example Richard (2007) defined mycotoxins as secondary metabolites produced by some fungal species that are associated with diseases. They are considered as 'natural products from fungi that evoke toxic responses when introduced in low concentrations in higher vertebrates and other animals by natural routes' (Bennett, 1987). They have also been defined as 'relatively low molecular weight, non-antigenic secondary metabolites capable of causing toxic effects in man and animals, which are synthesized generally, but not always, on grains and other plant materials' (Smith et al., 1984). From the above definitions it is clear that mycotoxins are secondary metabolites capable of causing disease to man and animals. They are considered as secondary metabolites because their biosynthesis is not required for the primary functions of growth and reproduction (Desjardins, 2006). Production of mycotoxins in cereals by *Fusarium* species can occur in the field (pre-harvest) and/or during storage (post-harvest) (Hope et al., 2005).

1.7.1. Pre-harvest mycotoxin production in cereals

Fusarium mycotoxin production in cereals is generally considered a pre-harvest problem and dependent on prevailing climatic conditions and fungal pathogenicity (Turner and Jennings, 1997; Doohan et al., 2003). When anthesis coincides with warm and wet conditions, infection by *Fusarium* species is high and some

mycotoxin production such as deoxynivalenol may also be high (Lacey et al., 1999, Xu, 2003). There is also evidence that optimal conditions for fungal growth are not necessarily optimal conditions for mycotoxin production (Hussein and Brusel, 2001). A three year study in Finland showed that although conditions for the formation of mycotoxins were not favourable, FPB symptoms appeared in the crop (Hietaniemi et al., 2004)

A number of agronomic factors influence mycotoxin production in cereals. The previous crop in a rotation is one of such influential factors. Levels of DON produced by *F. graminearum* for example, are known to increase if a cereal follows another cereal in a rotation (Champeil et al., 2004a). In America, Dill-Macky and Jones (2000) showed that DON contamination was high when wheat followed wheat or maize in a rotation than when wheat followed soybean. Crop debris serve as a source of infection for *Fusarium* species. This means that a tillage method that results in removal, destruction or burial of infected crop debris could reduce *Fusarium* inoculum available for infection the following year and subsequently reduce mycotoxin production (Edwards, 2004). In a study to compare mycotoxin production in wheat in four cropping systems in fields with natural contamination, Champeil et al. (2004b) reported that direct drilling increased the levels of the mycotoxins DON, ZEA and NIV when compared with other tillage systems

Free water is another factor that contributes to the growth of *Fusarium* species and subsequent mycotoxin production in the field with rains occurring at harvest time increasing the risk of *Fusarium* mycotoxins contamination in cereals (Hietaniemi et al., 2004).

1.7.2. Post-harvest mycotoxin production in cereals

Fusarium mycotoxin production in cereals can result from poor post-harvest practices such as inefficient drying and on farm buffer storage before drying which leads to proliferation of existing inoculum and thus mycotoxin production or can render grains more susceptible to *Fusarium* infection (Aldred and Magan, 2004). Moisture is the single most critical factor for growth of *Fusarium* species in the field (Sauer, 1978). In storage, *Fusarium* species require moisture content of 20-22% for growth and mycotoxin production on cereal grains (Christensen and Saur, 1982; Abramson, 1991). The moisture content at which grains are normally harvested (15-18%) is too low for fungal activity. If grains are well dried at harvest and stored under conditions that prevent re-wetting through condensation and insect damage then, mycotoxin production by *Fusarium* species will be at its barest minimum if not totally prevented (Sutton, 1982; Birzele et al., 2000).

1.8. Types of *Fusarium* mycotoxins

It is reported that over 300 different mycotoxins have been implicated in various forms and severity of toxicity in mammalian and avian species. It is also estimated that about 25% of the worlds total crop production is contaminated with mycotoxins (Fink-Gremmels, 1999).

Historically, mycotoxin associated diseases could be traced as far back as ancient Egyptian history where the last of the ten Plagues was linked to grain contamination with mycotoxins. The ‘St Anthony’s Fire’, a disease in the middle ages which was characterized by hallucinations, vomiting and terrible burning sensations in victims, was initially attributed to witchcraft or evil spirits. This disease was however, diagnosed as a result of ergot poisoning (Agrios, 2005). Alimentary Toxic Aleukia (ATA) is another mycotoxin associated disease in humans. It was a

devastating disease which was initially reported in the 1940s and 1950s in Russia. It affected the nervous system, finally resulting in death. This disease is attributed to T-2 toxins produced by *F. sporotrichioides* (Richard, 2007).

Of the mycotoxin producing genera, *Fusarium* is one of the most important, producing some of the most toxic and economically important mycotoxins. Mycotoxins produced by *Fusarium* species can be put into three main classes; fumonisins, zearalenones and trichothecenes. Other toxins such as beauvericin, fusaproliferin, fusarins and moniliformin are considered of minor importance (Desjardins, 2006).

1.8.1. Fumonisins

The group of mycotoxins called fumonisins derive their name from *F. moniliforme* from which they were isolated and structurally characterized in 1988 (Bezuidenhout et al., 1988). They are only produced by *Fusarium* species, among which *F. verticillioides* and *F. proliferatum* are the most consistent and high level producers. The detection of fumonisin contaminated food and feeds and fumonisin-associated diseases in humans and animals in parts of all five major continents (Africa, Asia, Europe, North America and South America) suggest that they are worldwide in distribution. Although different fumonisin levels have been detected in different plant species, maize seems to be the most important source (Desjardins, 2006).

1.8.1.1. Associated human and animal disease

Equine leukoencephalomalacia

Historically, fumonisin associated diseases have been reported as far back as the 1900s but it was not until the 1988 that the mycotoxin was isolated and structurally characterized (Bezuidenhout et al., 1988). Desjardins (2006) describes a mycotoxin associated disease in horses – leukoencephalomalacia, which killed

about 5,000 horses in central Illinois during the winter of 1934-1935. The disease was later confirmed to be a result of the ingestion of feed contaminated with fumonisins produced by *Fusarium verticillioides* (Kriek et al., 1981).

Pulmonary edema in swine

In the late 1980s and the early 1990s, another fumonisin associated disease, known as, pulmonary edema, was reported in pigs from the USA (Harrison et al., 1990). Characteristically, diseased animals showed difficulty in breathing, abortion, edema and death. It is reported that in 16 different outbreaks on farms along the eastern border of Iowa alone, about 1,100 pigs died in 1989 (Desjardins, 2006). Further investigations confirmed fumonisin B_1 produced by *F. verticillioides* and *F. proliferatum* to be the causal agent (Harrison *et al*., 1990; Osweiler et al., 1992). In view of the danger that fumonisin contaminated feed posed to the pig industry, the American Association of Veterinary Laboratory Diagnosticians, proposed that all pig feed should be tested for the presence of fumonisins and recommended that levels in feeds should be less than 10 µg g^{-1} (Miller et al., 1996).

Oesophageal cancer

In humans, oesophageal cancer has been closely linked to the consumption of fumonisin contaminated maize especially in South Africa (Rheeder et al., 1992, Sydenham et al., 1990). Oesophageal cancer occurs worldwide, although incidence rate varies from one geographic region to the other, with the highest incident rate in Kentani district in the Transkei region in South Africa (Marasas, 2001). Although the main cause/causes of oesophageal cancer still eludes scientists, the high incidence of the disease in areas where people consume high levels of fumonisins contamination makes these mycotoxins strong candidates as causal agents (Desjardins, 2006).

Neural tube defects

Another human disease which has been associated with fumonisins is a congenital abnormality known as neural tube defect. The neural tube is the precursor to the central nervous system in developing vertebrates. The disease is characterized by the inability of the neural tube to close during development of the foetus. In 1989, it was detected that there were fumonisins level in maize in the Texas-Mexico border region up to 70 µg g^{-1}. This coincided with outbreaks of equine leukoencephalomalacia. Maize in this region constituted a major part of the people's diet, prompting scientists to investigate whether the mycotoxin had any effect on human health (Desjardins, 2006). A survey in Mexican-American women who conceived during 1990-1991 in the region showed that 29 out of 10,000 live births had the congenital defect, about 8.9% higher than the average incidence in Mexican-Americans in the United States (Hendricks, 1999).

1.8.2. Zearalenone

Zearalenone was first isolated from *Gibberella zea* in 1996 and subsequently named after the species (Desjardins, 2006). ZON is among the fusarium mycotoxins with legal limits set by the European Comission in cereal grains and cereal-based products (Table 1.4).

Table 1.4. Maximum limits for ZON in unprocessed and finished cereal products in the EU.

Product	Maximum limit (µg kg^{-1})
Unprocessed cereals	100
Cereal flour	75
Bread, pastries, biscuits, cereal snacks and breakfast cereals	50
Processed cereal-based food for infants and young children	20

Source; EC, 2006

Although it was initially thought to be produced by a large number of *Fusarium* species, scientific evidence now shows that it is predominantly produced in large quantities by *F. graminearum*, *F. crookwellense* and *F. culmorum* (Desjardins, 2006). Production of ZON has been shown to be favoured by a delayed wet harvest of wheat (Edwards, 2011).

Among the three main classes of *Fusarium* mycotoxins, zearalenone seems to be the least toxic. Literature searches have shown no report of its association with any fatal human or animal disease. It has the ability to interact with oestrogen receptors resulting in hyperoestrogenism (EFSA, 2004). However, during the 1950s and 1960s the mycotoxin was associated with Swine Estrogenic Syndrome. The disease, although not fatal, was characterized by enlarged mammary glands, atrophy of ovaries and infertility. In cases where sows were fertile and littered, litter size and average litter weight was reduced (Tuite,et al., 1974).

1.8.3. Trichothecenes

Trichothecenes are a family of mycotoxins that have a common sesquiterpene nucleus. Characteristically, they have a general structure consisting of an epoxide

ring at the C-12, 13 with a double bond at C-9, 10 (Kraska et al., 2001; Deshpande, 2002). There are four main classes of trichothecenes; types A, B, C and D. Classification is mainly based on their functional groups. Type A has a functional group other than a ketone at carbon 8 (C8) e.g. HT-2+T-2. Type B has a carbonyl functional group at C7, 8 or C9, 10 and type D has macrocyclic ring system between C4 and C15 with 2 ester linkages (Koch, 2004; Deshpande, 2002). Trichothecenes are produced by a number of fungal genera such as *Trichoderma, Stachybotrytis*, *Myrothecium* and *Fusarium*. However, among these genera, *Fusarium* seems to be the most important, being the most consistent and largest producer (Desjardins, 2006). Of all the *Fusarium* mycotoxins, trichothecenes seem to be the most important accounting for a large and consistent number of chronic and fatal toxicosis of humans and animals (Desjardins, 2006). Apart from their ability to produce mycotoxins that have been implicated in various human and animal related mycotoxicosis, trichothecene producing *Fusarium* are plant pathogenic. They have the ability to infect a wide range of crops, most importantly in Europe; barley, wheat and oats (Venkatasubbaiah et al., 1995 and Desjardins, 2006). The grains of these cereals are the traditional source of trichothecene contamination, with levels of contamination increasing with wet conditions during harvest and storage under high moisture (Desjardins et al., 1993) Ingestion of contaminated grains by humans or animals as food or feed result in various forms of mycotoxicosis.

1.8.3.1. Associated human and animal diseases

Alimentary toxic aleukia

Alimentary toxic aleukia (ATA) is a fatal mycotoxicosis in humans. It results from the consumption of food prepared from grains contaminated with T-2 toxin. Lutsky and More (1981) quoted Mayer (1953) with the report of the outbreak of the

disease in poor Russian families in the 1930s. Desjardins 2006 also quotes a number of authors (Beardal and Miller, 1994; Gajdusek, 1953; Joffe, 1986 and Marasas et al., 1984) reporting the outbreak of the disease in Russia and Kazakhstan in 1932. The disease is characterized by vomiting, lack of coordination in muscle movement (ataxia), paralysis and finally death. It has also been reported that ATA causes a decrease in the number of white blood cells (leucopoenia) and enlarged lymph nodes in humans (Forgacs and Carll, 1962). In cats, ATA results in symptoms such as general weakness, bloody faeces, conjunctivitis, vomiting and fatality (Lustsky and Mor, 1981). Although there is no concrete evidence as to the *Fusarium* species responsible for the disease outbreaks (Desjardins, 2006), available literature points to *F. poae* and *F. sporotrichioides* as the T-2 producing species that may have been responsible for the outbreaks in Russia and Kazakhstan (Yagen and Joffe 1976; Marasas et al., 1984).

Akakabi-byo in Japan

Akakabi-byo, also known as red mould disease is a human toxicosis which was reported in Japan and Korea in 1890, 1901, 1914, 1920 and 1923 (Desjardins, 2006). The disease has been associated with the consumption of discoloured (red) cereals, mainly barley, wheat and rice and characterized by nausea, vomiting, congestion, diarrhoea, haemorrhage of internal organs such as the lungs and heart (Yoshizawa, 1983). Although there is no evidence for the role of trichothecenes and the *Fusarium* species associated with these earlier outbreaks in Asia as Desjardins (2006) describes it, available literature and information gathered from the recent outbreaks in Canada (1980 and 1982) as well as in the Kashmir valley in India makes DON and *F. graminearum* strong candidates for this mycotoxicosis (Yoshizawa, 1983; D'Mello et al., 1991; Bhart et al., 1989).

Swine feed refusal

Swine feed refusal, as the name implies, is a disease associated with pigs and characterized by loss of appetite resulting in total refusal of feed, emesis, weakness and in some cases death (Desjardins, 2006). Outbreaks of the disease occurred in the USA in 1928 (Mains et al., 1930), 1965 and 1972 (Tuite et al., 1974). It has also been reported in Europe (Marasas et al., 1984). Research has shown that these outbreaks were as a result of contamination of animal feed with DON produced by *F. graminearum.*

1.8.3.2. Role of Trichothecenes in plants

The reasons for the production of mycotoxins by fungi are not well understood (Desjardins, 2006). Mycotoxins produced by *Fusarium* species for example, are highly complex and their nature suggest that they are biologically active; producing species are mostly aggressive pathogens of agricultural plants, suggesting that they could possibly play a role in plant pathogenesis (Desjardins, 2006). The discussion of this subject is complicated and open to debate. This is because some mycotoxins are produced by more than one *Fusarium* species and available literature shows that the same mycotoxin may play different roles in different *Fusarium* species.

Fumonisins and *Alternaria alternata* f. sp *lycopersici* (AAL) toxins are structurally similar, but while the former has no effect on the ability of *F. verticillioides* to cause maize ear rot, the latter is considered a major virulence factor of *Alternaria alternata* on tomato. Although reasons for the production of these mycotoxins are not fully understood, their production is of great concern to man since they are mainly produced in grains which serve as a major source of food and feed for man and animals.

Some mycotoxins are known to be phytotoxins. Phytotoxins are secondary metabolites that have the ability to negatively affect plant metabolic activities causing death at concentrations below 10 mM (Beresteetskiy, 2008). Some trichothecenes, at low concentrations (1-10 µM) have been demonstrated to be phytotoxic, causing disturbances to vital activities of plant cells that result in wilting, chlorosis and necrosis (Desjardins, 2006; Beresteetskiy, 2008). Relative to animals, toxicity of trichothecenes to plants is a research neglected area. However, just as in animals, trichothecenes are known to inhibit ribosomal protein synthesis in plants (Beresteetskiy, 2008). It is worth noting that although the mode of action of trichothecenes seems to be similar in plants and animals, the level of toxicity differs when plant systems are compared with each other and with that of animals (Desjardins, 2006).

Trichothecenes may be important in plant pathogenic *Fusarium* species; most probably as virulence factors. Their production in infected plant tissues as well as their reported phytotoxic effect supports this assertion (Desjardins, 2006). Beresteetskiy (2008) reported that, of all the known phytotoxins, sesquiterpenes are the most prominent and trichothecenes as classical examples, induce chlorosis and wilting in plants.

The trichodiene synthase gene (*TRI5*) was the first trichothecene gene to be cloned. It was first isolated from *F. sporotrichioides* (Hon and Beremand, 1989) and subsequently cloned from *F. graminearum*, *F. poae*, *F. sambucinum* and *F. venenatum*. Most research into the role of trichothecenes in plant diseases has been done with *Fusarium* species with mutated *TRI5* genes. Studies using this gene have been possibly because the gene occurs as a single copy in all *Fusarium* species from which it has been cloned, showing up to 95% similarity and most importantly catalyzes the first step (sesquiterpene cyclization) in the trichothecene

biosynthetic pathway, thus, its disruption blocks the biosynthesis of trichodiene and consequently all trichothecenes (Hon and Desjardins, 1992; Royer et al., 1999; Desjardins and Proctor, 2007).

Mutational studies of *TRI5* in some *Fusarium* species have shown trichothecene to be a virulence factor. *Fusarium sambucinum* is a pathogen of parsnip and potato, causing rot in both plants. Disruption of the *TRI5* gene in *F. sambucinum* resulted in mutants that lacked the ability to produce any trichothecene. In parsnips, virulence of these mutants was highly reduced compared to the wild type (Desjardins, 2009). The virulence of *TRI5* mutants of *F. sporotrichioides* was also compromised on parsnip roots (Desjardins et al., 1989). *Fusarium graminearum* is a pathogen of wheat and maize causing head blight in the former and ear blight in the later. *TRI5* mutants of this fungus lack the ability to produce DON. The virulence of these mutants was found to be highly compromised when compared with the wild type (Proctor et al., 1997). Harris and colleagues also demonstrated that *TRI5* mutants of *F. graminearum* had reduced virulence on maize under field conditions (Harris et al., 1999)

Although trichothecenes seem to enhance the virulence of some *Fusarium* species, especially *F. graminearum*, there is evidence that its role varies from one plant species to another and within different *Fusarium* species. For example, whilst trichothecenes are known to enhance the virulence of *F. sambucinum* and *F. sporotrichioides* on parsnip roots, it seems not to play any vital role in the virulence of the same *Fusarium* species on potato tubers (Desjardins, 2006). Langevin and colleagues (data) demonstrated that trichothecene production in *F. graminearum* may not always be important in virulence. In an experiment with different cereal species the authors found that a *TRI*5 mutant of *F. graminearum* was able to infect heads of barley, oats, rye, triticale and durum wheat, with rate of spread being very

extensive only in the heads of durum wheat (Langevin et al., 2004). The ability of a trichothecene from one *Fusarium* species to enhance virulence in some plant species but not in others suggests that some plant species may have evolved mechanisms to overcome its phytotoxic activity since plants have over time evolved various and varied defence mechanisms against different plant pathogens (Desjardins et al., 1992). One such mechanism could be the metabolism of trichothecenes by plant enzymes into forms or products that are less toxic to the plant (Miller and Young, 1985; Desjardins et al., 1992)

1.8.4. Important trichothecenes

Within the trichothecenes mycotoxins, types A and B are known to be the most important and are of great public health concern. Members of these classes are shown in Table 1.5. Within these two groups however, members of type A are more potent toxins accounting for a number of chronic and acute mycotoxicosis (Krska et al., 2001; Smith et al., 1994). The potency of type A trichothecenes is probably due to the absence of the carbonyl group at position C-8 (Langseth and Rundberget, 1999). In terms of abundance and frequency of detection, DON is considered the most important trichothecenes (Krska et al., 2007; Yazaar and Omurtag, 2008; Edwards, 2009).

Table 1.5. Members of type A and type B trichothecenes

Type A	Type B
Diacetoxyscripenol (DAS)	3-Acetyldeoxynivalenol (3 AcDON)
Monoacetoxyscirpenol (MAS)	15-Acetyldeoxynivalenol (15 AcDON)
Neosolaniol (NEO)	Deoxynivalenol (DON)
HT-2	Fusarenon X
T-2	Nivalenol (NIV)

Source**:** Smith et al., 1994

1.8.4.1. Deoxynivalenol

Deoxynivalenol (DON) (Figure 1.7) also known as vomitoxin is a type B trichothecene. It is the most commonly occurring trichothecene in cereals (Krska et al., 2007; Edwards, 2009). It is soluble in water as well as organic solvents such as ethanol. Deoxynivalenol has a white crystalline powdery appearance, molecular weight of 296.3 and a melting range of 151^{o}C to 153^{o}C (Krska et al., 2007). It was called Rd-toxin when it was initially isolated in Japan (Moorooka et al., 1972). Deoxynivalenol is usually produced by *F. graminearum* and *F. culmorum*. It has a Tolerable Daily Intake (TDI) of 1 µg kg^{-1} body weight day^{-1} (EC, 2006) Although DON is not acutely toxic to farm animals, it has been implicated in some mycotoxin related animal diseases such as feed refusal in pigs, dogs and cats (Osweiler, 2000).

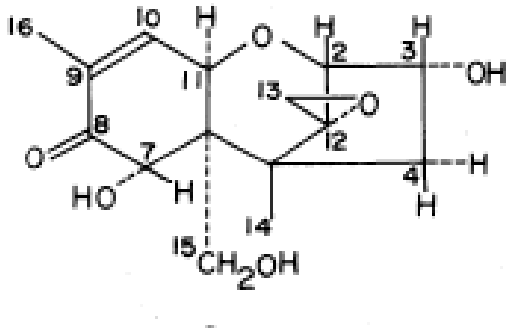

Figure 1.6. Structure of DON. Source: Yoshizawa et al., 1983

DON is one of the fusarium mycotoxins with legal limits in cereals and cereal-based products set by the EC (Table 1.6).

Table 1.6. Maximum limits for DON in unprocessed and finished cereal products in the EU

Product	Maximum limit (µg kg^{-1})
Unprocessed cereals other that durum wheat and oats	1250
Unprocessed durum wheat and oat	1750
Cereal flour	750
Bread, pastries, biscuits, cereal snacks and breakfast cereals	500
Processed cereal-based food for infants and young children and baby food	200

Source: EC, 2000

1.8.4.2. HT-2+T-2

HT-2 and T-2 (Figure 1.8) mycotoxins are produced by a number of *Fusarium* species. They are considered to be two of the most potent trichothecenes (SCF, 2002) with acute oral LD_{50} of 5-10 mg kg^{-1} body weight in rodents (SFCC, 2001) and tolerable daily intake of 0.1 µg kg^{-1} body weight day^{-1} (EFSA, 2011).

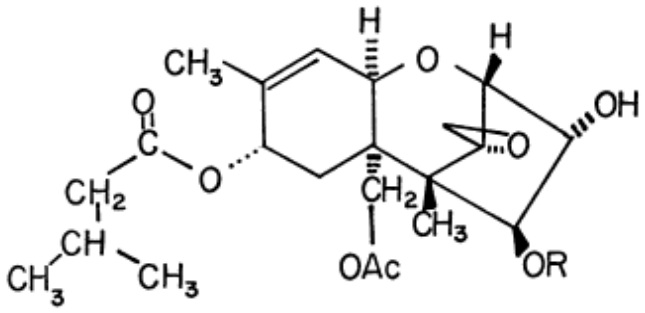

Figure 1.7. General structure of type A trichothecenes; T-2: R = Ac; HT-2: R = H. Source: Ellison and Kotsonis 1974

Until the identification and subsequent characterization of *F. langsethiae* in 2004 by Torp and colleagues (Torp and Nirenberg, 2004), *F. armeniacum*, *F. poae* and *F. sporotrichioides* were the main *Fusarium* species known to be responsible for HT-2+T-2 production in cereals in Europe (Eriksen and Alexander, 1998; Langseth and Rundberget 1999; Torp and Langseth, 1999; Edwards, 2009).

T-2 toxin also known as fusario toxin has a molecular weight of 466.58. It is readily metabolised into a deacetylated form (HT-2) in the gut and as a result their toxicology *in vivo* is considered equivalent (Eriksen and Alexander, 1998; WHO, 2001).

HT-2+T-2 are known DNA and RNA synthesis inhibitors. T-2 inhibits protein synthesis at the initiation phase by binding to the 60S ribosomal subunit thereby inhibiting the activity of peptidyl transferase (SCF, 2001), an enzyme that catalyzes the peptide bond formation during translation. *In-vitro* and *in vivo* experiments have shown HT-2+T-2 to have the ability to cause apoptosis; a process of programmed cell death that occurs in multicellular organisms (Ueno et al., 1995; Yang et al., 2000). Programmed cell death is an active genetically controlled process which is essential for plant growth and development (Gadjev et al., 2008). For example, programmed cell death is associated with embryo formation, degeneration of the aleurone layer during seed germination in monocotyledons, formation of root aerenchyma and epidermal trichomes and floral organ abscission among others (Gechev et al., 2006). The process by which HT-2+T-2 induces apoptosis in cells is not clearly understood. It has been suggested however, that HT-2+T-2 could activate the mitogen-activated protein kinases (MAPK) (Yang et al., 2000). Depending on activation conditions, activation of MAPK is known to either result in cell death or cell survival.

Convincing data from *in vivo* experiments on the effects of HT-2+T-2 on humans is limiting, however, available data suggests that symptoms of T-2 related diseases include abdominal pains resulting in vomiting and reduction in leukocytes (Moss, 2002; Pacin et al., 1994).

1.9. HT-2 and T-2, a concern in Europe

In Europe, HT-2 and T-2 occurrence is mainly in small grain cereals (Edwards et al., 2009). Since the late 1990s when HT-2 and T-2 were reported in Norwegian cereals (Langseth and Rundberget, 1999), their production has been consistently monitored. According to Edwards et al. (2009), the level of HT-2 and T-2 in European oats has increased dramatically over the last decade. Although HT-2 and T-2 can be produced by a number of *Fusarium* species such as *F. poae*, *F. sporotrichioides*, *F. armeniacum* and quite recently *F. sibiricum* in Asia (Yli-Mattila et al., 2011), *F. langsethiae* has been identified as the main producer in small grain cereals in Europe (Imathiu, 2008; Parikka et al., 2008)

Available data indicates that HT-2+T-2 in oats is higher than in barley and wheat. Reported high levels are found in unprocessed oat, but once processed, levels of HT-2+T-2 tend to reduce (Scudamore et al., 2007). Although actual concentrations have fluctuated in recent years (Edwards 2009a,b,c), HT-2 was reported to be the most commonly detected mycotoxin in oat based foods in Germany (Scholleberger et al., 2005).

Contamination by HT-2 and T-2 in food and feedstuff is a prime food safety issue. This is because HT-2 and T-2 are highly toxic and their complete elimination from agricultural commodities is almost impossible (FAO, 2004). A concerted effort is therefore geared towards their reduction in food and feed stuff to levels that have no significant impact on health (Desjardins, 2006).

Legal limits for deoxynivalenol, zearalenone and fumonisins have been set by the European Commission (EC, 2006). European legislation (EC, 2006) state ‘intake estimates indicate that the presence of HT-2+T-2 toxin can be of concern for public health. Therefore, the development of a reliable and sensitive method, collection of

more occurrence data and more investigations/research in the factors involved in the presence of HT-2+T-2 toxin in cereals and cereal products, in particular in oats and oat products, is necessary and of high priority'. In January 2012 discussion limits for HT-2+T-2 in unprocessed, processed and cereal products were proposed by the EC (Table 1.7)

Table 1.7. Maximum limits for HT+T-2 in unprocessed, processed and cereal products in the EU

Unprocessed cereals	**HT-2+T-2 (µg kg^{-1})**
Barley	200
Corn grain	150
Oats (with husk)	1000
Wheat (including durum wheat)	50
Processed cereal	
Barley grain for direct human consumption	25
Corn grain for direct human consumption	25
Oats grain for direct human consumption	50
Rye for direct human consumption	25
Wheat (including durum wheat) for direct human consumption	25
Cereal products	
All products derived from cereals	25
Except	
Cereal bran	50
Oat milling products	50
Breakfast cereal including cereal flakes	50

Source Anon. 2012

1.10. HT-2+T-2 producing *Fusarium* species

A number of *Fusarium* species have been implicated in the production of trichothecenes. This group of trichothecenes producing species have also been linked with various mycotoxin related diseases. Within the trichothecene producing species, some have the ability to produce HT-2+T-2, the two most potent trichothecenes. Notable among these species are *F. langsethiae* (Burgess and Summerell, 2000; Thrane et al., 2004) *F. sporotrichioides, F. armeniacum* and *F. sibiricum* sp. Nov. (Yli-Matilla, 2011)

1.10.1. *Fusarium langsethiae*

In 1999 Torp and Langseth first described a *Fusarium* species that closely resembled *F. poae* morphologically and *F. sporotrichioides* in terms of metabolite profile. This *Fusarium* species was initially called 'powdery' *F. poae* due to its powdery appearance on artificial growth media (Czapek-Dox Iprodine agar (CZID) and PDA) (Torp and Langseth, 1999). 'Powdery' *F. poae* was later named *F. langsethiae,* after Dr Wenche Langseth by her colleagues after she passed away during the identification and characterization process (Torp and Nirenberg, 2004).

Morphologically, both *F. langsethiae* and *F. poae* produce conidia that are globose to napiform in shape, however, *F. langsethiae* is differentiated from *F. poae* by its slower growth rate, producing less aerial mycelium. Conidia of *F. langsethiae* are borne on bent phialides as compared with straight monophialides of *F. poae*. When cultured on synthetic low-nutrient agar (SNA) *F. poae* produces napiform conidia in combination with falcate sporodochial conidia while *F. langsethiae* produces only napiform conidia (Figure 1.8). It also lacks the characteristic peach-like odour of *F. poae* when growing on artificial medium (Torp and Nirenberg, 2004).

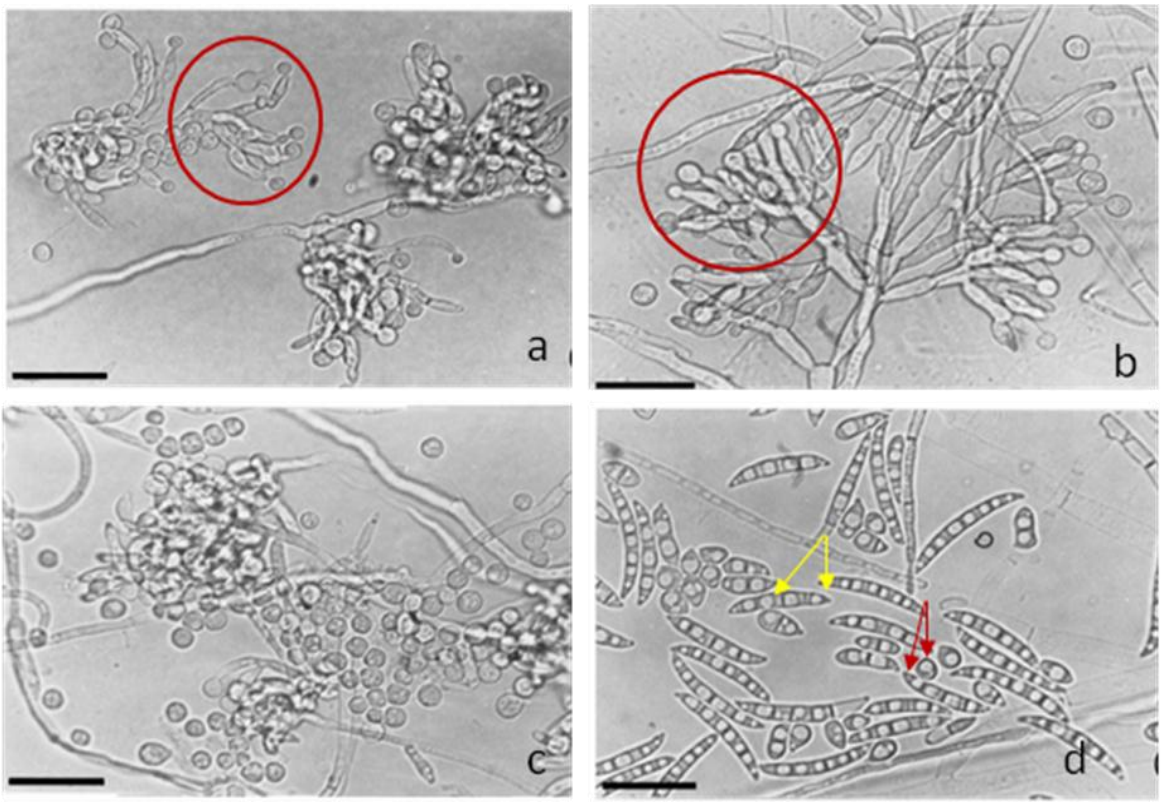

Figure 1.8 Morphological characteristics of *F. langsethiae* compared with *F. poae***. a:** conidiophore of *F. langsethiae* on SNA at 20^{o}C. Conidia are borne on branched conidiophores of the aerial mycelium with bent monophialides (red circle). **b:** conidia of *F. poae* are borne on conidiophores of the aerial mycelium with straight monophialides (red circle). **c:** sporulation of *F. langsethiae* isolate. Note the absence of sporodochial conidia. Conidia are solely napiform. d; sporulation of *F.poae*. Note the presence of both napiform conidia (red arrow) and sporodochial falcate conidia (yellow arrow). Scale bars = 25 µm. Source: Adapted from Torp and Nirenberg (2004).

Different isolates have different mycelia colour on artificial medium such as PDA. Its colour ranges from white to pink and light red (Torp and Nirenberg, 2004). The ability of *F. langsethiae* to produce different shades of colour on growing media has been linked to its ability to synthesise aurofusarin (Thrane et al., 2004). Aurofusarin has been associated with a number of *Fusarium* species including *F. acuminatum, F. avenaceum, F. crookwellense, F. culmorum*, *F. graminearum, F. poae, F. pseudograminearum, F. sambucinum, F. sporotrichioides* and *F. tricinctum* (Samson et al., 2000)**.** The pigment is produced during growth and development of

the fungal mycelium, increasing in concentration with fungal growth, leading to the staining of both mycelium and growth medium (Samson et al., 2000).

Although *F. langsethiae* bares some morphological resemblance to *F. sporotrichioides* (similar conidia;-napiform in aerial mycelium), *F. langsethiae* more closely resembles *F. sporotrichioides* in its mycotoxin profile. Both species produce a number of mycotoxins; trichothecenes (T-2, HT-2, DAS and NEO), culmorins, chrysogine and aurofusarin.

The geographic distribution of *F. langsethiae* cannot be described with certainty due to its recent identification and lack of experience in identifying this newly identified species (Edwards et al., 2009). Wilson et al. (2004) for example, indicated a situation where two isolates from Poland and Italy were initially identified as *F. sporotrichioides* but further analysis using PCR (ITS and *TRI5* sequences) confirmed that they were indeed *F. langsethiae*. This notwithstanding, *F. langsethiae* has been reported mainly in Europe; Austria, Czech Republic, Denmark, England, Germany and Norway (Torp and Adler, 2004; Torp and Nirenberg, 2004) and quite recently in Italy (Infantino et al., 2007), Poland (Lukanowski et al., 2008) and Serbia (Bocarov-Stancic et al., 2008).

1.10.2. *Fusarium sporotrichioides*

Fusarium sporotrichioides produces four main type A trichothecenes (DAS, NEO, HT-2+T-2) in cereals (Marasas et al., 1984 Chelkowski, 1998). Morphologically, *F. sporotrichioides* produces round conidia and fusiform conidia bearing aerial mycelium (Leslie and Summerell, 2006). It grows rapidly on PDA producing aerial mycelium with intense reddish colour with a yellowish tinge (Torp and Nirenberg, 2004). *Fusarium sporotrichioides* has been reported to occur in different plant species such as tiger nuts (*Cyperus esculentus* L), potato (*Solanum tuberosum*)

but most especially in cereals such as wheat and oats (Marasas et al., 1984; Miller, 1994; Mateo and Jimenez, 2000). It is a field as well as a post-harvest pathogen of cereals (Sudakin, 2003). Its occurrence is worldwide, although on an irregular basis (Bottalico, 1998).

A number of authors have indicated that *F. sporotrichioides* occurrence and subsequent mycotoxin production in cereals is influenced by water damage to grains especially when crops are left for long periods in the fields, or when grain is still wet when stored (Bottalico et al., 1989; Chelwkowski, 1998; Abramson et al., 2004).

1.10.3. *Fusarium poae*

Fusarium poae is widespread but is normally found in temperate soils. It has been isolated from seeds and seedlings and grain heads of cereals (De Nijs et al., 1996; Leslie and Summerell, 2006). *Fusarium poae* has been associated with head blight of wheat but is regarded as a weak pathogen within the FHB complex (Dohan et al., 1999). On PDA, it produces dense white mycelium. As cultures age, the mycelium changes colour from pale white to reddish brown. Cultures of *F. poae* may produce a distinct fruity odour (Leslie and Summerell, 2006). Microconidia which are globose to napiform are produced in abundance in aerial mycelium appearing in clusters that look like a bunch of grapes. Macroconidia are rarely produced, but when present they are curved, tapering and slender with three septa. (Leslie and Summerell, 2006).

Until the isolation and characterization of *F. langsethiae* which bears close morphological similarity to *F. poae, F. poae* was considered a potent producer of HT-2+T-2, but in recent times it has been considered a mild producer of HT-2+T-2 (Torp and Langseth, 1999; Thrane et al., 2004). It has been suggested that

previously identified high HT-2+T-2 strains of *F. poae* where actually *F. langsethiae* (Torp and Langseth, 1999). *F. poae* is also known to produce other mycotoxins including the type B trichothecene, nivalenol

1.10.4. *Fusarium armeniacum*

Fusarium armeniacum was previously known as *F. acuminatum* subsp. *armeniacum* due to the close resemblance between its macroconidial morphology and that of *F. acuminatum* (Leslie et al., 2006). It was elevated to a species level in 2000 based on toxicological studies and karyotyping (Burgess and Summerell, 2000). *Fusarium armeniacum* is regarded as a saprophyte in subtropical and tropical regions with a high incidence of occurrence in high rainfall areas (Leslie et al., 2006).

On carnation leaf agar, *F. armeniacum* produces macroconidia in bright orange sporodochia. Macroconidia are curved with foot shaped basal cell. On PDA, *F. armeniacum* is fast growing producing abundant white mycelium. It produces various pigments ranging from bright orange to red and reddish brown. *Fusarium armeniacum* produces no microconidia on artificial media (Leslie et al., 2006).

Fusarium armeniacum is a high HT-2+T-2 producer and according to Desjardins (2006), it has the ability to produce T-2 up to 500 µg g^{-1} of culture dry weight. *Fusarium armeniacum* has been reported to be toxigenic in chick assays (Wing et al., 1993).

1.10.5. *Fusarium sibiricum*

Fusarium sibiricum until recently has been confused with *F. langsethiae, F. poae*, and *F. sporotrichioides*. Initial European isolates were reported to be intermediate between *F. langsethiae* and *F. sporotrichioides* (Yli-Mittila, 2004). In Iran this species was initially reported to as *F*. cf. *langsethiae* (Kachuei et al., 2009).

Between 2000 and 2003 a number of isolates from Siberia and Russian Far East were identified as *F. poae* with the ability to produce high T-2 levels (Burkin et al., 2008). Morphologically, it is more closely related to *F. poae* and *F. langsethiae* and closely related to *F. sporotrichioides* phylogenetically (Yli-Mattila, 2011). Characteristically, *F. sibiricum* is not known to produce macroconidia, like *F. langsethiae*. It lacks the fruity odour of *F. poae* and has no pigmentation. It grows faster producing more aerial mycelium than *F. langsethiae* on Potato-Sucrose Agar but cannot be differentiated from *F. poae* and *F. sporotrichioides* based on growth rate (Yli-Mattila et al., 2011). Table 1.8 summarises the characteristics of *F. sibiricum* compared to *F. langsethiae*, *F. poae* and *F. sporotrichioides.*

Table.1.8. Similarities and differences between morphological characteristics of *F. sporotrichioides*, *F. poae*, *F. langsethiae* and *F. sibiricum*

Character	*F. sporotrichioides*	*F. poae*	*F. langsethiae*	*F. sibiricum*
Profuse mycelium growth	+	+	-	+
Pigmentation on PSA	+	±	±	-
Maximal growth rate (mm day^{-1})	7.7	7.4	6.3	7.4
Macroconidia	+	±	-	-
Ampulliform monophialides	-	+	+	+
Bent cylindrical monophialides	-	-	+	-
Conidiophores with long stides	-	-	-	+

Source: Adapted from Yli- Mattila et al. (2011).

It is of interest to note that *F. sibiricum* can also be differentiated from the above mentioned *Fusarium* species using PCR primers based on nucleotide polymorphisms within the IGS rDNA (Yli-Mattila et al., 2011).

1.11. The problem

HT-2 and T-2 are considered as two of the most potent trichothecenes and their distribution appears largely restricted to Europe (Edwards et al., 2009). Available data has shown that the level of HT-2+T-2 in oats is higher than in barley and wheat respectively (Edwards 2007a,b; Edwards, 2009a,b). A Tolerable Daily Intake (T-TDI) of 0.1 µg kg^{-1} body weight day^{-1} has been set for HT-2+T-2 (EFSA, 2011). Edwards, (2007a) reported that oats in the UK contained high concentrations of HT-2+T-2 and that 92% of the total samples analysed had levels greater than 10 µg kg^{-1} with a combined (HT-2+T-2) median, mean and maximum concentrations of 213, 570 and 9900 µg kg^{-1} respectively for samples collected from 2002 to 2005. Incidence and concentrations in barley and wheat were lower and 1% of total samples analysed had levels exceeding 100 µg kg^{-1} over the same time period (Edwards, 2007a and b).

Fusarium langsethiae, a newly identified species has been identified as the primary producer of HT-2+T-2 in European cereals (Imathiu, 2008; Edwards et al., 2012). There is limited data regarding this species' pathogenicity and mycotoxin production, but it is evident that its behaviour deviates from traditional trichothecenes producing *Fusarium* species (Imathiu, 2008). *Fusarium langsethiae* does not seem to produce any visible symptoms on oat plants or grains. Study of *F. langsethiae* has been hampered by the difficulty to artificially inoculate oats, wheat and barley and the lack of quick assays to quantify HT-2+T-2.

To enable the cereal industry and the European Commission to monitor and identify the factors associated with *F. langsethiae* and HT-2+T-2 production in UK cereals, the life-cycle and epidemiology of *F. langsethiae* will have to be fully understood.

Data obtained from field and through natural infection is difficult to explain due to factors beyond the control of the researcher. The development of artificial inoculation methods will allow for the study of *F. langsethiae* in the field and more importantly in the glasshouse allowing for a better understanding of *F. langsethiae*-cereal relationship.

Studying the differences in disease occurrence and severity in the different cereals will further enhance our understanding of *F. langsethiae*-cereal relationship and to determine if *F. langsethiae* infection and mycotoxins production is more prominent in any of these cereals.

Conducting field experiments with different cereals grown under identical agronomic conditions will identify if differences observed between *F. langsethiae* biomass and HT-2+T-2 within cereal species is due to genetic or agronomic differences.

Studying *F. langsethiae* development and subsequent HT-2+T-2 development both under field and glasshouse conditions will provide relevant information as to when (time/cereal growth stage), where (plant part), infection starts and how infection progresses through the plant during the different growth stages of the cereal. This will help in the understanding of the life-cycle of *F. langsethiae* and its epidemiology.

The main aim of this research was therefore to '**study the epidemiology and life-cycle of *F. langsethiae* in UK cereals**'. To achieve this main aim, the following specific objectives were set;

- To develop artificial inoculation method(s) to infect wheat, barley and oats with *F. langsethiae*

- To determine differences in disease development and severity in wheat, barley and oats caused by *F. langsethiae*

- To quantify *F. langsethiae* development and HT-2+T-2 production during crop development in artificially inoculated experiments of wheat, barley and oats

- To quantify *F. langsethiae* development and HT-2+T-2 production during crop development in commercial crops of wheat, barley, oats and triticale.

CHAPTER TWO

2. General materials and methods

2.1. Introduction

This chapter describes the methods that were routinely used in many of the experiments reported in this thesis.

2.1.2. Chemicals and reagents

All chemicals and reagents were sourced from Sigma (Dorset, UK) unless otherwise stated.

2.1.3. Isolation of *Fusarium langsethiae* from cereal grains

The method for isolating *F. langsethiae* from cereal grains was adopted from Imathiu (2008). Grains from each seed lot were mixed thoroughly after which about 20 g were taken into a 50 ml centrifuge tube and surface sterilized with sodium hypochlorite (1.2% available chlorine) amended with 0.05% Tween 20 for three minutes. Sterilized grain samples were then rinsed three times with sterile distilled water (SDW) and allowed to dry in Petri dishes in a laminar air flow cabinet. One hundred grains per seed lot were plated (five grains per plate) on potato dextrose agar (PDA, Merck, Germany) amended with streptomycin sulphate (130 µg ml^{-1}). Plates were incubated at room temperature (*ca*. 22^{o}C) for five to ten days. *Fusarium langsethiae* was sub-cultured onto fresh PDA plates, identified by micro- and macro-morphological characters (Torp and Nirenberg, 2004; Torp and Langseth, 1999) and confirmed using polymerase chain reaction (PCR) as described in Section 2.3.2.

2.1.4. Maintenance of fungal cultures

Actively growing mycelia of *F. langsethiae* isolated from cereal grains (Section 2.1.3.) were cut with a sterile a scalpel and inoculated onto PDA slopes. Inoculated slopes were then incubated at room temperature for three days after which they were stored at 4ºC.

2.1.5. Selection of single spore cultures

In order to ensure that isolates used for all experiments were pure, single spore cultures were made as described by Waller et al., (1998). *Fusarium langsethiae* isolates from PDA slopes (Section 2.1.4) were sub-cultured on PDA in Petri dishes. The spores were harvested from seven day old PDA plates as described in section 2.1.4. Spore suspension was then streaked onto water agar (20 g of agar litre^{-1} of tap water) along lines of an 'E' which had been made beneath the Petri dish with a sterile loop and incubated at room temperature for 24-48 h. Single germlings (representing a single germinating spore) were excised with a sterilized scalpel under a dissecting microscope onto freshly prepared PDA plates.

2.1.6. Production of inoculum (spore suspension)

Generally, unless stated, inoculum used for experiments was a conidial spore suspension. Inoculum was produced by culturing single spore isolates on PDA and incubating these at room temperature (*ca*. 22^{o}C) under natural light for 14 days. Spores were harvested by flooding individual cultures with SDW (*ca*. 5ml) and gently agitating culture surfaces with a sterilised L-shaped glass rod. The spore suspension was filtered through two layers of sterile muslin cloth to remove mycelia. The culture surface was then rinsed with SDW (*ca*. 2.5 ml), filtered and added to spore suspension. Spore concentration (spores ml^{-1}) was determined using a haemocytometer (Weber Scientific International, UK). Spore suspensions

were aliquoted into 5 ml volumes and stored at -20^{o}C. Viability of spores was assessed by culturing a 10-fold dilution series of spore suspension on Rose Bengal Chloramphenicol Agar (Merck, Germany).

2.2.1. DNA extraction from fungal cultures cultivated on PDA plates

To extract DNA from fungal cultures growing on PDA, mycelia were gently removed from the PDA surface, avoiding any agar, with a sterilized scalpel into 1.9 ml Eppendorf tubes. Two hundred and fifty microliters of chelex carbon buffer (1 g chelex 100 and 0.25 g charcoal (granular 20-60 mesh activated) made up to 20 ml using distilled water) was added and mycelia crushed using a sterile micropestle, incubated at 56^{o}C for 20 min, vortexed and allowed to cool at room temperature for 30 min. The tube content was vortexed, centrifuged at 12,000 x g for 15 min and 50 µl supernatant removed and added to 50 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and vortexed. Extracted DNA was stored at 4ºC and used directly for PCR reactions.

2.2.2. DNA extraction from fungal cultures cultivated in broth medium

To produce large quantities of pure fungal DNA, two *F. langsethiae* isolates (FL/2004/11 and FL/0/09/009/3) were cultured in potato dextrose broth (PDB). Fifty milliliters of PDA was autoclaved in a 1 L Erlenmeyer conical flask. The flask content was cooled at room temperature and inoculated with *F. langsethiae* spore suspension (5 x 10^{6} spores ml^{-1}). The content of the flask was mixed and incubated in an orbital incubator (80 rpm) (Sanyo, UK) at 20ºC for 5 days in darkness after which the contents was transferred into a sterile 50 ml tube and spun in a bench-top centrifuge for 5 min at 3,000 x g. The pellet formed was recovered by pouring of the supernatant and washing twice with SDW. The pellet was frozen at -20^{o}C for 24 hrs and freeze dried in an Edwards Modulyo drier (Edwards, Sussex, UK). The

freeze-dried sample was shaken by hand to powder with three sterile 8-mm diameter stainless steel ball bearings. Thirty milliliters of CTAB buffer (175.4 g NaCl, 46 g sorbitol, 20 g N-lauryl sarcosine, 16 g hexadecyl trimethylammonium bromide (CTAB), 15 g ethylenediamine tetraacetic acid (EDTA) and 20 g polyvinylpolypyrolidone (PVPP), made up to 2 L with distilled water) was added to the powdered sample, mixed and incubated at 65^{o}C for 1 hr. Ten milliliters of potassium acetate (5M) was then added to resulting sample, mixed, cooled on ice and frozen at -20^{o}C overnight.

Samples were thawed, 10 ml chloroform added, mixed for 1 min by gentle inversion and then centrifuged at 3,000 x g for 15 min. The aqueous phase was removed and added to an equal volume of 100% isopropanol in fresh 50 ml centrifuge tubes. The content was gently mixed by inversion and centrifuged at 3,000 x g for 15 min. The resulting DNA pellet was washed twice with 44% isopropanol, air-dried, re-suspended in 1 ml TE buffer and incubated at 65ºC for 25 min. Resulting DNA suspension was transferred into 1.9 ml Eppendorf tube and the 50 ml tubes washed twice with 0.5 ml TE buffer and the wash added to the Eppendorf tubes. The DNA suspension was vortexed and left at room temperature overnight before centrifuging at 12,000 x g for 5 min and stored at 4ºC.

2.2.3. DNA extraction from plant material

Plant materials were milled in a sample mill (Cyclotec 1093). Milled samples were mixed thoroughly by hand and 1.5 to 3 g (leaf and stem samples depending on total sample weight) and 5 g (grain samples) were weighed into 50 ml centrifuge tubes for DNA extraction. To each 50 ml centrifuge tube, 30 ml of CTAB buffer was added with adjustments made for leaf and stem samples. Tube content was then mixed thoroughly by hand and with an Hs501 digital shaker (IKA Labortechnik) for 20 minutes and incubated at 65ºC for 1 hour. Tubes were then shaken by hand

and centrifuged at 3,000 x g for 15 minutes after which 0.9 ml of the supernatant was removed and added to 0.3 ml potassium acetate (5M) in a sterile 1.9 ml Eppendorf tube, mixed for 1 minute and frozen at -20^{o}C for 1 hour. Tube contents were thawed at room temperature, 0.6 ml chloroform added, mixed for 1 min and centrifuged at 12,000 x g for 15 min. One milliliter of the aqueous phase was removed and added to a sterile 1.9 ml Eppendorf tube containing 0.8 ml of 100% isopropanol and mixed for 1 min before centrifuging at 12,000 x g for 15 min. Resulting DNA pellets were washed twice with 1 ml 44% isopropanol. Pellets were air dried before re-suspending in 0.2 ml TE buffer and incubating at 65ºC for 25 min. Tube contents were vortexed and left at room temperature overnight before spinning at 12,000 x g for 5 min. Extracts were stored either at 4°C (short term) or -20°C (long term).

2.2.4. Spectrophotometric determination of DNA concentration

With the exception of DNA extracted from stems and leaves of plants at Zadoks growth stages 90, 92 (Zadoks et al., 1974) and harvested plants which were diluted 10-fold, all others were diluted 50-fold before determining their concentrations. DNA concentration was determined by measuring absorbance using a scanning spectrophotometer (Beckman Instruments, USA) and using the Warburg-Christian equation, that is;

Nucleic Acid = (-36 x (Abs280 – Abs328)) + (62.9 x (Abs260 – Abs328)

DNA concentration was calculated. DNA extractions were diluted to 40 ng μl^{-1}. DNA from stems and leaves of plants at Zadoks growth stages 90 and at harvest were diluted to 20 ng μl^{-1} due to the low levels of initial total DNA recovered. After dilution DNA concentrations were checked again to determine the final working concentration.

2.3.1. Internal transcribed spacer (ITS) amplification

An initial control PCR was carried out on all DNA samples prior to any diagnostic and/or quantitative PCR to ensure the presence and amplification potential of DNA in the samples. This involved amplification with ITS4 and ITS5 primers (TCC TCC GCT TAT TGA TAT GC and GGA AGT AAA AGT CGT AAC AAG G respectively). These primers amplify any fungal or plant DNA present in a sample at an annealing temperature of 50°C. PCR was carried out using a 25 µl reaction mixture made up of 100 µM of each nucleotide, 100 nM of each primer, 20 U of *Taq* polymerase (ABgene, Epsom, UK) ml^{-1}, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 100 µg of gelatine ml^{-1}, 0.5 mg ml^{-1} of Tween 20, 0.5 mg ml^{-1} of Nonidet P-40 and 5 µl of DNA sample. Water (PCR grade) was used as negative control. Samples were amplified using a PTC-100 thermal cycler (MJ Research Inc., Minnesota, USA) programmed for initial denaturation at 94°C for 75 sec followed by 35 cycles of 15 seconds at 94°C, 15 sec at 50°C and 45 sec at 72°C. There was a final extension step at 72°C for 4 min 25 sec before cooling to 4°C until sample recovery. Amplicon gel electrophoresis was carried out on 2% agarose gels stained with ethidium bromide (0.05%). PCR products (*ca.* 650 bp amplicons in size) were viewed on a Gel Doc 1000 system (Bio-Rad, Buckinghamshire, UK) under UV light.

2.3.2. Quantitative PCR

DNA samples were amplified with a real-time PCR instrument (iCycler Bio-Rad, UK) with *F. langsethiae* primers; FlangF3 5'-CAAAGTTCAGGGCGAAAACT-3' and LanspoR1 5'-TACAAGAAGACGTGGCGATAT-3' (Wilson et al., 2004) as detailed previously (Edwards et al., 2012). qPCR MasterMix Plus for SYBR® Green I with flourescein (Eurogentec, USA) reagent was used according to manufacturer's instructions with a 25 µl reaction volume which included 5 µl template DNA. PCR water (5 µl) was used instead of template as a negative control. A 10-fold dilution

series of *F. langsethiae* DNA (10^{0}– 10^{-4}ng μl^{-1}) was included in each PCR run to provide a standard curve. Concentrations of *F. langsethiae* DNA were divided by the total DNA concentration within a sample to give values per ng of total DNA.

The real-time PCR conditions entailed an initial activation step (UNG) of 50^{o}C for 2:30 min and an initial melt of 10 min at 95^{o}C followed by 45 cycles with a melting step of 95^{o}C for 10 sec, annealing temperature of 65^{o}C for 10 sec, extension at 72^{o}C for 30 sec, and a hold at 82^{o}C for 10 sec during which fluorescence was measured. Melting curve florescence was determined by holding at 95^{o}C for 1 min, cooling to 55^{o}C for 1 min and then raising the temperature to 95^{o}C at a ramp rate of 0.05^{o}C sec^{-1}.

2.3.3. HT-2+T-2 estimation

T-2 in milled cereal head sub-samples was measured using the Ridascreen® T-2 ELISA assay (R-Biopharm AG,Darmstadt, Germany) following manufacturer's instructions. Total HT-2+T-2 was estimated based on the known ratio of HT-2 and T-2 in UK oats and the cross-reactivity of the T-2 antibody with HT-2 (Edwards, 2012).

CHAPTER THREE

3. Development of *Fusarium langsethiae* in commercial cereal production; a field survey.

3.1. Introduction

To understand the interaction between *F. langsethiae* and the different cereal species, assessment of the fungus at different physiological growth stages of the cereal is very important and according to Cooke (2006), data from field samples provide reliable information on the incidence, severity and spatial pattern of a disease under study. Data from field samples on the development of *F. langsethiae* in cereals is lacking. Researchers in the past few years have concentrated their efforts on the identification and quantification of *F. langsethiae* DNA and HT-2+T-2 mycotoxin levels in harvested cereal grains from the field (Edwards 2009a,b,c; Lukanowski et al., 2008; Infantino et al., 2007; Torp and Nirenberg, 2004; Torp and Langseth, 1999).

Apart from common problems associated with field studies such as the difficulty in explaining data as a result of a number of variables which the researcher has no or little control over, there are no known symptoms of *F. langsethiae* infection in the field, compounding the problems of field study of this fungus.

The aim of this work was to quantify *F. langsethiae* development and HT-2+T-2 production during crop development in commercial cereal rotations of wheat, barley, triticale and oats.

Null hypotheses

1. *Fusarium langsethiae* is not a pathogen of commercial crops of wheat, barley, triticale and oats in the UK.

2. *Fusarium langsethiae* has no preference between cereal species.

3. *Fusarium langsethiae* and the mycotoxins HT-2+T-2 cannot be detected in commercial crops of wheat, barley, triticale and oats in the UK

3.2. Materials and Methods

3.2.1. Study fields and general sampling

To assess the infection and development of *F. langsethiae* in cereals in the UK, a survey was carried out in commercial fields of wheat, barley, oats and triticale in Shropshire and Staffordshire during the 2009, 2010 and 2011 cropping seasons. All fields were within 30 km of Harper Adams University College, Newport, Shropshire UK. In all cropping seasons sampling was done between April and August. A survey was carried out in 25, 27 and 26 different fields in the 2009, 2010 and 2011 cropping seasons respectively (Table 3.1). Samples were taken between Zadoks growth stages (GS) 22 to GS92 (Zadoks et al., 1974).

Table 3.1. Commercial fields sampled in 2009 and the previous crop cultivated in the field

Field	Cereal	Variety	Previous crop
O1	Winter oats	Gerald	potatoes
O2	Winter oats	Gerald	winter wheat
O3	Winter oats	Gerald	potatoes
O4	Winter oats	Gerald	winter wheat
O5	Winter oats	Gerald	winter wheat
O6	Winter oats	Gerald	winter oats
O7	Winter oats	Gerald	winter wheat
O8	Winter oats	Gerald	winter wheat
O9	Winter oats	Gerald	winter wheat
O10	Winter oats	Gerald	winter oats
SB1	Spring barley	Tipple	winter wheat
SB2	Spring barley	Quench	potatoes
SB3	Spring barley	Quench	winter wheat
SB4	Spring barley	Quench	winter wheat
SB5	Spring barley	Quench	maize
WB1	Winter barley	Carat	winter wheat
WB2	Winter barley	Saffron	winter wheat
WB3	Winter barley	Saffron	winter wheat
WB4	Winter barley	Pearl	winter barley
WB5	Winter barley	Sequel	oil seed rape
WW1	Winter wheat	Alchemy	winter oats
WW2	Winter wheat	Alchemy	winter oats
WW3	Winter wheat	Alchemy	winter wheat
WW4	Winter wheat	Alchemy	winter wheat
WW5	Winter wheat	Alchemy	winter wheat

In the 2010 cropping season, 33 fields comprising seven oats, six spring wheat, six winter wheat, five winter barley, five spring barley and four triticale fields were sampled (Table 3 2).

Table 3.2. Commercial fields sampled in 2010 and the previous cultivated in thefield

Field	Cereal	Variety	Previous crop
O1	Winter oats	Gerald	Winter oats
O2	Winter oats	Gerald	Spring wheat
O3	Winter oats	Gerald	Spring wheat
O4	Winter oats	Gerald	Spring wheat
O5	Winter oats	Gerald	Spring wheat
O6	Winter oats	Gerald	Spring wheat
O7	Winter oats	Gerald	Winter oats
SB1	Spring barley	Propino	Maize
SB2	Spring barley	Quench	Spring barley
SB3	Spring barley	Quench	Spring barley
SB4	Spring barley	Quench	Spring barley
SB5	Spring barley	Quench	Grass
T1	Triticale	Grenado	Spring barley
T2	Triticale	Grenado	Spring barley
T3	Triticale	Grenado	Maize
T4	Triticale	Grenado	Maize
WB1	Winter barley	Saffron	Wheat
WB2	Winter barley	Carat	Wheat
WB3	Winter barley	Saffron	Fallow
WB4	Winter barley	Saffron	Potatoes
WB5	Winter barley	Saffron	Winter wheat
WW1	Winter wheat	Alchemy	Winter oats
WW2	Winter wheat	Alchemy	Winter oats
WW3	Winter wheat	Alchemy	Winter oats
WW4	Winter wheat	Alchemy	Winter oats
WW5	Winter wheat	Alchemy	Spring barley
SW1	Spring wheat	AC Barrie	Winter wheat
SW2	Spring wheat	AC Barrie	Winter wheat
SW3	Spring wheat	AC Barrie	Winter wheat
SW4	Spring wheat	AC Barrie	Winter wheat
SW5	Spring wheat	AC Barrie	Winter wheat
WW6	Winter wheat	Alchemy	Winter barley
SW6	Spring wheat	AC Barrie	Winter wheat

In 2011, 27 fields were sampled comprising of seven oat, five spring barley, five winter barley, five winter wheat and four triticale fields (Table 3.3).

Table 3.3. Commercial fields sampled in 2011 and previous crop cultivated in the field

Field	Cereal	Variety	Previous crop
O1	Winter oats	Gerald	Winter oats
O2	Winter oats	Gerald	Winter wheat
O3	Winter oats	Gerald	Spring wheat
O4	Winter oats	Gerald	Spring wheat
O5	Winter oats	Gerald	Spring wheat
O6	Winter oats	Gerald	Spring wheat
O7	Winter oats	Gerald	Winter oats
SB1	Spring barley	Propino	Maize
SB2	Spring barley	Quench	Spring barley
SB3	Spring barley	Quench	Spring barley
SB4	Spring barley	Quench	Spring barley
SB5	Spring barley	Quench	Grass
T1	Triticale	Grenado	Spring barley
T2	Triticale	Grenado	Spring barley
T3	Triticale	Grenado	Maize
T4	Triticale	Grenado	Maize
WB1	Winter barley	Saffron	Wheat
WB2	Winter barley	Carat	Wheat
WB3	Winter barley	Saffron	Fallow
WB4	Winter barley	Saffron	Potatoes
WB5	Winter barley	Saffron	Winter wheat
WW1	Winter wheat	Alchemy	Winter oats
WW2	Winter wheat	Alchemy	Winter oats
WW3	Winter wheat	Alchemy	Winter oats
WW4	Winter wheat	Alchemy	Winter oats
WW5	Winter wheat	Alchemy	Winter oats

In each field, a plot of 10 x 10 m was marked out from which samples were taken. Twenty whole plants were taken randomly from each plot. At harvest (GS92) weeds from three oat fields, predominantly Italian rye grass from two oat fields and wild oats from one field were also sampled at harvest. Samples were visually assessed for any disease symptoms before processing.

3.2.2. Processing of samples

Plant stem bases and roots were washed free of soil and divided into sub-samples depending on the growth stage of the plant. At GS23 to GS29 about 4 cm of the lower stem was taken as a sub-sample. Between GS30 and GS39, two sub-samples were taken, lower stem base and leaves. Leaves, lower stem base and upper stem were taken for plants at GS40 to GS49. For plants at GS59–GS92 lower stem base, leaves and inflorescence or heads were taken as sub-samples. All cereal heads were examined for fusarium head blight symptoms before processing. Sub-samples were put in labelled paper bags, frozen (-18^{o}C) for at least 24 hours after which they were freeze-dried for five days in a Modulyo freeze drier (Edwards, Sussex, UK). Freeze-dried samples were milled in a laboratory mill with a 1 mm screen (Cyclotec 1093 or IKA MF10).

DNA extraction, spectrophotometric determination of DNA concentration, ITS and QPCR were carried out as detailed in Section 2.2.3-2.3.1, except that for 2011 samples DNA concentrations were determined using a Nanodrop 2000 Spectrophotometer (Thermo Scientific). HT-2+T-2 quantification was carried out as detailed in Section 2.3.3

Weather data was obtained from the Harper Adams University College weather station (http://weather.harper-adams.ac.uk/)

3.2.3. Data analysis

Mean DNA values were calculated using Microsoft Excel (V. 2010, Microsoft). In sub-samples with detectable but not quantifiable (i.e. below the limit of quantification (LoQ)) *F. langsethiae* DNA were given a value of half the LoQ (0.0005 pg ng^{-1}) to allow for the estimation of mean values. A linear regression of *F. langsethiae* DNA against HT-2+T-2 concentration grouped by cereal and year of sampling was carried out to determine if there was a relationship between the parameters under study using Genstat (V.13 VSN International Ltd.).

3.3. Results

Head blight symptoms were not observed on any of the cereal head sub-samples.

The initial control PCR (ITS4 and ITS5) showed that DNA from all stem, leaf and head sub-samples was amplifiable. However, DNA could only be amplified in 60% and 65% of the root samples from 2010 and 2011 respectively. There was no detectable *F. langsethiae* DNA in any of the root samples with amplifiable DNA. There was also no *F. langsethiae* DNA in any of the weeds sampled at harvest. *Fusarium langsethiae* DNA could be detected in a small number of sub-samples but could not be quantified in any sub-samples before GS30.

There was a yearly variation in *F. langsethiae* DNA levels in head sub-samples at harvest. Levels in oat decreased from 2009 to 2011 whilst levels in the other cereals did not follow a particular pattern over time (Figure 3.1).

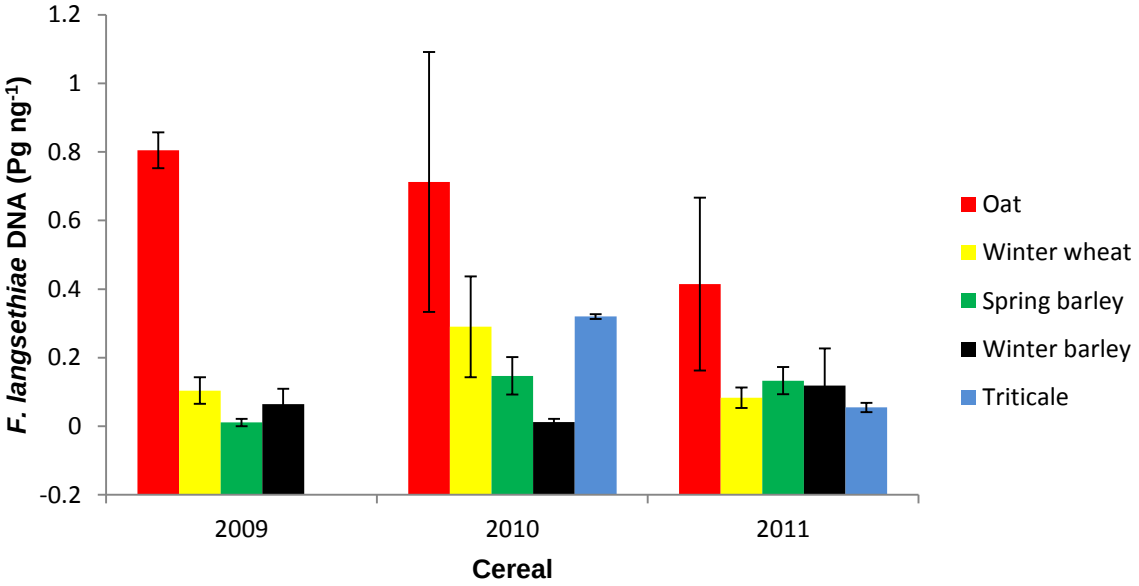

Figure 3.1. Mean *F. langsethiae* DNA in cereal head sub-samples of oat, winter wheat, spring barley, winter barley and triticale at harvest (G92) in 2009, 2010 and 2011 cropping seasons. Bars represent standard error.

Development of *F. langsethiae* in all cereals studied was similar (based on pattern *of F. langsethiae* DNA in different plant parts over three years) but most differences were distinct for oats due to the higher levels of *F. langsethiae* DNA observed in this cereal (Figure 3.2).

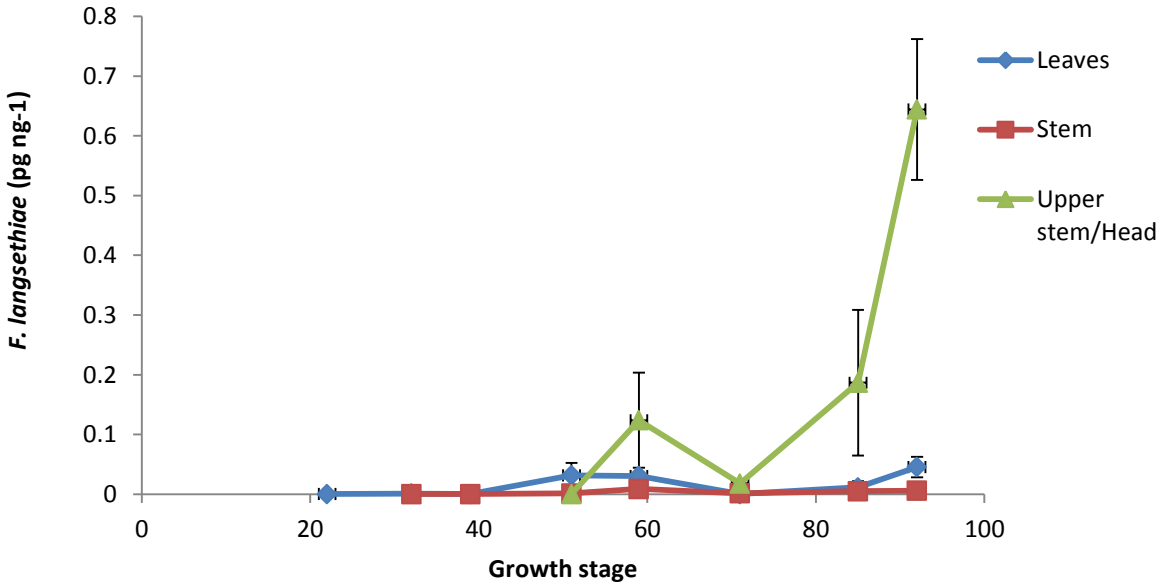

Figure 3.2. Mean *F. langsethiae* DNA in oat plants under commercial cultivation in 2009, 2010 and 2011 cropping seasons. (n = 24). Bars represent standard error

In each year of sampling, the level of HT-2+T-2 in the heads of wheat, barley and oat followed the same trend as *F. langsethiae* DNA that was amplified in the heads. Analysis of variance (at 95% confidence level and using Tukey test to separate the means) revealed significant differences in HT-2+T-2 concentration for each year of sampling with oats (P=0.005), winter wheat (P=0.033) and spring barley (P=0.007). In winter barley, however, the difference in HT-2+T-2 concentrations over the three years was not statistically significant (P=0.11) (see detailed results on wheat, barley and oat for mean HT-2+T-2 values; pages 65-75).

Weather data showed yearly differences in maximum and minimum temperatures as well as relative humidity values, especially during sampling weeks of the two important growth stages (GS59 and GS92), based on *F. langsethiae* DNA concentrations in head sub-samples. In 2009 winter barley reached GS59 at sampling point 3 whilst the other cereals reached the same growth stage at sampling point 4. GS92 was reached at sampling points 5 and 6 for winter barley

and all the other cereals respectively (Figure 3.3A). In 2010 GS59 was reached at sampling points 2 and 3 for winter barley and the other cereals respectively and GS92 at sampling points 4 and 5 respectively (Figure 3.3B). In 2011 at first sampling (1) winter barley was at GS59 whilst the same GS was reached by the other cereals at sampling point 3. GS92 was reached at sampling points 5 and 6 for winter wheat and the other cereals respectively (Figure 3.3C). Due to the relevance of these two growth stages (GS59 and 92), weekly mean maximum, minimum and relative humidity values were calculated by taking the day of sampling as the last day of the week (Table 3.4).

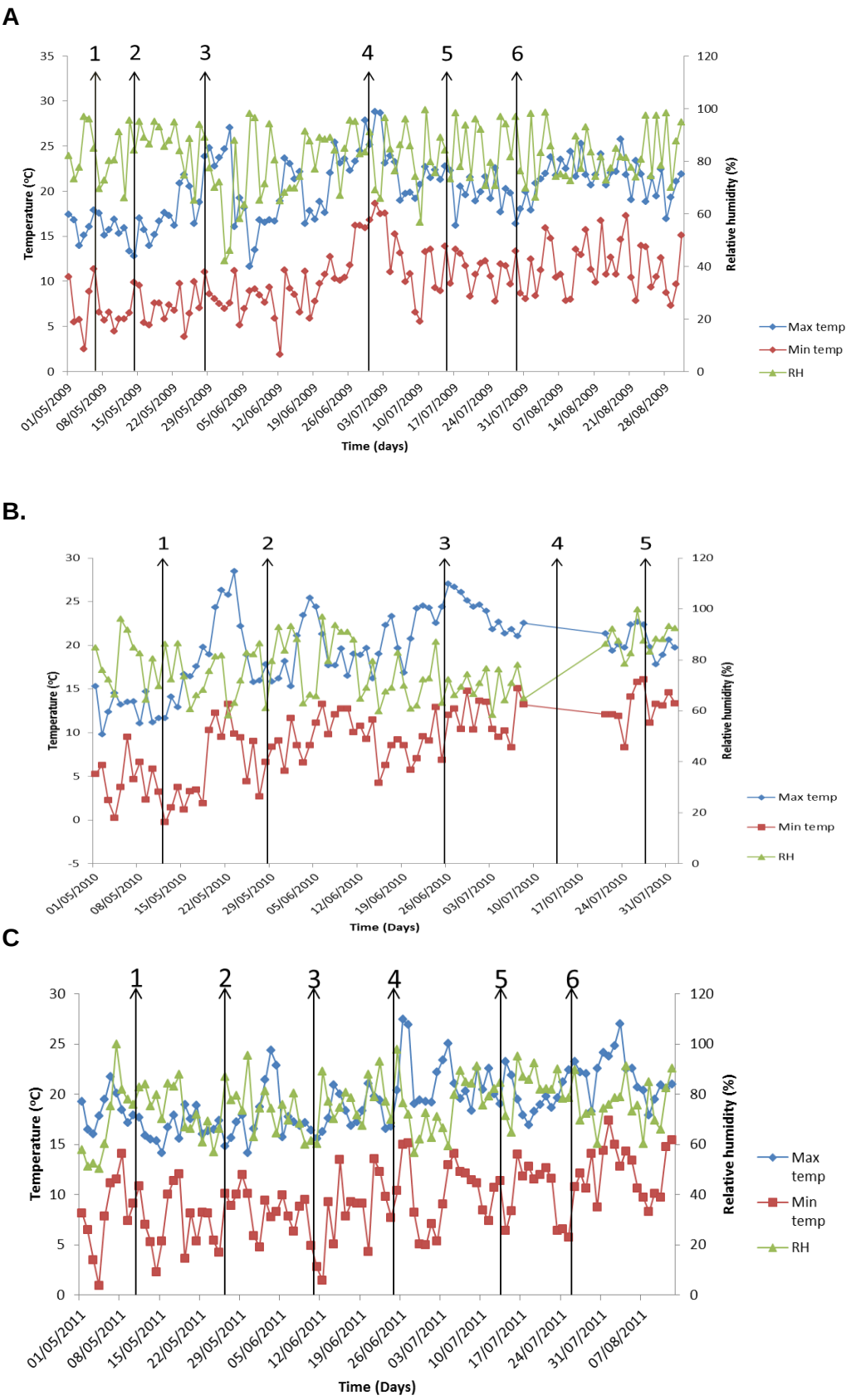

Figure 3.3 Weather data for sample years and periods A, 2009, B 2010 and C 2011.

Table 3.4. Mean temperatures and mean relative humidity at GS59 and GS92

Week of sampling	Max. temperature (°C)			Min. temperature (°C)			Relative humidity (%)		
Oat	2009	2010	2011	2009	2010	2011	2009	2010	2011
GS59	24.2	23.9	17.7	13.9	9.0	7.9	85.6	69.9	68.6
GS92	19.1	20.9	20.5	10.5	12.8	9.4	85.2	87.1	83.3
Winter barley									
GS59	22.6	21.9	18.4	7.9	8.3	10.2	64.7	75.0	82.4
GS92	21.9	21.0	20.6	10.6	_13.0	10.5	79.7	74.0	83.8

Oats

In all three years of sampling *F. langsethiae* DNA in sub-samples between GS22 and GS39 were below the LoQ except in upper stem sub-samples in 2009 and leaf sub-samples in 2011 (Table 3.5).

At GS59, relative to the previous growth stage sampled, there was an increase in *F. langsethiae* DNA in all sub-samples in all sampling years except in 2010 where there was a 55% decrease in leaf sub-samples and in 2011 where there was a decrease of 4% in stem sub-samples (Table 3.5). In both 2010 and 2011 sampling years *F. langsethiae* DNA in head sub-samples increased by over a 100% between GS49/51 and GS59. Mean *F. langsethiae* DNA in head sub-samples at GS59 ranged between 0.027 and 0.679 pg ng^{-1} in 2009, between 0.01 and 0.29 pg ng^{-1} in 2010 and between 0.0005 and 0.0496 in 2011 (Table 3.5). After GS59 *F. langsethiae* DNA in head sub-samples increased gradually over the growing period peaking at GS92 with values ranging from 0.449 to 1.544 pg ng^{-1} in 2009, 0.046 to 2.849 pg ng^{-1} in 2010 and 0.033 to 1.877 pg ng^{-1} in 2011. In leaf and stem sub-samples however, although relatively high *F. langsethiae* DNA was recorded at GS92 increases over the growing period was not linear. For example, in 2009 there was a decrease in *F. langsethiae* DNA in stem sub-samples from GS59 through to GS92 (see Table 3.5).

Table 3.5. Mean *F. langsethiae* DNA (pg ng^{-1}) in oat sub-samples in 2009, 2010 and 2011

	2009			2010			2011		
Growth stage	Leaves	Stem	Upper stem/head	Leaves	Stem	Upper stem/head	Leaves	Stem	Upper stem/head
22	<LoQ	N/A	N/A	_	_	_	_	_	_
32	<LoQ	<LoQ	N/A	<LoQ	<LoQ	<LoQ	0.0024	<LoQ	N/A
39	<LoQ	<LoQ	0.0230	_	_	_	_	_	_
49/51	_	_	_	0.0630	<LoQ	<LoQ	<LoQ	0.0023	0.0006
59	0.0410	0.0240	0.2830	0.0280	0.0010	0.0860	0.0235	0.0022	0.0031
71	_	_	_	_	_	_	<LoQ	0.0019	0.0180
80/85	<LoQ	0.0150	0.4300	0.0340	<LoQ	0.0760	<LoQ	<LoQ	0.0546
92	0.0700	<LoQ	0.8050	0.0586	0.0038	0.7120	0.0084	0.0014	0.4152

N/A = no sub-sample taken, - = no sampling.

F. langsethiae DNA in head-sub-samples at harvest decreased gradually over the sampling years with that in 2011 sub-samples having the least.

HT-2+T-2 values ranged between 1232-10777, 75-2905 and 425-2598 µg kg^{-1} with means of 4300, 1505 and 1229 ug kg^{-1} in 2009, 2010 and 2011 respectively. A simple linear regression showed a good correlation between HT-2+T-2 and *F. langsethiae* DNA with the regression being highly significant (P<0.001). A coefficient of determination (r^2) of 0.52% was obtained (Figure 3.4).

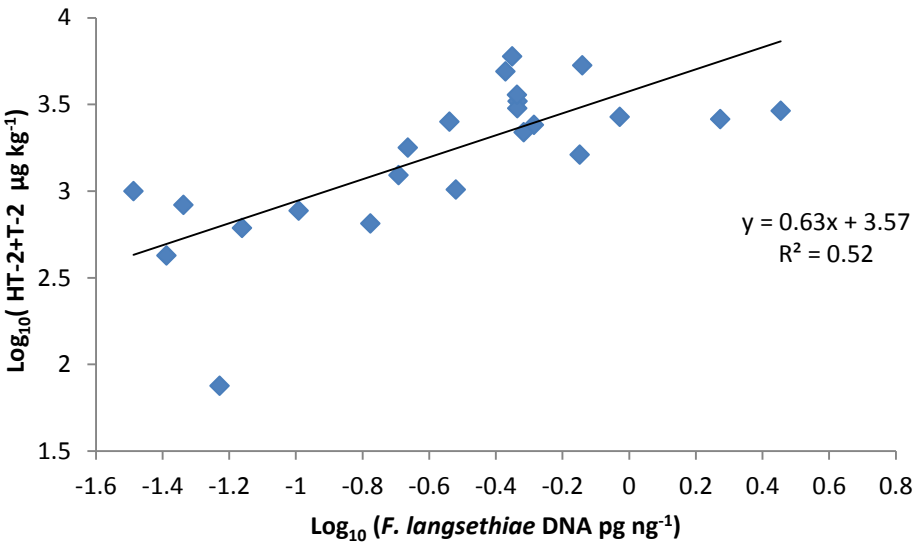

Figure 3.4. Relationship between *F. langsethiae* DNA and HT-2+T-2 in oat heads from 24 fields in Shropshire and Staffordshire from 2009 to 2011 cropping seasons

Winter wheat

With the exception of 2009, sampling for winter wheat started at GS33. In 2009, *F. langsethiae* DNA recovered from all sub-samples between GS22 and GS55 were below the LoQ. In 2010 and 2011, however, a number of fields had levels above the LoQ at these growth stages (Table 4.6). In 2009 quantifiable levels of *F. langsethiae* DNA was detected in sub-samples at GS59. During the same year there was over a 100% increase in *F. langsethiae* DNA in all sub-samples between GS51/55 and GS59/60. At GS59, mean *F. langsethiae* DNA ranged between 0.0005 and 0.183 pg ng^{-1} in leaf sub-samples, below 0.0005 to 0.028 pg ng^{-1} in stem sub-samples and below 0.0005 to 0.066 pg ng^{-1} in head sub-samples.

F. langsethiae DNA increased gradually from GS59 through to GS92 in stem and head sub-samples in 2009 and in head sub-samples in 2010 (Table 4.6). This was not the case in the other sub-samples during the sampling years. However, there was a consistent increase in *F. langsethiae* DNA between GS80/85 and GS92 in all sub-samples in all sampling years except in stem sub-samples in 2011. The increase in *F. langsethiae* DNA between GS80/85 and GS92 in head sub-samples was 1% in 2009, 90% in 2010 and above 100% in 2011 sampling years (Table 3.6).

The highest mean *F. langsethiae* DNA in head sub-samples at harvest was recorded in 2010 with values ranging between 0.0450 and 0.7377 pg ng^{-1}, with samples collected in 2011 having the lowest *F. langsethiae* DNA concentration with values ranging between 0.0005 and 0.1803 pg ng^{-1}.

Table 3.6. Mean *F. langsethiae* DNA (pg ng^{-1}) in winter wheat sub-samples in 2009, 2010 and 2011

	2009			2010			2011		
Growth stage	Leaves	Stem	Upper stem/head	Leaves	Stem	Upper stem/head	Leaves	Stem	Upper stem/head
22		<LoQ	N/A	_	_	_	_	_	_
33/35	<LoQ	<LoQ	N/A	0.0200	0.0180	N/A	<LoQ	<LoQ	N/A
51/55	<LoQ	<LoQ	<LoQ	0.0530	0.0500	<LoQ	0.0032	<LoQ	<LoQ
59/60	0.0650	0.0110	0.0240	_	_	_	0.0030	0.0119	0.0239
69	_	_	_	0.2290	0.0030	0.0210	0.0190	<LoQ	0.1278
80/85	0.0165	0.0140	0.1030	0.0210	<LoQ	0.1260	0.0006	0.0059	0.0129
92	0.0290	0.0370	0.1040	0.1600	0.0200	0.2400	0.0168	0.0037	0.0835

N/A = no sub-sample taken, - = no sampling.

HT-2 and T-2 levels followed a similar trend as that observed in *F. langsethiae* DNA over the sampling years. Mean HT-2+T-2 values were 363, 556 and 96 µg kg^{-1} and ranged between 111-600, 142-920 and 51-142 µg kg^{-1} in 2009, 2010 and 2011 respectively. A positive correlation was observed between log-transformed *F. langsethiae* DNA levels and HT-2+T-2 levels in harvested heads with a significant regression (P=0.017, r^2=0.42) (Figure 3.5)

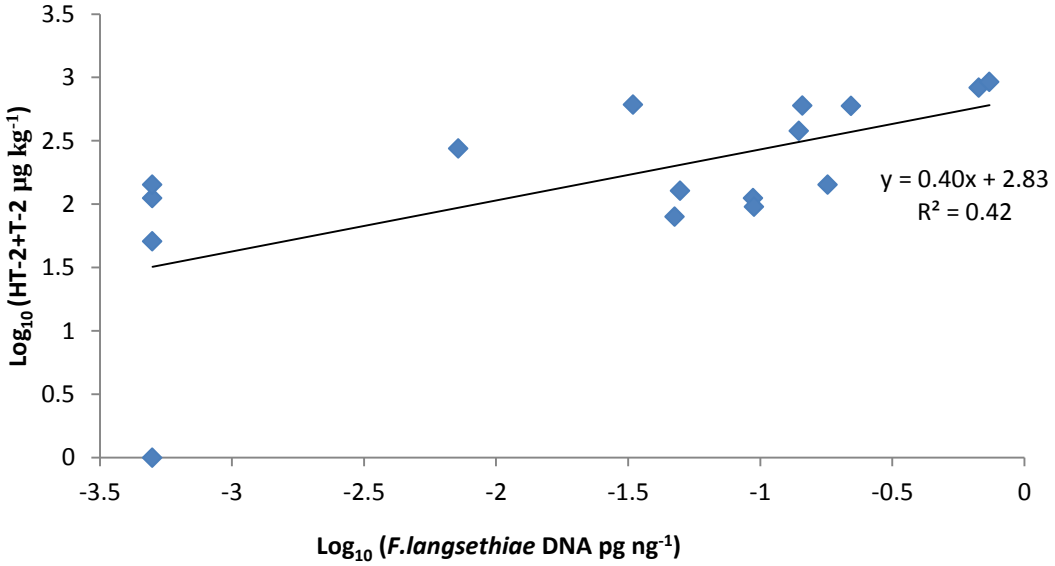

Figure 3.5. Relationship between *F. langsethiae* DNA and HT-2+T-2 in winter wheat heads from all 16 fields in Shropshire and Staffordshire from 2009 to 2011 cropping seasons

Spring wheat

Spring wheat fields were sampled in 2010 only. The pattern of *F. langsethiae* biomass development did not differ much from that in winter wheat. A significant observation was the high levels of *F. langsethiae* DNA in leaf and stem sub-samples at GS34. This however decreased gradually over the growing period to a mean of 0.022, 0.020 and 0.030 pg ng^{-1} in leaf, stem and head sub-samples respectively at plant maturity (Table 3.7).

Table 3.7. Mean *F. langsethiae* DNA (pg ng^{-1}) in spring wheat sub-samples in 2010

	2010		
Growth stage	leaves	stem	Upper stem/head
34	0.2980	0.0480	
51	0.1140	0.0005	0.0005
59	0.1513	0.0030	0.0013
69	0.1890	0.0020	0.0005
85	0.0110	0.0005	0.0050
92	0.0220	0.0200	0.0295

Winter Barley

Sampling of winter barley fields started at different growth stages over the three year period. The first sampling was done at GS32 in 2009, at GS49/51 in 2010 and GS59 in 2011 (Table 3.8).

Although there was a general increase in *F. langsethiae* DNA over the growing season in the three years of sampling (except in stem sub-samples in 2009) (Table 3.8), this increase was not linear in any sub-sample. However, there was a consistent increase in *F. langsethiae* in leaf and head sub-samples between GS49/51 and 59 and GS85 and GS92 except in 2010 where there was a decrease of 70% in leaf sub-samples and 14% in head sub-samples between GS85 and GS92. In 2009, increase in *F. langsethiae* DNA in leaf and head sub-samples between GS85 and GS92 was above 100%. In 2011 between the same growth stages, increase in *F. langsethiae* DNA was 82% in leaf sub-samples and again above a 100% in head sub-samples (Table 3.8). *F. langsethiae* DNA in winter barley heads at harvest was highest in 2011 with values ranging from 0.0035 to 0.5503 pg ng^{-1} with samples collected in 2010 having the lowest concentration with values ranging from 0.0005 to 0.05 pg ng^{-1} (Table 3.8.)

Table 3.8. Mean *F. langsethiae* DNA (pg ng^{-1}) in winter barley sub-samples in 2009, 2010 and 2011

	2009			2010			2011		
Growth stage	Leaves	Stem	Upper stem/head	Leaves	Stem	Upper stem/head	Leaves	Stem	Upper stem/head
32	<LoQ	<LoQ	N/A	_	_	_	_	_	_
49/51	0.0148	<LoQ	<LoQ	0.0270	0.0080	0.0010	_	_	_
59	0.0698	<LoQ	0.0070	0.0805	<LoQ	0.1395	0.0295	<LoQ	0.0514
69	_	_	_	0.0078	0.0026	0.0088	<LoQ	0.0035	0.0143
85	<LoQ	<LoQ	0.0042	0.1070	0.0430	0.0140	0.0028	0.0164	0.0128
92	0.2200	<LoQ	0.0650	0.0320	0.0001	0.0120	0.0051	0.0340	0.1185

N/A = no sub-sample taken, - = no sampling.

The highest HT-2+T-2 values were recorded in 2010 with the lowest recorded in 2009. Mean HT-2+T-2 values were 278, 559 and 403 µg kg^{-1} with values ranging between 124-639, 152-735 and 246-462 µg kg^{-1} in 2009, 2010 and 2011 respectively. Although a positive correlation was seen between log-transformed *F. langsethiae* DNA and HT-2+T-2 levels in heads, the regression was not significant ($P=0.075$, $r^2=0.22$) (Figure 3.6).

Spring Barley

In spring barley, samples taken before GS59 had *F. langsethiae* DNA below the LoQ except in leaf sup-samples taken in 2010 (Table 3.9).

From GS59, when quantifiable *F. langsethiae* DNA was observed, there was an increase in all sub-samples over the sampling period (although this was not linear) peaking at GS92 except in 2009 where there was 88% decrease in *F. langsethiae* DNA in stem sub-samples between GS85 and GS92. Increase in *F. langsethiae* DNA between GS85 and GS92 in leaf and head sub-samples respectively was above 100% and 22% in 2009 and above 100% in both sub-samples in 2010. In 2011 however, although there was an increase of 73% in leaf sub-samples, *F. langsethiae* DNA in head sub-samples decreased by 20% between GS85 and GS92 (Table 3.9).

Table 3.9. Mean *F. langsethiae* DNA (pg ng-1) in spring barley sub-samples in 2009, 2010 and 2011

	2009			2010			2011		
Growth stage	leaves	stem	Up.stem/head	leaves	stem	Up.stem/head	leaves	stem	Up.stem/head
32	0.0005	0.0005	N/A				0.0005	0.0005	N/A
51	_	_	_	0.0150	0.0005	0.0005	0.0005	0.0005	0.0005
59	0.0005	0.0005	0.0100				0.0347	0.0072	0.0114
69	_	_	_	0.1970	0.0200	0.0060	0.0005	0.0053	0.1073
85	0.0230	0.0340	0.0090	0.2410	0.0005	0.0070	0.0189	0.0045	0.1677
92	0.2730	0.0010	0.0110	0.5190	0.1180	0.1470	0.0328	0.0276	0.1330

N/A = no sub-sample taken, - = no sampling.

F. langsethiae DNA in head sub-samples at harvest was highest in samples collected in 2010 with values ranging between 0.0028 and 0.3342 pg ng^{-1} with samples collected in 2009 having the lowest with values ranging between 0.0005 and 0.0560 pg ng^{-1}.

HT-2+T-2 values over the sampling years followed a similar pattern to *F. langsethiae* DNA values at harvest. Values ranged between 75-568, 531-625 and 75-568 µg kg^{-1} with mean values of 186, 573 and 208 µg kg^{-1} in 2009, 2010 and 2011 respectively. A correlation was obtained between log-transformed *F. langsethiae* DNA in head sub-samples at maturity and HT-2+T-2 levels (Figure 3.6), with a statistically significant regression (P=0.004, r^2=0.48).

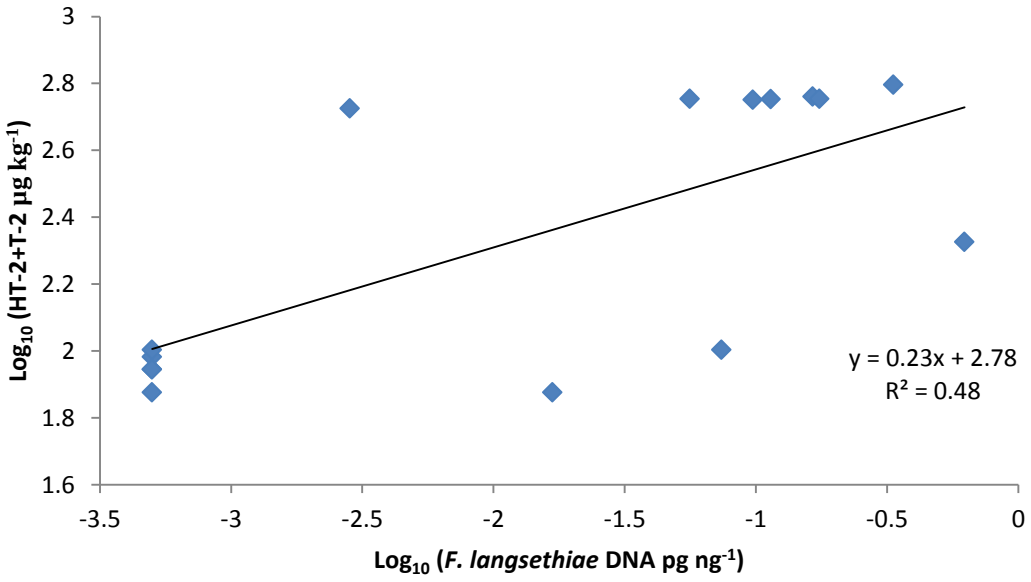

Figure 3.6. Relationship between *F. langsethiae* DNA and HT-2+T-2 in spring barley heads from 15 fields in Shropshire and Staffordshire from 2009 to 2011 cropping seasons

Triticale

Triticale fields were sampled in 2010 and 2011 only. In 2010, *F. langsethiae* DNA in samples collected before GS59 had values below the LoQ except in stem sub-samples whilst all samples collected in 2010 had values below the LoQ even at GS59 (Table 3.10).

Table 3.10. Mean *F. langsethiae* DNA (pg ng^{-1}) in triticale sub-samples in 2010 and 2011

Triticale	2010			2011		
Growth stage	Leaves	Stem	Upper stem/head	Leaves	Stem	Upper stem/head
49	_	_	_	<LoQ	<LoQ	<LoQ
55	<LoQ	0.0030	<LoQ	_	_	_
59	0.0270	<LoQ	0.0010	<LoQ	<LoQ	<LoQ
69	0.3270	0.0020	0.0020	<LoQ	0.0018	<LoQ
71	_	_	_	0.1658	<LoQ	0.0055
85	0.0040	<LoQ	0.0100	<LoQ	0.0018	0.0078
92	0.4470	0.0120	0.3200	0.0268	0.0198	0.0552

- = no sampling.

From the growth stage when quantifiable *F. langsethiae* DNA was observed in sub-samples, it increased in all sub-samples over time with highest values being recorded at GS92 in all sampling years. These increases were however, not linear in leaf and stem but they were in head sub-samples (Table 3.10). At harvest *F. langsethiae* DNA in head sub-samples was higher in 2010 with values ranging from 0.0180 to 0.8190 pg ng^{-1} and 0.0367 to 0.0947 pg ng^{-1} in 2011.

Recorded HT-2+T-2 values were highest in 2010 with values ranging from 320-599 µg kg^{-1} and a mean of 510 µg kg^{-1} HT-2+T-2 values for 2011 ranged between 97-212 µg kg^{-1} , with a mean of 124 µg kg^{-1}. The regression between *F. langsethiae* DNA and HT-2+T-2 was not significant ($P = 0.13$, $r^2 = 0.34$) (Figure 3.7).

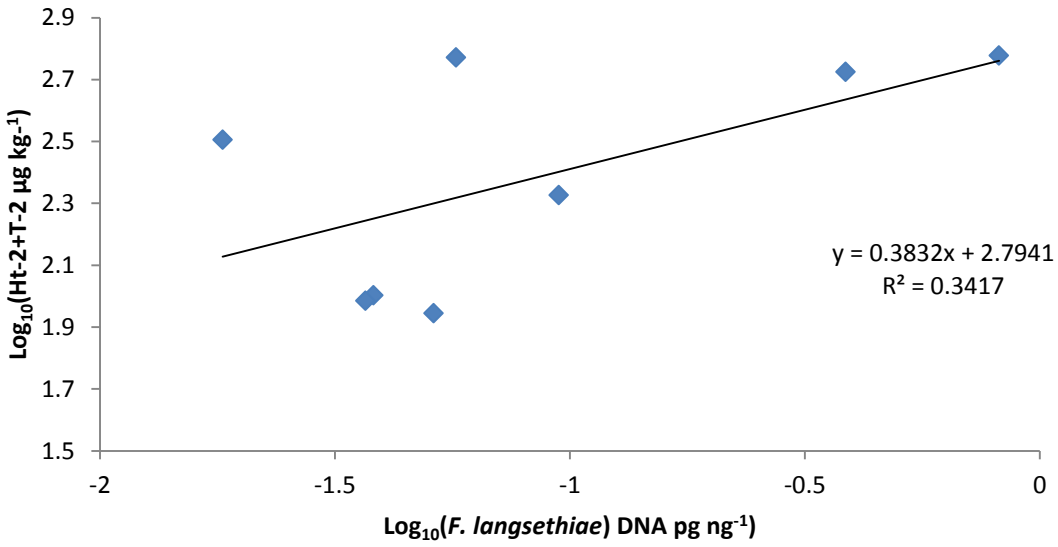

Figure 3.7. Relationship between *F. langsethiae* DNA and HT-2+T-2 in triticale heads from 10 fields in Shropshire and Staffordshire from 2010 to 2011 cropping seasons

3.4. Discussion

Results from this study have highlighted three important facts: 1. *Fusarium langsethiae* biomass and HT-2+T-2 in harvested heads of the cereals differed, with oats having the highest concentrations in all three years of the study. 2. There was a yearly difference in *F. langsethiae* biomass as well as HT-2+T-2 concentrations and 3. The development of *F. langsethiae* in the cereals studied over the sampling periods followed a similar pattern.

The absence of *F. langsethiae* DNA in sampled weeds suggests that the weeds may not be alternative hosts to *F. langsethiae*. However, since the sampling size was small, there is the need for further sampling of a broader range of weed species to confirm this.

Fusarium langsethiae biomass in the cereals was estimated using the amount of fungal DNA quantified per unit of plant DNA. The amount of *F. langsethiae* DNA in the heads of cereals at harvest was found to be highest in oat followed by that in

wheat, barley and triticale. These levels correlated well with HT-2+T-2 levels. This indicates that there may be a stronger association between *F. langsethiae* and oats than other cereal species. Associations between *Fusarium* species and different plant species is not a new phenomenon and this trend may even vary from region to region. *Fusarium moniliforme* (*verticillioides*) and *F. subgutinans* tend to be more associated with infected maize heads than wheat, barley or oat (Doohan et al. 2003). Again it is on record that in the USA, *F. graminearum* is the predominant causal agent of FHB in wheat (Doohan et al., 1998; Xu et al., 2005). However, in Europe it is caused by a number of *Fusarium* species under different conditions. Dry and warm conditions favour infection by *F. poae,* whereas warm and humid conditions tend to favour *F. graminearum* infection and *F. culmorum* infection is favoured by cool, wet or humid conditions. The two *Microdochium* species that are involved in head blight infection occur in cool to moderate temperatures with frequent rain showers (Xu et al., 2008).

Field data regarding the occurrence of *F. langsethiae* is lacking, but there is some data on levels of HT-2+T-2 in cereals. Occurrence data from three European countries (Sweden, Finland and Norway) in 2007 showed that among wheat, barley and oat, lower levels of HT-2 and T-2 occurred in wheat, moderate levels in barley and high levels in oat (Edwards et al., 2009). In contrast, a study carried out in the UK between 2001 and 2005 showed that the concentration of HT-2+T-2 was highest in oats followed by wheat and then barley (Edwards, 2009a,b,c). This is in agreement with the findings from the present study.

The reason for the stronger association between *F. langsethiae* and oats is not yet understood, but some speculations can be made. The occurrence of FHB is not as obvious in oat as it is in wheat and barley (Brown and Cooke, 2005). Some authors have attributed this to the architecture of the oat panicle. The oat panicle is

composed of open rachis from which loose spikelets arise. This structure results in single spikelet infection and thus a slower rate of spread of *Fusarium* infection compared to the denser head architecture found in wheat and barley (Kosova et al., 2009). *F. langsethiae* is thought to be a poor competitor compared to the other *Fusarium* species responsible for FHB (Yli-Mattila et al., 2009) and it has been suggested that it takes advantage of the absence of these species. This means that in the event of competition for infection in these three cereals, single spikelet infection coupled with a slow rate of infection in oat provides *F. langsethiae* with greater scope to infect and colonize. Consequently, this may be one of the reasons for the observed trend.

The effect of the previous crop in a rotation is known to impact on the occurrence of FHB pathogens and mycotoxin production. Deoxynivalenol production and subsequent loss of grain quality is high when wheat, barley and oat follow maize in a rotation (Dill-Macky and Jones, 2000). Edwards (2007a) also showed that previous crop was an important factor in levels of HT-2+T-2 in UK oats and indicated that the lowest levels were detected when oats followed a non-cereal in a rotation. In the present study, however, rotations in all fields studied were similar and so rotation is unlikely to be the reason for the high level of *F. langsethiae* biomass and corresponding high HT-2+T-2 levels in oats.

Observed yearly differences in *F. langsethiae* DNA were not the same in all cereal species studied. Whilst there was a clear pattern of *F. langsethiae* DNA levels in oat head sub-samples at harvest, levels in wheat, barley and triticale did not show a similar pattern.

In oats, *F. langsethiae* biomass found in the heads at harvest was highest in 2009 and gradually decreased over the subsequent sampling years. Xu et. al. (2008), quoted De Wolf et al. (2000) that weather conditions, often 7-14 days before

flowering are known to generally influence sporulation of FHB pathogens and subsequent disease occurrence. To understand the change in *F. langsethiae* biomass over the sampling years in this study, consideration was given to the temperature and relative humidity conditions during the week of sampling at GS59 and GS92 when a large increase in *F. langsethiae* DNA occurred. Climatic factors coupled with host factors have a profound influence on the growth and survival of *Fusarium* species (Doohan et al., 2003). Moreover, temperature and moisture are the most important climatic conditions influencing the infection and distribution of FHB pathogens. Fusarium head blight infection in cereals tends to be severe when anthesis coincides with warm and humid conditions (Edwards, 2004; Xu, 2003). It is also known that inoculum production and dispersal are also influenced by climatic conditions such as temperature, humidity and wind (Parry et al., 1995). *Fusarium langsethiae* infection appeared to take place at head emergence but before flowering (around GS59). At this growth stage mean weekly temperature and humidity were found to be highest in 2009 followed by 2010 with the lowest being recorded in 2011. It is of interest to note that the range between the maximum and minimum temperatures in 2009 were relatively narrow compared to the other two years at GS59. *Fusarium langsethiae* biomass at GS59 followed the same pattern. This suggests that warm temperatures coupled with a relatively high relative humidity favour *F. langsethiae* infection in oats at GS59. Other field data to support this trend is lacking. However, Medina and Magan (2010), showed that the growth rate of *F. langsethiae* isolates from England, Finland, Norway and Sweden were faster at 20-25^{o}C and at 0.98-0.995 water activity (a_w) in *in-vitro* studies. These authors also indicated that at low a_w (0.90), *F. langsethiae* did not grow irrespective of temperature, highlighting the importance of moisture for the growth of *F. langsethiae*. This is in agreement with data from this work where high *F. langsethiae*

biomass in oat heads corresponded well with high temperatures (ca. 24^{o}C) and relative humidity (70-85%) during the infection period.

The temperature and relative humidity data at harvest does not appear to influence the observed *F. langsethiae* biomass and corresponding HT-2+T-2 levels in oat heads at harvest suggesting that once infection has been favoured at earlier growth stages, conditions at harvest may play a minor role in the final levels of *F. langsethiae* biomass and HT-2+T-2 concentration. This may be reasonable because according to Medina and Magan (2011), the production of HT-2+T-2 by *F. langsethiae* occurs over a wide range of temperatures (10-30^{o}C) and once infection has been established, relative humidity has little effect on the pathogen already in the grain which would be mainly associated with the internal moisture conditions of the plant. There is therefore the need for data from longer periods of sampling to determine the association between pathogen development and weather parameters.

Triticale was sampled in 2010 and 2011, and the pattern of *F. langsethiae* biomass in heads at harvest followed a similar pattern to that observed with oats, therefore it is reasonable to say that factors that accounted for this observation are likely to be the same as that already discussed. However, in wheat and barley, the pattern observed did not match that observed in oat, especially in 2009. This was not as expected as, apart from winter barley which reached GS59 and GS92 earlier in all sampling years, all the other cereals studied reached these two growth stages at similar dates in all sampling years and thus it could have been expected that the pattern of *F. langsethiae* in the heads to be similar even if concentrations present were different.

At the time of sampling when winter barley was at GS59 in all three years, the average temperature was highest (although not much different) in 2009 followed by

that in 2010 and 2011, just like it was when oat plants were being sampled. Relative humidity at this growth stage on the other hand increased over the years unlike the case of oat where relative humidity decreased over the years. This can, however, not be used to fully explain the pattern of *F. langsethiae* biomass observed in winter barley heads over the sampling years. This is because if relative humidity was the deciding factor, then one would expect a gradual increase in *F. langsethiae* biomass in heads of winter barley from 2009 to 2011, but this was not the case. The temperature data at harvest again does not explain the observed *F. langsethiae* biomass in winter barley, but rather follows a similar trend as the relative humidity data. The relative humidity data alone cannot be used to adequately explain the observed *F. langsethiae* and HT-2+T-2 levels in winter barley heads at harvest. This is because although moisture is known to influence fungal growth and type A trichothecene production, at plant maturity fungal growth is minimal and the effect of environmental condition on the production of mycotoxin, is not entirely a direct effect of moisture but a function of temperature and moisture (Doohan et al., 2003)

On the basis of the findings from this study the development of *F. langsethiae* in cereals is postulated to follow a specific pattern: At growth stages before head emergence concentrations of *F. langsethiae* DNA in leaf and stem sub-samples were low and in almost all cases were below the limit of quantification. This suggests the presence of fungal spores on these plant parts rather than mycelial growth. As the cereals develop through head emergence, *F. langsethiae* DNA concentrations reach quantifiable levels especially in the leaves and head sub-samples with high levels at GS59. This would suggest that head infection takes place at head emergence, peaking around GS59. Fusarium head blight infection in cereals generally occurs during anthesis (Parry et al., 1995). The infection of cereal heads by *F. langsethiae* before flowering could be one of the major differences between the infection process of *F. langsethiae* and the other FHB pathogens. After

infection, the concentration *F. langsethiae* DNA in leaves generally tends to go down, only to increase again at GS92. This observation suggests the possibility of an increase in air-borne *F. langsethiae* spores around GS59. These spores may land on the leaves and heads of the cereals thus explaining the high DNA concentrations recorded at this growth stage. On the leaves spores may start to grow but the rate of growth decreases as the plant develops. It has been suggested that the first line of defence against pathogen infection through the leaf is the resistance provided by the cuticle and cell wall which tends to serve as a physical penetration and colonization (Huckelhoven, 2008). This defence mechanism could probably resist mycelial growth on the leaf and coupled with the probable absence/reduction of fungal spores in the air could have contributed to the observed reduced *F. langsethiae* DNA in the leaves before GS92. In head sub-samples, however, *F. langsethiae* DNA concentration continue to increase after GS59 (although this increase is not linear) reaching a maximum at GS92 when plants are about to be harvested. Levels of *F. langsethiae* DNA tend to increase slightly on leaf sub-samples as well and to a lesser extent on stem sub-samples at GS92. This pattern suggest that in heads, once infection has started, the fungus grows and colonizes the head tissues as the plant develops resulting in an increase in fungal biomass in the head. As the plant senesces (GS92) spore production increases, further increasing DNA concentrations in the head. This is understandable in that as a plant dies, phytopathogenic fungi sporulate and disseminate its spores for propagation the following season (Agrios, 2005). The resulting spore dissemination at GS92 is what contributes to the increase in *F. langsethiae* biomass on leaves and stems at this growth stage. Further studies such as microscopy and spore trap monitoring would be necessary to confirm this proposed epidemiology

3.5. Conclusions

This study has shown that symptomless grains or heads of oats can contain high levels of *F. langsethiae* DNA and HT-2+T-2 which is in agreement with initial work done by Imathiu (2008). Head infection, when it occurs, takes place during head emergence but before flowering and it is favoured by high temperatures and relative humidity. The source of inoculum for infection and the conditions that stimulate the release of spores for infection are still not clear, but spores are likely to be airborne. It will therefore be interesting to study the dynamics of *F. langsethiae* spores in the air in commercial fields during a cropping season to see if some, if not all, factors that influence spore release could be identified and if seasonal fluctuations match the proposed development above.

Fusarium langsethiae has a closer association with oats compared to wheat, barley and triticale. The reasons for this preference are not well understood and although head architecture and weather conditions at head emergence may play a critical role, this study has not fully confirmed that. Further studies are required to help understand the differences in cereal host species susceptibility to *F. langsethiae*. It will also be of interest to study the form of resistance mechanism involved in *F. langsethiae* infection in cereals. The role of agronomic factors in FHB disease occurrence is important in cereals. Since the fields sampled in this study were not under the same agronomic conditions there is the need for further studies to determine if under similar field and agronomic conditions, *F. langsethiae* infection and subsequent HT-2+T-2 production will be similar in the different cereal species.

Chapter Four

4. Study of *F. langsethiae* in Cereals under identical field and agronomic conditions

4. 1. Introduction

In the UK, the little data available shows that levels of HT-2+T-2 has over the years been highest in oats compared with wheat and barley (Edwards et al., 2009). Data from a two year commercial field survey showed that *F. langsethiae* DNA as well as HT-2+T-2 was highest in oats compared to wheat, barley and triticale (Chapter 3). The fields sampled, although they were in close proximity (within 30 Km), did not have the same agronomy. In the UK, agronomic factors such as differences between previous crop and cultivation are known to influence mycotoxins such as DON, HT-2+T-2 levels in cereal grains (Edwards et al., 2009). The authors showed that when oats were drilled following a cereal HT-2+T-2 levels were higher in grains than when oats followed a non-cereal in the rotation. There was therefore the need to carry out a field experiment to determine if under the same agronomic conditions *F. langsethiae* DNA would be higher in oats than wheat and barley. Higher *F. langsethiae* DNA and subsequent high HT-2 and T-2 levels in oats compared to wheat and barley under identical agronomic conditions would suggest that the high infection rate of *F. langsethiae* and subsequent HT-2 and T-2 production in oats is more of an inherent cereal character and probable under the influence of genetic control. Again in the field survey (Chapter 3), DNA and mycotoxin analysis were carried out on whole heads (not threshed). In the UK cereals are harvested mechanically and are threshed in the process. It was therefore necessary to determine if a similar trend of results as in the field survey will be obtained when cereal heads are threshed and what proportion of *F. langsethiae* DNA and HT-2+T-2 will be in the different cereal head fractions. When wheat is harvested the grains thresh free, thus the rest of the head is made of the rachis, lemma and glume.

Barley varieties used in this study were hulled, thus after threshing the lemma and palea was still attached to the grain with the rest of the head mainly consisting of the awns and the rachis. After harvesting of oats the rest of the head consisted mainly of the rachis, rachis branches and the glumes (Welch 1995). The objectives of this experiment was therefore to determine if there would be a difference in *F. langsethiae* DNA and HT-2+T-2 concentration in wheat, barley and oat heads and grains at maturity when cultivated under identical growing conditions.

Null hypotheses:

1. There is no difference in *F. langsethiae* DNA in wheat, barley and oat heads at maturity when they are cultivated under the same agronomic conditions
2. There is no difference in HT-2+T-2 concentration in wheat, barley and oat heads at maturity when cultivated under the same agronomic conditions
3. There is no difference between *F. langsethiae* DNA concentrations in grains and the rest of the cereal head of wheat, barley and oats.
4. There is no difference between HT+T-2 concentrations in grains and the rest of the cereal head of wheat barley and oats.

4.2. Materials and Methods

Experiments were carried out on two different sites; Woodseaves located in Staffordshire, UK and Harper Adams University College located in Shropshire, UK. Experimental sites were about 12 km apart. Each site comprised of two experiments within the same field, one for winter cereal varieties and the other for spring cereal varieties. Table 4.1 shows the list of varieties used for the experiments.

Table 4.1. List of winter and spring varieties of wheat, barley and oats used for field experiment

	Variety (sub-plot)	
Cereal (whole plot)	Winter varieties	Spring varieties
Wheat variety 1	Alchemy	Belvoir
Wheat variety 2	Robigus	Paragon
Wheat variety 3	Solstice	Granary
Barley variety 1	Cassia	Propino
Barley variety 2	Flagon	Quench
Barley variety 3	Suzuka	Tipple
Oats variety 1	Dalguise	Firth
Oats variety 2	Gerald	Atego
Oats variety 3	Mascani	Husky

The experiment was laid out in a split plot design with cereal as the main plot and the variety as the sub-plot. Each experiment comprised of four blocks with three plots (each with dimensions 6 x 6 m) which were divided into three sub-plots (2 x 6 m). Cereal species were randomized within each block and the cereal variety randomised within each plot (Fig. 4.1).

Guard Plot			
Block 1		**Block 2**	
PLOT 1	Wheat Variety 3	**PLOT 1**	Wheat Variety 2
	Wheat Variety 1		Wheat Variety 3
	Wheat Variety 2		Wheat Variety 1
PLOT 2	Oats Variety 1	**PLOT 2**	Barley Variety 2
	Oats Variety 3		Barley Variety 1
	Oats Variety 2		Barley Variety 3
PLOT 3	Barley Variety 1	**PLOT 3**	Oats Variety 3
	Barley Variety 2		Oats Variety 1
	Barley Variety 3		Oats Variety 2
PLOT 1	Oats Variety 2	**PLOT 1**	Oats Variety 1
	Oats Variety 3		Oats Variety 3
	Oats Variety 1		Oats Variety 2
PLOT 2	Barley Variety 3	**PLOT 2**	Barley Variety 2
	Barley Variety 2		Barley Variety 3
	Barley Variety 1		Barley Variety 1
PLOT 3	Wheat Variety 3	**PLOT 3**	Wheat Variety 2
	Wheat Variety 1		Wheat Variety 3
	Wheat Variety 2		Wheat Variety 1
Block 3		**Block 4**	
Guard Plot			

Figure 4.1. Field experimental layout showing blocks and plots with the different cereal species and varieties randomized within the plots and sub-plots respectively

Winter varieties were drilled in mid-October 2010 and spring varieties in mid-March 2011 using a plot drill (Plotman, Wintersteiger Austria) (Fig. 4.2). For winter cereal varieties a rate of 250 seeds m^{-2} was used for wheat and oats and 300 m^{-2} for barley. All spring cereal varieties were drilled at 350 seeds m^{-2}. Standard agronomic practices were carried out on both fields.

Figure 4.2. Drilling of cereal seeds using a plot drill (Plotman, Wintersteiger Austria) for the the winter cereals at woodseaves in October 2010.

At maturity cereal heads were harvested manually and air dried after which they were threshed using a mini-threshing machine (F. Walter-H. Wintersteiger, Austria). Grains and the rest of cereal heads (comprising of rachis, lemma and glume in wheat, the awns and the rachis in barley and rachis, rachis branches and the glumes in oats) were collected and milled as detailed in Section 2.2.3. DNA extraction and HT-2+T-2 analysis were carried out as detailed in Section 2.2.3 – 2.3.2

Data were $\log_{10}$ transformed before analysis. Data from each field was analysed first separately, and then combined due to the small difference between their residual standard errors. A split plot ANOVA was carried out with block nested within trial in the design structure and significant differences between means determined by LSD ($p = 0.05$) using Genstat (V.13 VSN International Ltd.). Regression analysis with groups (Genstat) was used to compare the relationship between *F. langsethiae*

DNA and HT-2+T-2 concentration between cereal species and cereal parts (grain and rest of head).

4.3. Results

In both fields, levels of *F. langsethiae* DNA in oat heads were higher than in wheat and barley. This was true for both winter and spring varieties. With the exception of barley, *F. langsethiae* DNA was higher in winter varieties than in spring varieties (Table 4.2).

Table 4.2. Log transformed *F. langsethiae* DNA (pg ng^{-1}) for heads of wheat barley and oat at harvest. Back transformed data in parentheses**.**

	Winter varieties	Spring varieties
Wheat	2.371 (0.0043)	2.42 (0.0037)
Barley	2.762 (0.0017)	2.597 (0.0025)
Oat	1.754 (0.0176)	2.239 (0.0058)
P-value	<0.001	0.01
%CV	7.1	8.4
s.e	0.48	0.22
LSD (5%)	0.176	0.234

In winter cereal varieties, there was no significant interaction between cereal and cereal part (P = 0.165). The interaction between cereal, variety and cereal part was also not significant (P = 0.778). It was observed that *F. langsethiae* DNA recovered from the rest of the cereal heads was significantly higher than that recovered from the grains (P < 0.001). *F. langsethiae* DNA in the rest of the cereal heads was about five times higher than that in the grains only (Figure 4.3).

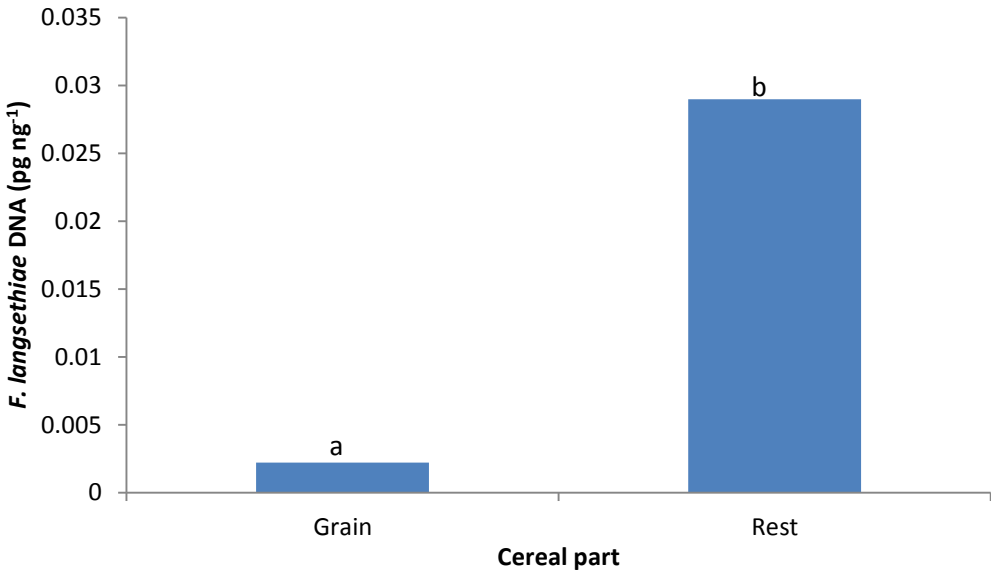

Figure 4.3. Back transformed mean *F. langsethiae* DNA recovered from grains and rest of the winter cereal heads. Bars with different letters are significantly different from each other (LSD = 0.17)

F. langsethiae DNA recovered from the different varieties of wheat, barley and oat differed with a significant interaction between cereal and variety ($P < 0.001$).There was no significant difference in *F. langsethiae* DNA recovered from heads of the different winter wheat varieties (Figure 4.4) which had a grand back-transformed *F. langsethiae* mean of 0.0043 pg ng^{-1}.

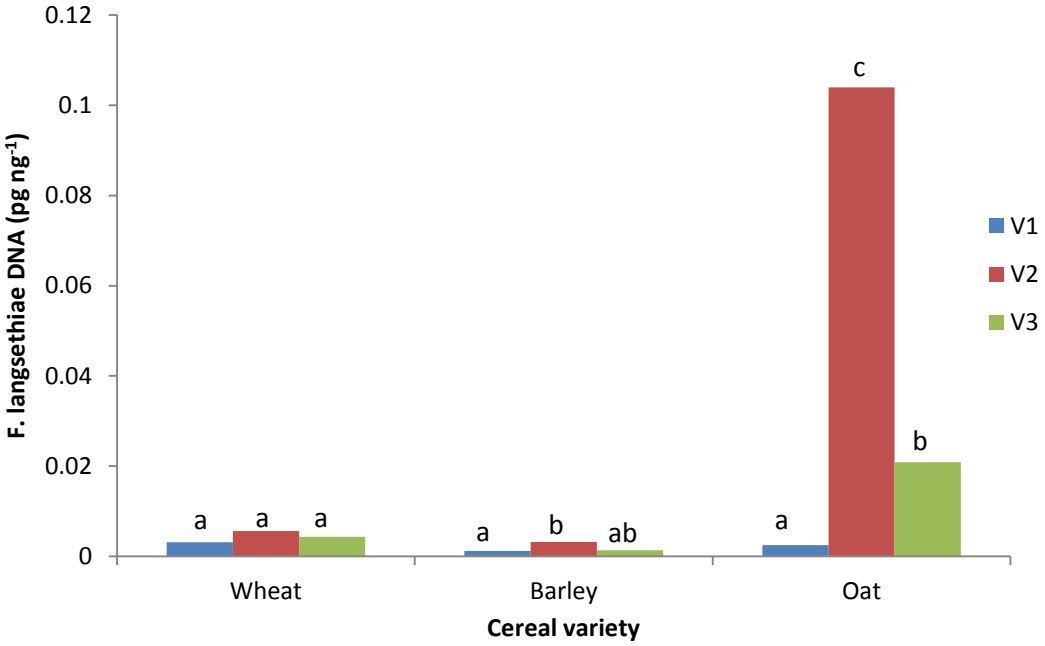

Figure 4.4. Back-transformed *F. langsethiae* DNA recovered from the different winter cereal varieties. For wheat V1 = Alchemy, V2 = Robigus and V3 = Solstice. For barley V1 = Cassia, V2 = Flagon and V3 = Suzuka. For oat V1 = Dalguise, V2 = Gerald and V3 = Mascani. Varieties of the same cereal species with same letters are statistically not different from each other (%CV = 8.8, s.e = 0.19, LSD = 0.18).

Flagon had the highest *F. langsethiae* DNA among the winter barley varieties with a mean of 0.032 pg ng^{-1}. This was significantly ($P < 0.05$) different from that recovered from Cassia which had a mean of 0.0012 pg ng^{-1} (Figure 4.4). In winter oats, back transformed mean *F. langsethiae* DNA in heads of the different varieties were 0.104, 0.020 and 0.002 pg ng^{-1}, for Gerald, Mascani and Dalguise respectively and these means were significantly ($p < 0.05$) different from each another (Figure 4.4).

In spring varieties, the interaction between cereal species and part was significant ($P < 0.001$). In all three cereals *F. langsethiae* DNA in the rest of the head was significantly ($P < 0.05$) higher than that in the grains. Log_{10} transformed mean *F. langsethiae* DNA in the rest of the head was about 38 times higher than in the grains alone for wheat, eight times higher for barley and 17 times higher for oat compared to the rest of the head (Figure 4.5).

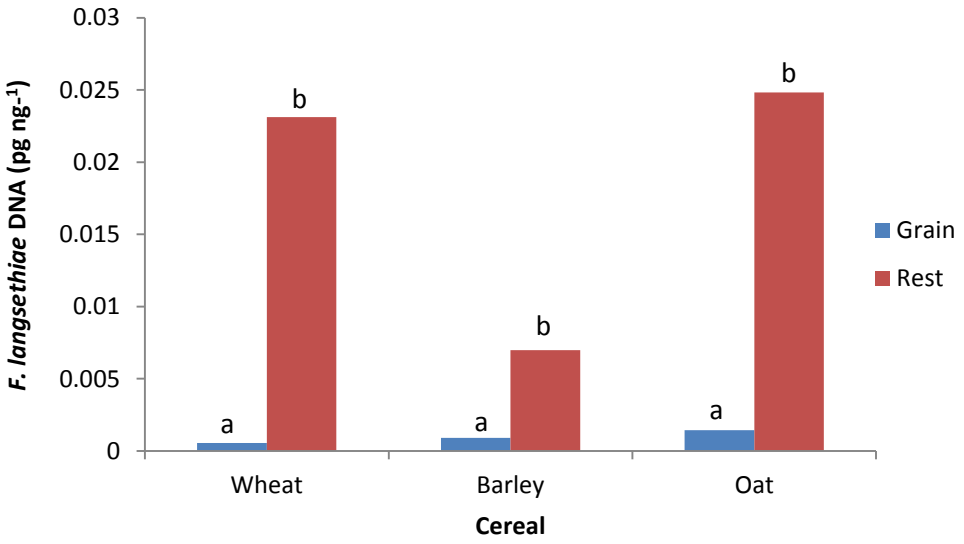

Figure 4.5. *F. langsethiae* DNA recovered from grains and the rest of the heads of spring wheat barley and oats. Parts of cereal species with different letters are statistically different from each other (LSD = 0.28).

The interaction between cereal and variety was significant ($P < 0.001$). In wheat, *F. langsethiae* DNA recovered from the different varieties were not significantly ($P > 0.05$) different from each other (Figure 4.6).

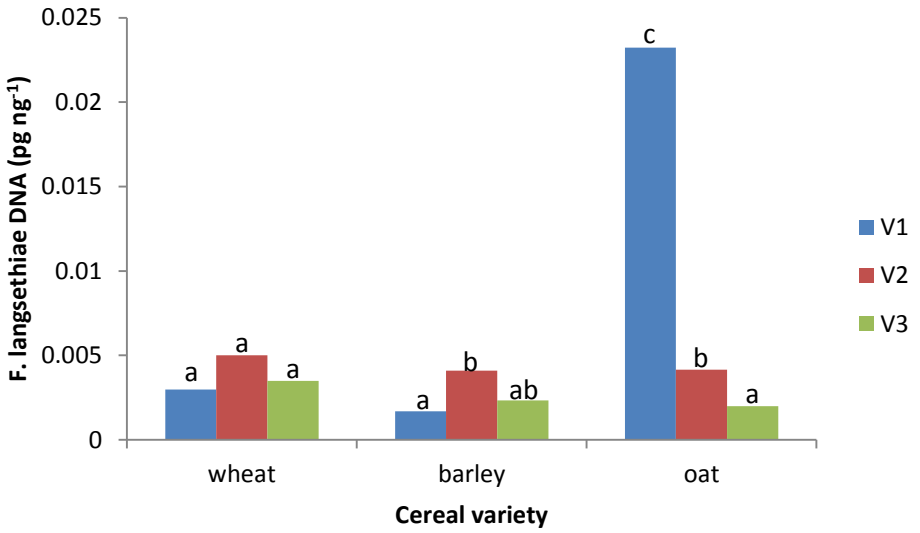

Figure 4.6. Back-transformed mean *F. langsethiae* DNA recovered from the different spring cereal varieties. For wheat V1 = Belvoir, V2 = Paragon and V3 = Granary. For barley V1 = Propino, V2 = Quench and V3 = Tipple. For oat V1 = Firth, V2 = Atego and V3 = Husky. Varieties of the same cereal species with same letters are statistically not different from each other based on the LSD (%CV = 12.20, s.e = 0.29, LSD = 0.33).

Quench had a mean *F. langsethiae* DNA of 0.004 pg ng^{-1}. This was the highest *F. langsethiae* DNA recovered from the spring barley varieties and was significantly ($P < 0.05$) higher than that recovered from Propino which had the lowest mean *F. langsethiae* DNA of 0.0017 pg ng^{-1} (Figure 4.6).

In oats, the highest *F. langsethiae* DNA was recovered from Firth with a mean *F. langsethiae* DNA of 0.23 pg ng^{-1} followed by that from Atego and Husky with mean *F. langsethiae* DNA of 0.004 and 0.002 pg ng^{-1} respectively. These means were all significantly ($P < 0.05$) different from each other (Figure 4.6).

Levels of HT-2+T-2 in cereals followed similar pattern as *F. langsethiae* DNA. Levels in winter varieties were higher than that observed in spring varieties except in barley (Table 4.3).

Table 4.3. Log_{10}-transformed HT-2+T-2 concentrations (µg kg^{-1}) in heads of wheat, barley and oats at harvest**.** Back-transformed data in parentheses.

	HT-2+T-2 in winter cereals	HT-2+T-2 in spring cereals
Wheat	1.910 (81)	1.865 (73)
Barley	1.790 (62)	1.792 (62)
Oat	2.905 (804)	2.285 (193)
P-value	< 0.001	0.04
%CV	2.5	3.4
s.e	0.05	0.07
LSD	0.24	0.29

In winter cereal species there was no significant interaction between cereal and cereal part (P= 0.059) and the interaction between cereal, variety and part was not significant (P = 0.25). HT-2+T-2 levels in different cereal parts analysed were different with the grains having significantly lower levels than that in the rest of the head (P < 0.001). HT-2+T-2 level in the rest of the head was about two times higher than that found in the grains (Figure 4.7).

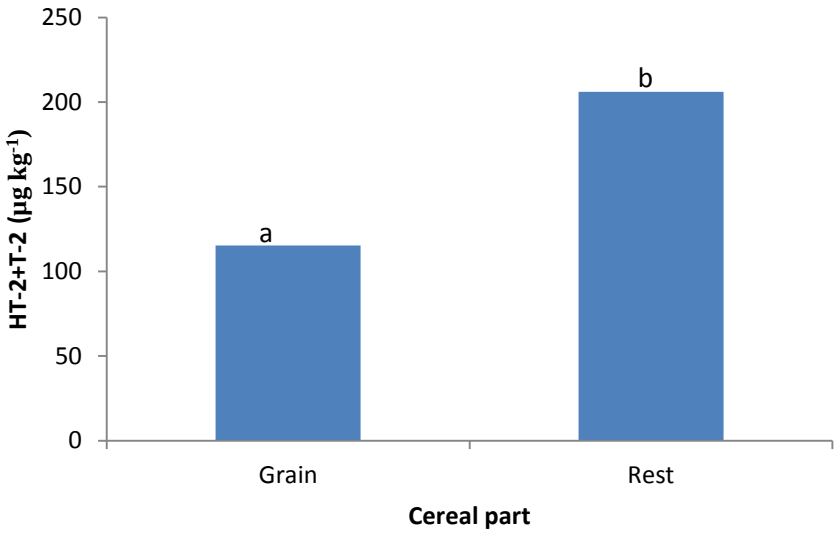

Figure 4.7. Back-transformed HT-2+T-2 in grains and rest of the winter cereal heads. Bars with different letters are significantly different from each other (LSD = 0.07).

Levels of HT-2+T-2 in the different winter cereal varieties differed with a statistically significant interaction between cereal and variety (P = 0.001). There was no

significant (P > 0.05) difference in HT-2+T-2 concentration in the different winter wheat varieties (Figure 4.8) with an overall mean HT-2+T-2 concentration of 159 µg kg^{-1}.

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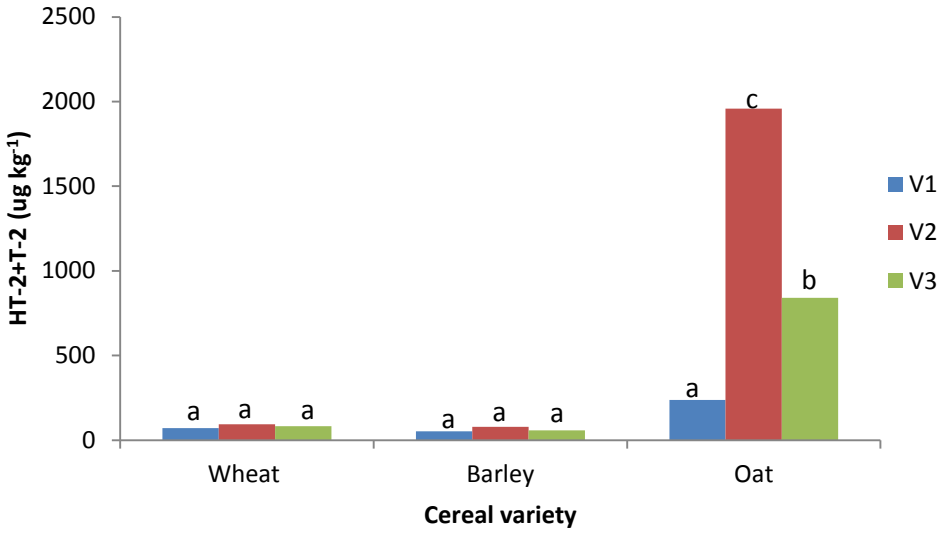

Figure 4.8. HT-2+T-2 levels in the different winter cereal varieties. For wheat V1 = Alchamy, V2 = Robigus and V3 = Solstice. For barley V1 = Cassia, V2 = Flagon and V3 = Suzaka. For oat V1 = Dalguise, V2 = Gerald and V3 = Mascani. Varieties of the same cereal species with same letters are statistically not different from each other based on the LSD (%CV = 4.2, s.e = 0.09, LSD = 0.22)

An overall mean HT-2+T-2 of (Back-transformed) was recorded for winter barley. No significant difference was observed in HT-2+T-2 levels within the different winter barley varieties (LSD P = 0.05) (Figure 4.8) with an overall mean concentration of 154 µg kg^{-1}

Within the winter oat varieties, Gerald had the highest HT-2+T-2 concentration with a back-transformed mean of 1956 µg kg^{-1} followed by Mascani which had a back-transformed mean of 841 µg kg^{-1} and Dalguise which had a back-transformed HT-2+T-2 mean of 237 µg kg^{-1}. These means were all significantly (P < 0.05) different from each other.

A simple linear regression between *F. langsethiae* DNA recovered from winter cereal heads and their corresponding HT-2+T-2 grouped by cereal species showed a highly significant ($P < 0.001$) regression best fitted by separate non-parallel lines. However for wheat and barley alone a single line accounted for 68% of the variance and parallel lines although significant accounted for only an additional 1.5% of the total variance observed. Thus two lines where fitted, one for oat ($P < 0.001$, $r^2 = 0.79$) and another for wheat and barley ($P < 0.001$, $r^2 = 0.68$) (Figure 4.9)

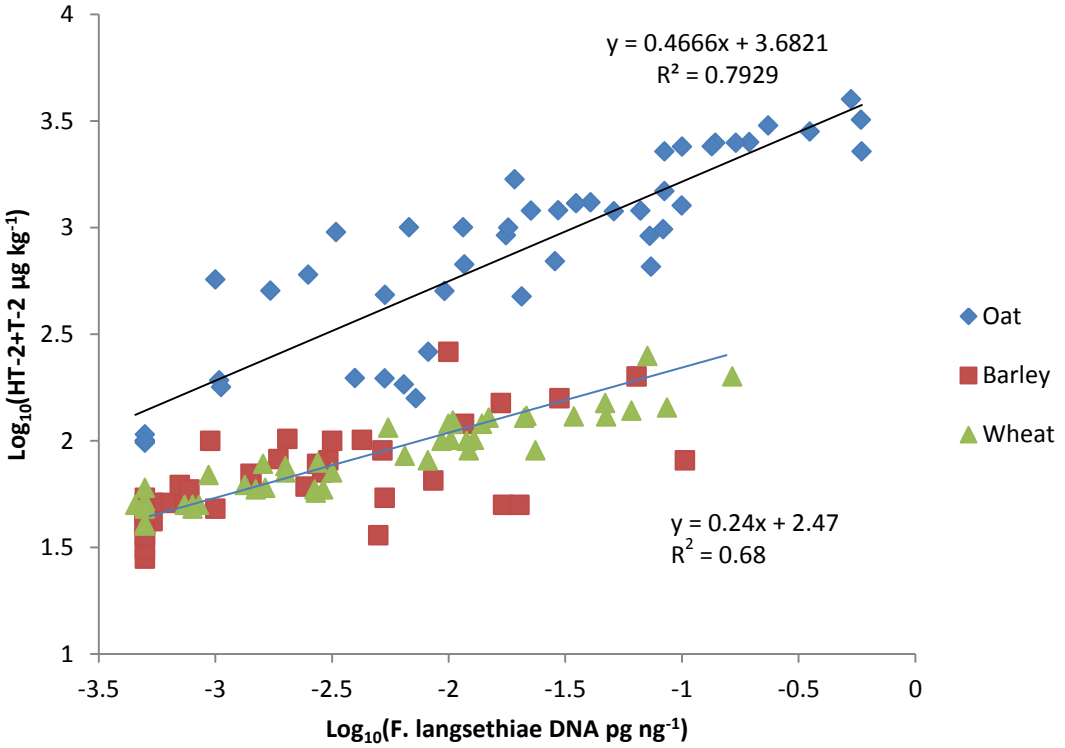

Figure 4.9. The relationship between *F. langsethiae* DNA and HT-2+T-2 concentration in winter cereal heads

For HT-2+T-2 in spring cereal species, a significant ($P < 0.001$) interaction was found between cereal species and cereal part. In spring wheat, HT-2+T-2 in the rest of the head was about three times higher than that found within the grains, whilst it

was about one and half times higher in barley and about five times higher in oats (Fig. 4.10).

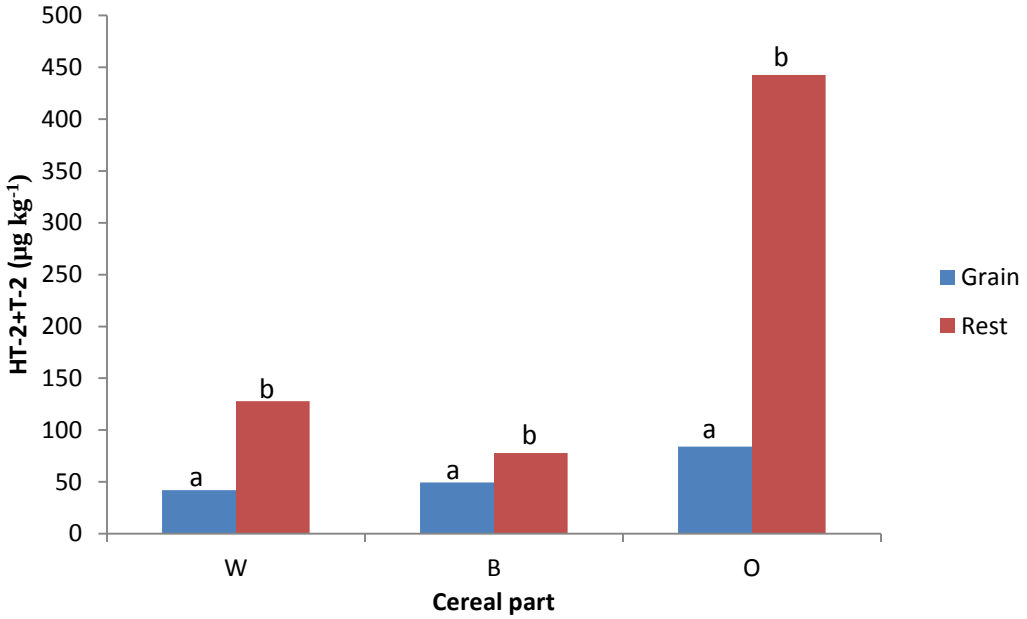

Figure 4.10. Back-transformed HT-2+T-2 concentration for grains and the rest of the heads of spring wheat, barley and oats. For each cereal species parts of cereal with different letters are statistically different from each other (LSD = 0.22)

HT-2+T-2 levels in the different spring cereal varieties differed with a significant interaction between cereal and variety ($P < 0.001$). HT-2+T-2 concentration for the three spring wheat varieties were not significantly ($P > 0.05$) different from each other (Figure 4.11) with an overall back-transformed mean of 87 µg kg^{-1}.

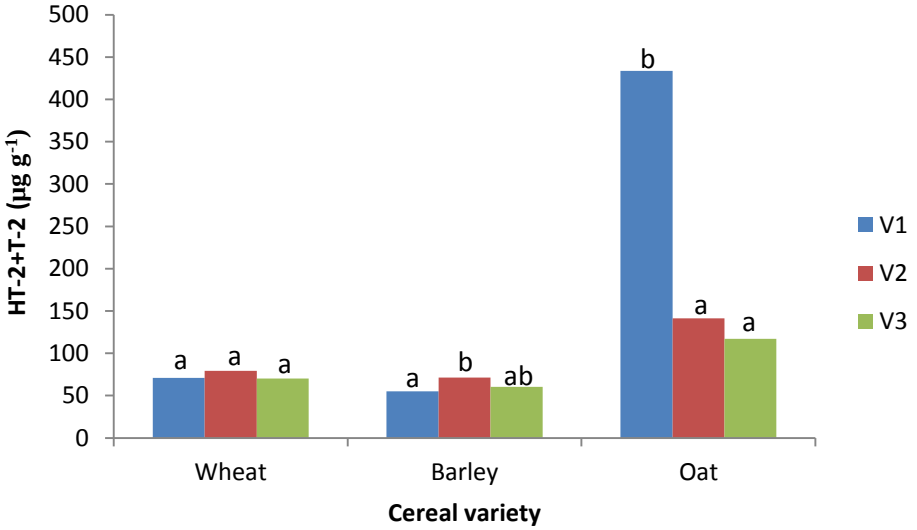

Figure 4.11. Back-transformed HT-2+T-2 levels in the different spring cereal varieties. For wheat, V1 = Belvoir, V2 = Paragon and V3 = Granary. For barley V1 = Propino, V2 = Quench and V3 = Tipple. For oat V1 = Firth, V2 = Atego and V3 = Husky. Varieties of the same cereal species with same letters are statistically not different from each other based on the LSD (%CV = 5.1, se = 0.10, LSD = 0.26)

HT-2+T-2 concentration in Quench was the highest among the three spring barley varieties (back-transformed mean of 71 µg kg^{-1}). This was significantly ($P < 0.05$) higher than that for Propino (back-transformed mean of 55 µg kg^{-1}) which had the least HT-2+T-2 concentration among the three varieties (Figure 4.11).

In oats, the highest HT-2+T-2 concentration was recorded in Firth with a back-transformed mean of 433 µg kg^{-1}. This was significantly ($P < 0.05$) higher than for Atego and Husky with a back-transformed HT-2+T-2 mean of 141 and 117 µg kg^{-1} respectively (Figure 4.11).

A simple linear regression between *F. langsethiae* DNA recovered from spring cereal heads and their corresponding HT-2+T-2 grouped by cereal species was highly significant ($P < 0.001$) and was best fitted by separate non-parallel lines. However for wheat and barley alone, a single line account for 73% of the variance and parallel lines although significant accounted for only an additional 5.3% of the

total variance observed. Thus two lines where fitted, one for oat ($P < 0.001$, $r^2 = 0.78$) and another for wheat and barley ($P < 0.001$, $r^2 = 0.73$) (Figure 4.12)

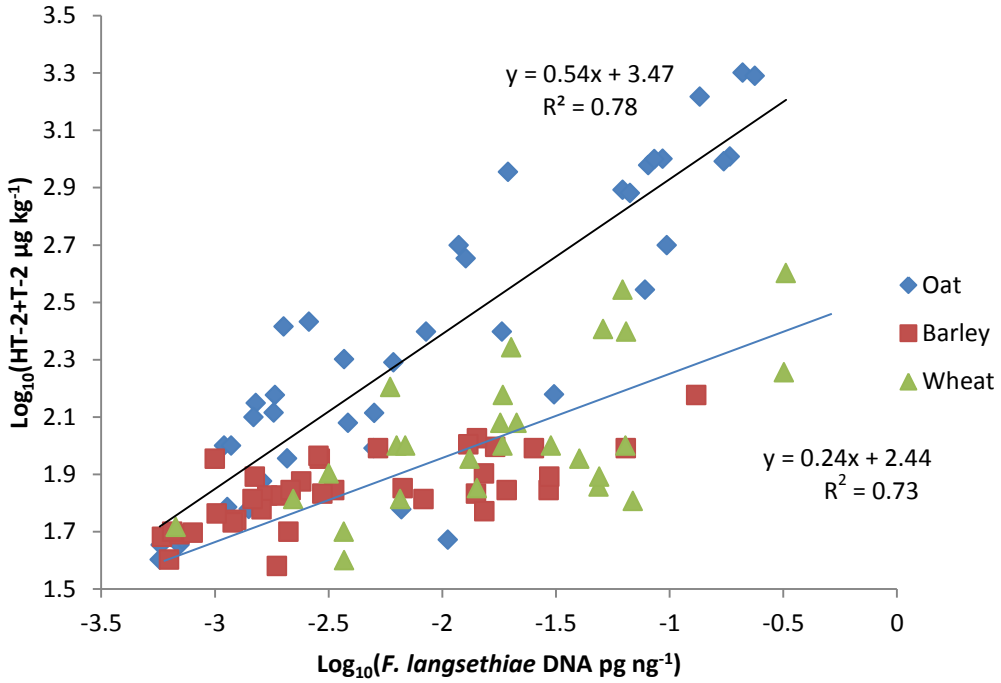

Figure 4.12. Relationship between *F. langsethiae* DNA and HT-2+T-2 concentration in spring cereal heads**.**

A visual comparison of Figure 4.9 and 4.12 shows that the regressions for winter and spring oats were similar and the regression for winter and spring wheat and barley were nearly identical. Regression analysis was repeated for all oats grouped by sowing date (winter and spring). The data was best fitted by separate parallel lines (Figure 4.13) which accounted for 84% of the observed variance.

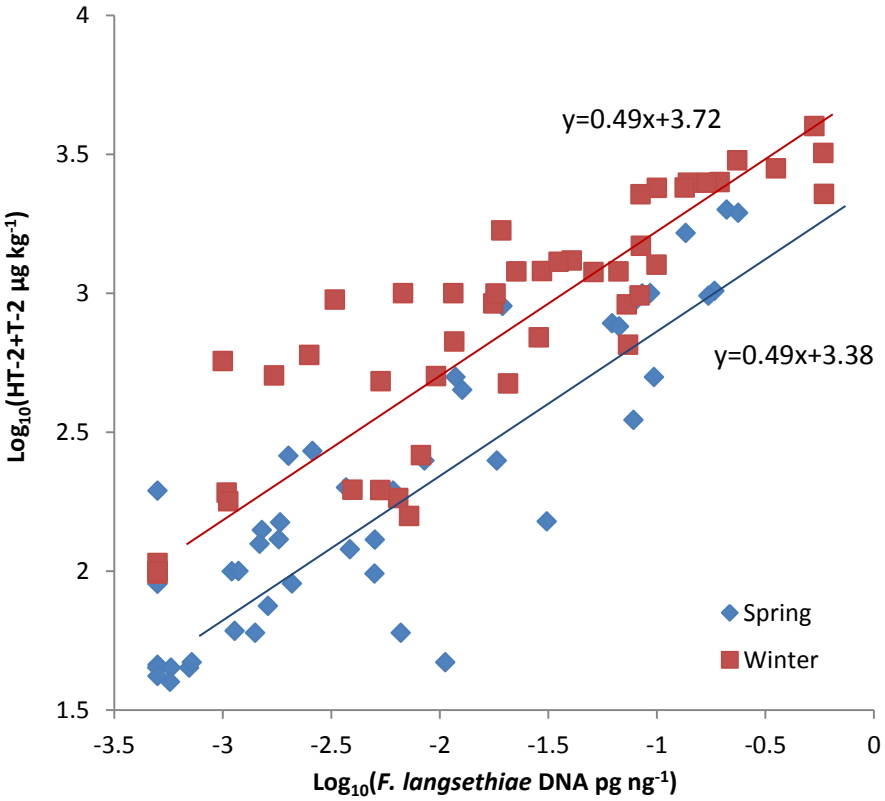

Figure 4.13. Relationship between *F. langsethiae* DNA and HT-2+T-2 concentration in winter and spring sown oats

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The regression plots shows that for a fixed concentration of *F. langsethiae* DNA there is a higher concentration of HT-2+T-2 on winter oats compared to spring oats.

Regression analysis was also repeated for all barley and wheat samples grouped by drilling date. This regression showed drilling date had no significant effect on the relationship of HT-2+T2 to *F. langsethiae* DNA concentration and was best fitted by a single line (y = 0.024x + 2.45). The regression accounted for 64% of the observed variance.

4.5. Discussion

This study has confirmed the preference of *F. langsethiae* for oats in both winter and spring sown cereals. Since *F. langsethiae* was identified as the main producer of HT-2 and T-2 in cereals in Europe and more especially in the UK, there has been growing evidence to suggest its preference for oats compared with wheat and barley (Edwards et al., 2009). HT-2+T-2 concentration data from four European countries in 2007 showed oats to have the highest concentration among wheat, barley and oats (Edwards et al., 2009).

Generally *F. langsethiae* DNA and HT-2+T-2 concentrations were higher in winter varieties except in barley where *F. langsethiae* DNA was higher in spring than in winter varieties. This is consistent with data from a three year commercial field survey (Chapter 3), available data on UK oat (Edwards, 2009b) and French malting barley (Barrier-Guillot, 2008). The reason for this observation is not fully understood, however it has been suggested that the agronomy used for these crops may be important (Edwards et al., 2009). In this study, the agronomy used in the cultivation of these crops was similar except the sowing time and seed rate. The winter varieties were drilled in mid-October 2010 and the spring varieties in mid-March 2011. Sowing times have been suggested to be a probable factor accounting for the differences in HT-2+T-2 concentrations observed in winter and spring drilled cereals. It has been reported that when French barley, regardless of the cultivar, are sown in autumn, HT-2 and T-2 levels are half the level compared to that sown in winter and three times less than after spring sowing (Orlando et al., 2010). In the UK, however, winter sown oats have a higher HT-2 and T-2 contamination than spring sown oats (Edwards et al., 2009). Although there were some differences in the seeding rate between spring and winter varieties, there is evidence which suggest that seed rate has no significant effect of FHB severity and subsequent DON production in both spring and winter wheat (Fernandez, et al., 2005;

Schaafsma and Tamburic, 2005). Apart from the observed differences in *F. langsethiae* DNA and HT-2+T-2 concentration within the cereal species, differences in the different cereal varieties were also observed. Differences in HT-2+T-2 concentrations in different cereal varieties have been previously reported by Edwards et al. (2009).

In winter barley varieties Flagon had the highest *F. langsethiae* DNA concentration followed by Suzaka and Cassia respectively whilst in the spring varieties highest *F. langsethiae* DNA was recovered from Quench followed by Tipple and Propino. Edwards (2012) reported that within winter and spring varieties of barley, Flagon had more resistance to DON producing FHB pathogens than Quench and Tipple. Data from this study is in agreement to some extent with this report. This is because in this study Flagon had a lower mean *F. langsethiae* DNA than Quench but not Tipple. This study is based on just a single year's data whilst that of Edward (2012) is from about six years of field data. This difference may account for the difference observed in *F. langsethiae* DNA and thus resistance in the different varieties.

In winter oats, this study showed Gerald to have the highest *F. langsethiae* DNA and HT-2+T-2 concentration followed by Mascani and then Dalguise and these differences were statistically significant. This is in agreement with data from a 6 year survey of UK oats by Edwards (2012). The author reported Gerald to have the highest and statistically significantly higher HT-2+T-2 concentration followed by Mascani and Dalguise. Within spring oat varieties the author indicated that HT-2+T-2 concentrations differed among the varieties studied, although to a lesser extent compared to winter varieties and reported that Firth had a higher HT-2+T-2 concentration than Atego and Husky. In this case however, the difference in HT-2+T-2 concentration between Firth and Atego was not significantly different but was

significant between Firth and Husky This is in agreement with *F. langsethiae* DNA and HT-2+T-2 concentration recorded in these varieties in this study.

Results of this study have showed that a significant difference existed between *F. langsethiae* DNA and HT-2+T-2 levels in grains and the rest of the head. In spring varieties where there was a significant interaction between cereals and cereal parts, it was observed that the ratio between *F. langsethiae* DNA in rest of the head to that in the grains was highest in wheat with the lowest ratio in barley. With HT-2+T-2 concentrations, the highest ratio was estimated in oat with barley again having the least ratio. This observation may be as a result of the lesser amount of materials constituting the rest of the head of barley after threshing (awns and the rachis) as compared to that that in wheat and oats. Reduction in *F. langsethiae* DNA and HT-2+T-2 concentration in grains after threshing was expected. This is because processing in general is known to reduce HT-2+T-2 content in cereal heads (Edwards et al., 2009). Data from de-hulling experiments have shown that the process reduces HT-2+T-2 levels in oat grains and that this reduction is typically around 90% (Edwards 2007a; Scudamore et al., 2007).

It is important to note that not only does *F. langsethiae* has a stronger preference for oats, but the production of HT-2 and T-2 per unit fungal biomass is far higher in oats than the other two cereals and higher for winter compared to spring oat varieties. For example, in winter cereal varieties when the concentration of *F. langsethiae* DNA recovered from cereal heads is about 0.01 pg ng^{-1} corresponding HT-2+T-2 concentration is about 100 µg kg^{-1} in both wheat and barley heads and about 250 µg kg^{-1} in oat heads. A ten-fold increase in *F. langsethiae* DNA concentration (0.1 pg ng^{-1}) in cereal heads resulted in about a 50% increase in HT-2+T-2 concentration (150 µg kg^{-1}) in wheat and barley and a 400% increase (1000 µg kg^{-1}) in winter oat heads. The reasons for these observed differences are not clearly understood,

however, there is evidence that some plant species are able to metabolise some trichothecenes to other derivatives. For example Lemmens et al. (2005) reported that the resistance of a wheat genotype (CM82036) to FHB and DON was due to its ability to convert DON to DON-3-glucoside, a less toxic derivative. The possibility thus exist that the observed relatively lower HT-2+T-2 concentration in wheat and barley heads per unit *F. langsethiae* DNA compared to oats could be due to the ability of wheat and barley to metabolise HT-2 and T-2 into other derivatives. It could also be that the oat head provides certain conditions that stimulate greater HT-2 and T-2 production. The observed difference between winter and spring oats may be due to either the varietal differences or due to the fact that winter oats develop earlier with heads emerging earlier than spring oats. This means that panicles of winter sown oats are exposed to *F. langsethiae* infection earlier and for a longer period (till harvest) allowing for much more HT-2 and T-2 production compared to spring oats. This assumption however, does not hold for wheat and barley since no significant difference was seen between winter and spring sown.

4.6. Conclusions

Within the experimental limitations of this study, results have confirmed the preference of *F. langsethiae* for oats and the subsequent production of high HT-2 and T-2 in oat heads even when wheat, barley and oats are cultivated under similar field and agronomic conditions. Not only are oats more susceptible to *F. langsethiae* compared with the other cereal species but they also accumulate more HT-2+T-2 compared to other cereal species per unit *F. langsethiae* biomass (as measured by DNA).

Results from this Chapter suggests that the genetics of the cereal species studied may have a greater influence on their resistance to *F. langsethiae* although some agronomic factors may be important in *F. langsethiae* cereal infection and

subsequent HT-2 and T-2 occurrence in cereal heads. This study was based on one year field data and only three varieties of each cereal. There is therefore the need to carry out further experiments and more importantly with a larger number of cereal varieties to confirm this observation. This will be more feasible under glasshouse conditions. Glasshouse experiments would require artificial inoculation, however, there is no effective artificial inoculation method for *F. langsethiae* in cereals that can result in head infection with corresponding high HT-2 and T-2 levels in cereal heads. There is therefore the need to develop or to optimize existing inoculation methods for traditional FHB species for *F. langsethiae*. This study has also shown that there is variability in *F. langsethiae* DNA and HT-2+T-2 concentration within the cereal varieties used for the study. This suggests that there is variability in the resistance of these cereal varieties to *F. langsethiae* infection. However, only three varieties of each cereal were used. It will therefore be of interest to screen a larger number of UK winter and spring varieties of wheat, barley and oats to determine what variation exist across all UK.

Chapter Five

5. Pathogenicity and aggressiveness of *Fusarium langsethiae* isolates towards wheat, barley and oat and resistance of UK wheat, barley and oat varieties to *F. langsethiae*

5.1. Introduction

In plant pathological studies, pathogenicity, virulence and aggressiveness are three important and distinct terms that have over the years created some confusion as to their definitions (Shaner et al., 1992). Whilst there is some level of consensus on the definition of pathogenicity, the same cannot be said about aggressiveness and virulence.

Shaner et al. (1992) refers to a number of definitions for virulence, ranging from standard English usage through to medical and scientific definitions. These definitions include the quality and property of being virulent, the relative capacity of a microorganism to overcome the defence mechanism of a host (Gove, 1986), the relative power and degree of pathogenicity possessed by an organism to produce disease (Thomas, 1989). The Commissie voor de Terminologie of the Netherlands Society of Plant Pathology (Subcommittee, F.B.P.P.T., 173) defines it as the severity of a disease in a host. Agrios (2005) defines it as the rate at which a pathogen causes disease or reaches a 'disease situation.' Whilst Shaner et al. (1992) consider virulence a quantitative description; Bos and Parlevliet (1995) think otherwise and describe it as a qualitative phenomenon.

The definition for aggressiveness of a plant pathogen has been more inconsistent than virulence and some authors such as Thomas and Elkinton (2004) have even suggested its elimination from plant pathological terminologies and replaced with 'infectivity'. In their proposed 'hierarchy of terms for describing pathogenicity', Shaner et al. (1992) also eliminated 'aggressiveness' and replaced it with parasite

fitness. The Webster's Third New International Dictionary defines it as 'spreading with vigour'. It is the extent to which a plant pathogen can attack a susceptible host and it is measured by the amount of pathogen developing on or in a given host species (Bos and Parlevliet, 1995). According to Bos and Parlevliet, proponents for continued use of this term, aggressiveness of different strains or isolates of a given plant pathogenic species may vary, with the more aggressive ones attacking plants faster and/or more intensively. For example in the 1970s the Dutch elm disease caused by *Ophiostoma ulmi* reoccurred in Europe attacking previously known resistant Elm cultivars. This reoccurrence was attributed to the emergence of a new and a more aggressive strain *O. novo-ulmi* which attacked plants faster and more intensely (Gibbs and Brasier, 1973).

There is much more consensus on the definition for pathogenicity; 'the ability of an organism to cause disease' (Agrios, 2005; Bos and Parlevliet, 1995; Shaner et al., 1992). It is determined jointly by aggressiveness and the virulence (Bos and Parlevliet, 1995).

In this study, I define pathogenicity as the ability of an organism to cause disease (Agrios, 2005; Bos and Parlevliet, 1995 and Shaner et al., 1992) and aggressiveness as the extent to which a pathogen attacks a susceptible host (Bos and Parlevliet, 1995) and assessed by the lesion length caused on a wounded leaf by a plant pathogenic species.

When a plant pathogen comes into contact with a plant, disease only occurs when environmental conditions are favourable, the plant is susceptible and the pathogen is virulent. Thus, disease occurs only when these three components (pathogen, environment and host) interact. This interaction is best described as the disease triangle model (Agrios, 2005)

When environmental conditions are favourable for fungal infection, the spores of pathogenic fungi come into contact with a susceptible host plant, attach to the host surface and then develop structures such as appressoria that will enable it to penetrate the host. Host penetration may also occur through natural openings such as the stomata or artificial openings such as wounds. Appressoria have the ability to exert pressure on the host surface thus aiding penetration. Mechanical penetration may also be aided by cell wall degrading enzymes (van den Ende and Linskens, 1974; Perfect and Green, 2001; Agrios 2005; Bakar et al., 2005). Once the fungus has been able to penetrate the host surface into the underlying tissues, it begins to feed, grow and colonize. These processes put together result in different disease symptoms.

Over time plants have developed various defence mechanisms to combat fungal attack at various stages of the disease cycle. When a plant does not fall within the host range of the pathogen, the plant is naturally resistant to the pathogen. This is known as non-host resistance (Agrios, 2005). A plant can become or have resistance to a pathogen simply because it has the ability or some means to escape or tolerate infection by the pathogen, a mechanism known as apparent resistance. A susceptible plant variety may 'escape' from a disease when factors in the disease triangle do not interact properly or for sufficient time. When a plant variety sustains less damage or injury compared with a susceptible variety, although the biomass of the pathogen is the same, it is said to be tolerant (Agrios, 2005). Plants are also able to combat disease because they have 'true' resistance. There are two types of true resistance, *R* gene resistance and partial resistance.

R gene resistance, also known as race specific, monogenic or vertical resistance has one underlying principle; the plant has a resistance (*R*) gene that is directed against an avirulence (*AVR*) gene of the pathogen. *R* genes encode plant

resistance proteins which are able to directly or indirectly detect plant pathogenic specific elicitor proteins known as avirulence proteins encoded by *AVR* genes, a theory described by Flor, (1946) as the gene-for-gene interaction (Kruijt et al., 2005). Plant varieties with *R* gene resistance generally show complete resistance to a specific pathogen under most environmental conditions, however, this form of resistance is not very stable since a single mutation in the pathogen for example, will make the previously avirulent pathogen virulent. Partial resistance, also known as race non-specific, general or quantitative resistance is controlled by a number of genes, although there are a few examples controlled by single genes (Agrios, 2005). A plant variety exhibiting partial resistance has similar intermediate degrees of resistance against a number of infecting pathogenic races. This type of resistance does not provide complete resistance to all the infecting pathogens. Although more stable, it is not as effective as *R* gene resistance (Agrios, 2005).

The different reactions of plants to invading pathogens during the infection process lead to changes in the plant that are normally observed as disease symptoms. These symptoms such as lesions and their sizes, incubation period (time from inoculation to first appearance of lesions) and latent period (time from inoculation to the first appearance of sporulation) are part of partial disease resistance (PDR) components and have been used in *in-vitro* detached leaf assays to investigate disease such as *Septoria tritici* (Arraiano et al., 2001; Chartrain et al., 2004) and FHB resistance of wheat (Diamond and Cooke, 1999). *In-vitro* detached leaf assays have been used to study FHB resistance in cereals using various FHB pathogens (Browne and Cooke, 2004; Diamond and Cooke, 1999; Brown et al., 2006) and has been shown to be a useful tool in predicting reaction of matured plants to FHB pathogens (Diamond and Cooke, 1999).

In the study of FHB resistance in cereals, field trials are commonly used, but these studies are expensive and time consuming (requiring at least 3 years to ensure that plant possess genuine resistance). In addition, data obtained from such studies are difficult to explain as a result of the complex relationship between disease development and environmental conditions which cannot be controlled in the field. These difficulties make an *in-vitro* detached leaf assay a preferred method since it takes a shorter time to produce results, conditions are easier to control and assays are readily repeatable (Diamond and Cooke, 1999). *In-vitro* detached leaf assays have been used by a number of authors to assess pathogenicity and resistance in different crops such as faba bean (Bouhassan et al., 2003), soybean (Twizeymana, 2007), chrysanthemum (Pettitt et al., 2011), pear (Yessad et al., 1992) and in cereals such as wheat (Brown, et al., 2006; Imathiu et al., 2009).

Harper Adams University College keeps a culture collection of *F. langsethiae* isolates originally isolated from oat and wheat at different periods and have been kept over time. There is however, very little information on the characteristics of these different isolates. Two experiments were therefore carried out to assess these isolates and to identify a highly aggressive isolate to be used to compare varieties of wheat, barley and oat varieties in an *in-vitro* detached leaf assay. The lesion length formed by the different *F. langsethiae* isolates on the different cereal species and varieties was used as a measure of aggressiveness with the basic assumption that more aggressive *F. langsethiae* isolates would form longer lesions on leaves of the different cereal species and more resistant cereal varieties would have shorter lesion lengths.

The objectives of these experiments were therefore;

1. To assess the aggressiveness of 20 *F. langsethiae* isolates using an *in-vitro* detached leaf assay

2. To assess the resistance of different UK varieties of wheat, barley and oats to *F. langsethiae*

Null hypotheses

1. There is no difference in aggressiveness of *F. langsethiae* isolates towards wheat, barley and oats

2. Storage period (age) has no effect on aggressiveness of *F. langsethiae* isolates towards wheat, barley and oats

3. The original host of the isolates has no effect on aggressiveness of *F. langsethiae* towards wheat, barley and oats

4. There is no difference in the resistance of different UK varieties of wheat, barley and oats to *F. langsethiae* infection.

5. An *in-vitro* leaf assay cannot be used to assess the resistance of different UK oat varieties to *F. langsethiae* and subsequent HT-2+T-2 in grains after harvest under field conditions.

5.2. Materials and Methods

Two experiments were carried out. The first was to test hypotheses 1, 2 and 3. The second was test hypothesis 4 and 5.

5.2.1. Fungal species and spore production

For the first experiment twenty isolates of *F. langsethiae* were selected (Table 5.1). These were originally isolated from wheat or oats between the periods of 2001 and 2009. For the second experiment, an isolate was selected based on the results from experiment one.

Table 5.1. UK *Fusarium langsethiae* isolates used in detached leaf assay

F. langsethiae isolate	Year of isolation	Host
FL/0/09/015	2009	Oats
FL/0/09/050	2009	Oats
FL/0/09/009/1*	2009	Oats
FL/0/09/009/2	2009	Oats
FL/0/09/009/3	2009	Oats
FL/07/062/1	2007	Oats
FL/07/062/2	2007	Oats
FL/2004/01	2004	Oats
FL/2004/02	2004	Oats
FL/2004/03	2004	Oats
FL/2004/11	2004	Oats
FL/2004/09	2004	Oats
FL/07/3	2007	Wheat
FL/2004/171(a)	2004	Wheat
FL/2004/171(b)	2004	Wheat
Fl/2004/170	2004	Wheat
FL/2004/140(a)	2004	Wheat
FL/2001/69(a)	2001	Wheat
FL/2001/69(b)	2001	Wheat
FL/2001/1	2001	Wheat

*Isolate used for experiment two.

Isolates were sub-cultured from PDA slopes onto PDA plates as described in Section 2.1.2. Spores were harvested after 14 days, spore concentration determined and diluted to a working concentration of 5 x 10^5 spores ml^{-1} as detailed in Section 2.1.5.

5.2.2. Production of leaf material

One variety each of oats (Gerald), wheat (Claire) and barley (Tipple) were used for the first experiment. These varieties were selected for the experiment because they are currently among the most important winter varieties in the UK and secondly Imathiu *et al*. (2009) used the same varieties in their experiments, thus they were selected to allow consistency and easy comparison of data. For the second experiment, UK varieties under testing for the HGCA Recommended list in 2010 of wheat, barley and oats were provided by the National Institute of Agricultural Botany, UK (Table 5.2)

Table 5.2. Number of wheat, barley and oat varieties used for the *in-vitro* leaf assay

Cereal	Number of varieties
Winter wheat	42
Spring wheat	10
Winter barley	22
Spring barley	21
Winter oats	10
Spring oats	14

Seeds were surface sterilized as detailed in Section 2.1.2, allowed to dry in an air flow cabinet. Thirty seeds were sown in plastic trays (21.5 x 15 cm) containing sterilized John Innes Compost Number 2 at a depth of 1 cm. Experimental trays were placed in a growth cabinet (Sanyo Versatile Environmental Test Chamber, Japan) at 20^{o}C and a 12 h photoperiod. Leaves were harvested 14 days after sowing by cutting 4 cm length segments from the tip of the primary leaves.

Each cereal species was assessed in separate experiments with Gerald included in all experiments to serve as a negative control and to allow comparison between experiments.

5.2.3. Leaf inoculation and disease assessment

Harvested leaves were wounded at the middle of the upper surface with a sterile 10 µl micropipette tip. Wounded leaves were carefully placed in a Petri dish with 0.5% water agar amended with kinetin (10 mg l^{-1}) with the adaxial surface facing up. In experiment one, each Petri dish had a wounded leaf segment of wheat, barley and oat representing one replicate within a split plot design with three blocks. In experiment two, each Petri dish contained three leaf segments of one variety and was replicated in three blocks. A 5 µl conidial suspension was placed on each wound. The same volume of SDW was used as control inoculation. Petri dishes were arranged in a randomized complete block design in a growth cabinet (Sanyo

Versatile Environmental Test Chamber, Japan); 20^{o}C, 10 h light 14 h darkness with a block on each shelf of the cabinet.

Seven days post inoculation, lesion lengths were measured on a light box to determine the aggressiveness of the different *F. langsethiae* isolates and resistance of the different cereal varieties to *F. langsethiae* infection. Lesions were identified as a water-soaked necrotic and/or chlorotic area.

Data was analysed with Genstat using linear regression with groups and as a split plot ANOVA (experiment 1). For experiment two, data was normalised using the formulae **(X/Y)*A** (X = overall mean Gerald lesion length, Y = mean Gerald lesion length in an experiment and A = mean lesion length of each leaf segment within a replicate) and analysed using a general ANOVA. Where significant differences were observed, means were separated by Tukeys post hoc test. A regression analysis was done for winter and oat varieties to test if there was a relationship between lesion length and HT-2+T-2 data obtained from field trials (Edwards, 2012).

5.3. RESULTS.

No lesions developed on negative control leaf segments (SDW inoculated) in both experiments. Lesions formed on all three cereals were similar and identified as water-soaked and/or chlorotic area (Figure 5.1.)

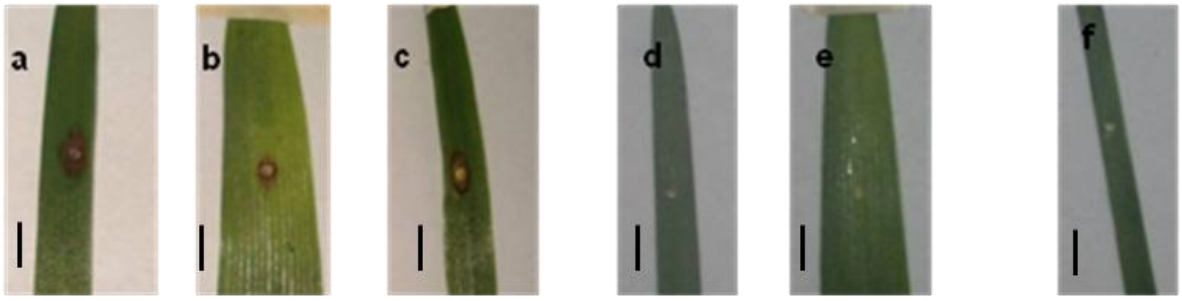


Figure 5.1. Wounded detached cereal leaves seven days post inoculation a, b and c are *F. langsethiae* inoculated oat, barley and wheat leaves and d, e and f are SDW (control) inoculated oat, barley and wheat leaves respectively. Bar = 8 mm.

5.3.1 Experiment 1. Assessing the pathogenicity and aggressiveness of 20 *F. langsethiae* isolates towards wheat, barley and oat.

All *F. langsethiae* isolates used for the experiment caused visible lesions on wheat, barley and oat.

A regression analysis of isolate age (length of time that each isolate had been stored) against isolate lesion length grouped by isolate host (cereal from which the isolate was originally isolated) and cereal (cereal species leaf inoculated) showed that both the age of isolate and the original isolate host did not have a significant effect on lesion length (P=0.38 and P=0.07 respectively). There was a highly significant difference (P<0.001) in the length of the lesion that developed on wheat, barley or oats. There was no significant interaction between any of these factors (P>0.05). Based on these results a split plot ANOVA (where each Petri dish was considered as a plot with each cereal leaf as a sub-plot) was carried out. This showed that cereal/isolate interaction was not significant (P=0.37). There was a highly significant (P<0.001) difference between lesion lengths formed by different *F. langsethiae* isolates used (Table 5.3). Isolate Fl/2004/17(a) caused the shortest lesion (3.8 mm) on all cereals and isolate Fl/0/08/009/1 caused the longest (9.4 mm) lesion on all cereals used. Results from individual isolates showed a continuum of lesion lengths with no obvious segregation of isolates into distinct groups.

A highly significant difference (P<0.001) was also observed between lesions on the different cereals (wheat, barley and oats) (Table 5.3). Lesions developed on oats were the longest, followed by barley with the shortest lesions developing on wheat. Lesion lengths on all cereals were significantly different from one another (P < 0.05).

Table 5.3. Lesion length caused by 20 *F. langsethiae* isolates in wounded detached leaf assay of wheat, barley and oats

Isolate	Cereal			Isolate mean
	Wheat	Barley	Oats	
FL/2004/171(a)	3.00	3.33	5.17	3.83[a]
FL/07/062/2	4.00	4.00	5.33	4.44[ab]
FL/07/3	3.67	4.67	5.33	4.56[ab]
FL/2004/09	4.33	5.33	7.00	5.56[abc]
FL/2001/1	4.33	5.33	7.00	5.56[abc]
FL/2004/140(a)	4.33	6.00	6.67	5.67[abc]
FL/2004/03	4.67	4.00	8.67	5.78[abc]
FL/0/09/009/2	4.33	4.33	9.00	5.89[abc]
FL/2004/01	4.67	5.67	7.33	5.89[abc]
FL/2004/02	3.67	5.67	8.33	5.89[abc]
FL/2004/171(b)	4.33	5.00	8.33	5.89[abc]
FL/0/09/050	4.33	6.00	8.33	6.22[abc]
Fl/2004/170	5.33	5.33	8.00	6.22[abc]
FL/0/09/015	4.67	6.00	9.00	6.56[abcd]
FL/2001/69(b)	3.67	9.33	6.67	6.56[abcd]
FL/0/09/009/3	4.33	7.67	9.00	7.00[bcd]
FL/07/062/1	7.00	6.00	8.33	7.11[bcd]
FL/2004/11	5.33	8.67	9.00	7.67[cd]
FL/2001/69(a)	6.67	9.00	9.33	8.33[cd]
FL/0/09/009/1*	7.33	10.33	10.67	9.44[d]
Cereal mean	4.70[a]	6.08[b]	7.83[c]	
P-value cereal		< 0.001		
P-value isolate		< 0.001		
P-value interaction		0.37		
%CV		27.8		

Values are means of three replicates. Values with the same superscript letter are not statistically different based on the Tukeys test (P = 0.05). * = isolate selected for experiment 2.

5.3.2. Experiment 2. Assessing the resistance of UK wheat, barley and oat varieties to *F. langsethiae* infection.

Fusarium langsethiae isolate FL/0/09/009/1 caused lesions on all varieties of all the three cereals studied. Lesions caused on wheat, barley and oat were similar to that described above (Section 5.3). In general, there was no significant difference in the lesion length of Gerald between experiments (P = 0.19) with lesion length ranging between 5.0 mm – 6.4 mm and a mean of 5.5 mm. There was a significant

difference in lesion lengths formed on the different cereal species (P<0.001). Winter barley and spring wheat had significantly (p < 0.05) lower lesion lengths compared to the other cereal species screened (Figure 5.2).

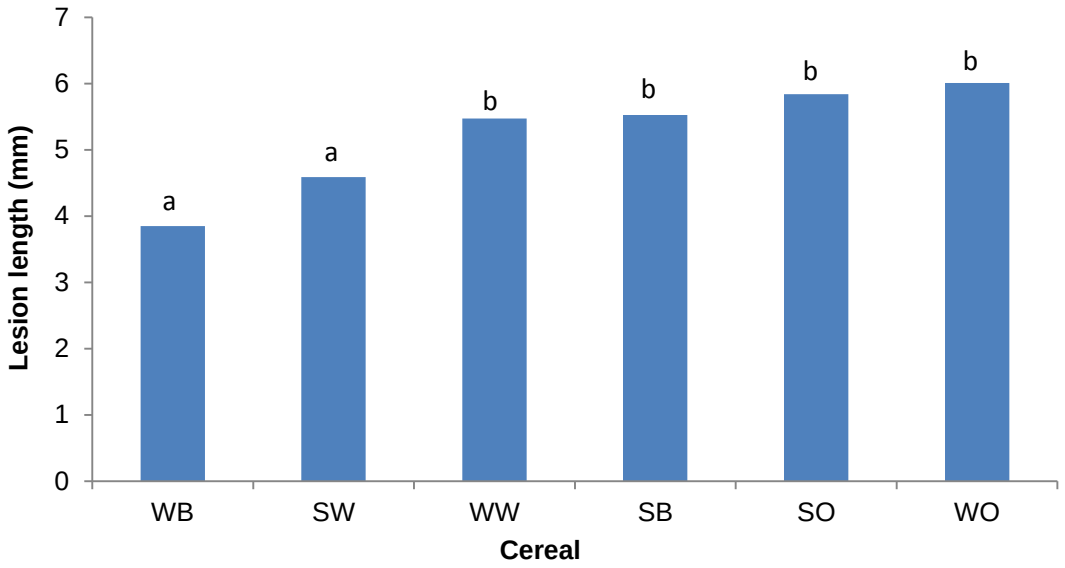


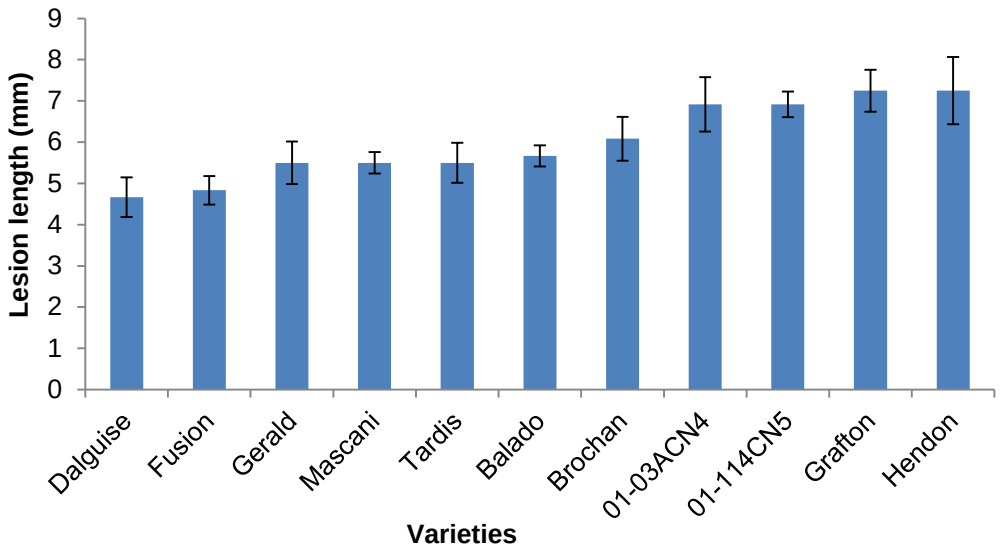
Figure 5.2. Lesion length caused by *F. langsethiae* on different cereal species. Columns with the same letters are not statistically different from each other (P = 0.05, %CV = 45 SEM = 2.40). WB = winter barley, SW = spring wheat, WW = winter wheat, SB = spring barley, SO = spring oats and WO = winter oats.

5.3.2.1. Oats

There was a highly significant difference (P<0.001) within the length of lesions formed on the different oat varieties. Lesion length varied from 4.7 mm – 7.3 mm with a grand mean of 6.0 mm for winter oat varieties. Within these varieties, lesion length on Dalguise was the shortest which was significantly (P = 0.05)) lower than those formed on Grafton and Hendon which had the longest mean leaf lesion lengths (Figure 5.3a). With spring oat varieties, Dominik had the shortest mean lesion length which was was significantly (P<0.05) lower than the mean of Ascot which had the longest mean lesion length (Figure 5.3b). Lesion lengths on spring oat varieties ranged between 3.9 to 7.1 mm with a grand mean of 5.8 mm.

A regression of all oat varieties (winter and spring) was not significant (P = 0.9). A regression analysis of lesion length against HT-2+T-2 from three years field data (Edwards, 2012)for five winter varieties (Brochan, Dalgise, Gerald, Mascani and Tardis) which are conventional tall varieties showed a significant regression (P=0.05) and accounted for 76% of the variance observed. Short and naked oat varieties were not include in the regression because lesion lengths formed on these varieties did not correspond well with the HT-2+T-2 field data. A similar analysis done for eight spring varieties (Argyle, Ascot, Atego, Canyon, Firth, Husky, Leven and Rozmar) again showed a significant regression (P=0.04) with 56% of variance accounted for (Figure 5.4 a and b).

a



b

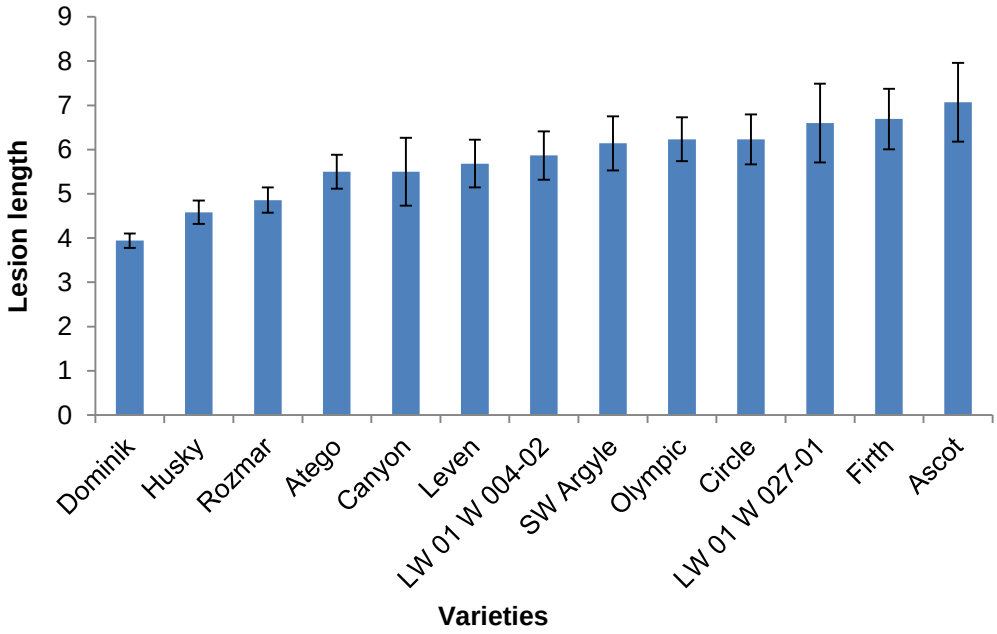
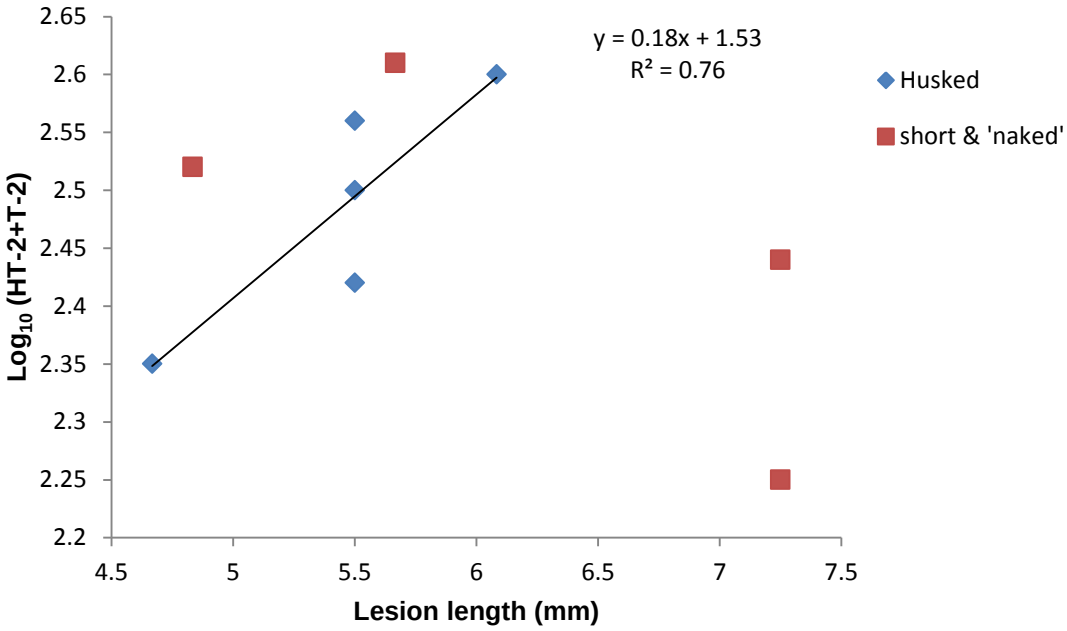


Figure 5.3. Lesion length on winter oat **a** (P<0.001 and %CV = 27.6) and spring oat **b.** (P<0.001 and %CV = 34.8).

a



b

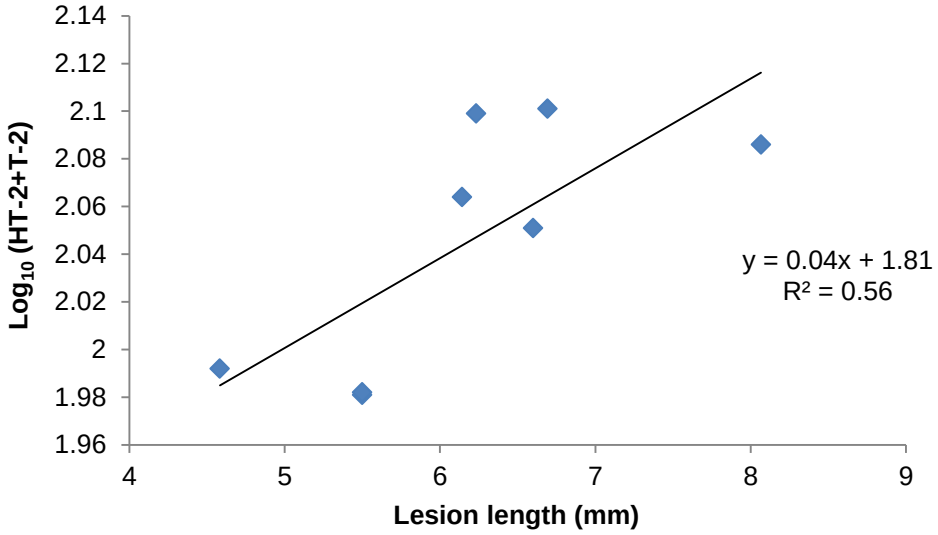


Figure 5.4. Relationship between lesion length and three years field data for HT-2+T-2 (Edwards 2012) for five husked winter (a) and seven spring (b) oat varieties

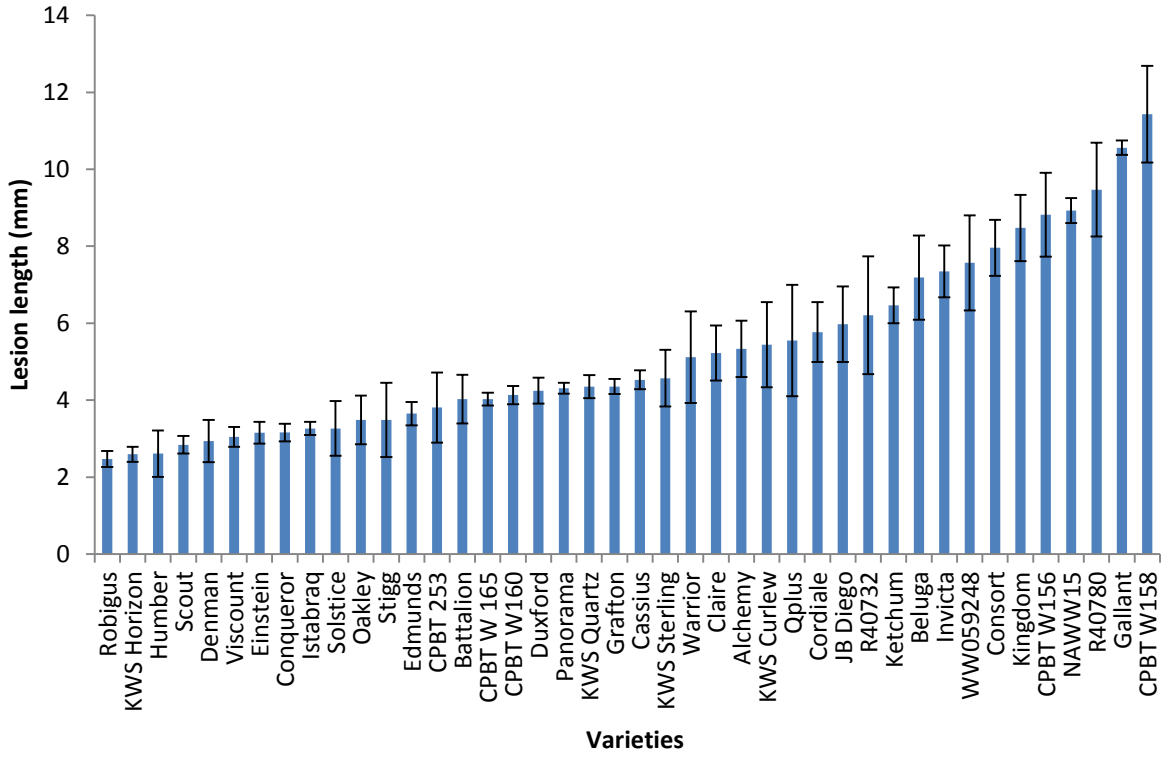
5.3.2.2. Wheat.

ANOVA showed a significant (P<0.001) difference among the lesion lengths formed on all wheat varieties. However, lesion lengths overlapped and it was not possible to

differentiate varieties into distinct groups. Although no distinct groups were formed the first five winter wheat varieties with the shortest lesion (Robigus to Denman) had lesion lengths ranging from 2.5 to 3.0 mm were significantly different ($P<0.05$) from the last ten varieties (Beluga to CPBTW 158) with the longest lesion lengths ranging from 7.4 to 11.8 mm. Between these two sets of varieties was a larger set (Viscount to Ketchum) whose lesion lengths were not significantly different from the two sets described. Lesion lengths of this set of varieties ranged between 3.1 and 6.7 mm (Figure 5.5 a).

Differences in lesion length of spring wheat varieties were statistically significant ($P<0.001$). Statistically, the difference ($P<0.05$) in means of the lesion lengths of the different varieties was between Paragon which had the longest lesion (7.6 mm) and the rest of the varieties. Among the spring wheat varieties, B4W3 had the shortest lesion length (3.8 mm) (Figure 5.5 b).

a



b.

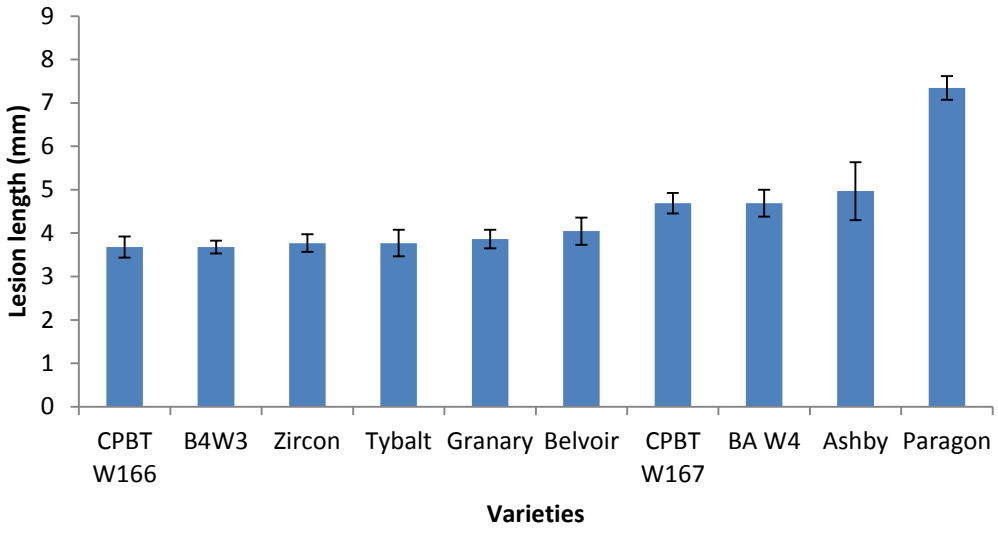


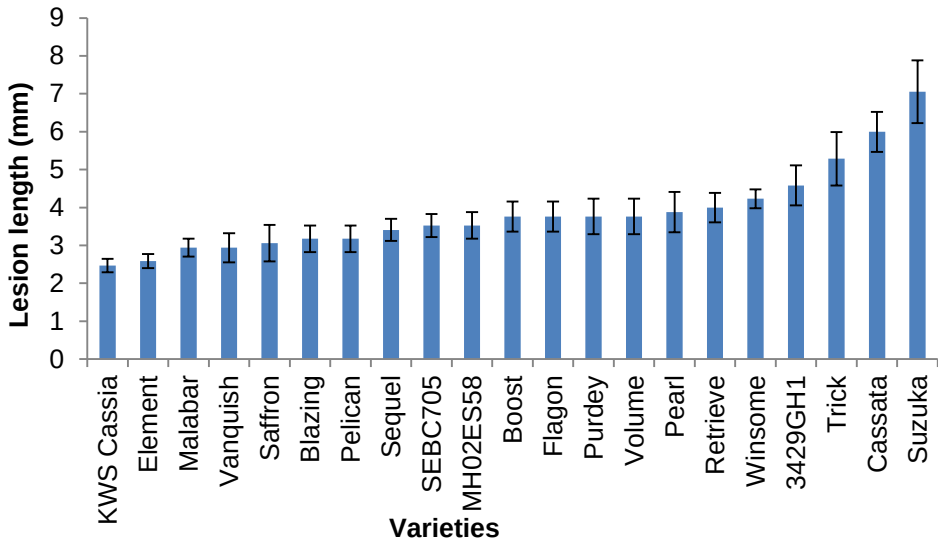
Figure 5.5. Lesion length on (a) winter wheat ($P < 0.001$ and %CV = 43.2) and (b) spring wheat ($P < 0.001$ and %CV = 21.1) Error bars represent standard error of means.

5.3.2.3. Barley

Lesions formed on winter barley varieties had lengths ranging from 2.5-7.0 mm and were highly significant from each other ($P<0.001$). Lesion lengths on varieties although significantly different formed a continuum that could not be put into distinct groups. KWS Cassia, Element, Malabar Vanquish and Saffron which had lesion lengths ranging from 2.5 to 3.1 mm were significantly shorter ($P<0.05$) than Trick, Cassata and Suzuka with lesion lengths ranging between 5.3 and 7.0 mm (Fig. 5.6a)

Highly significant differences ($P<0.001$) were observed among lesion lengths formed on spring barley varieties. Spring barley just like the winter varieties could not also be put into groups based on their lesion length. However Forensic and Decanter with lesion lengths 3.6 and 3.7 mm respectively were significantly ($P<0.05$) shorter than Oxbridge, Wagon, Checkmate, Moonshire, Quench and Shuffle which had lesion lengths between 6.1 and 8.1 mm (Figure 5.6b).

a



b.

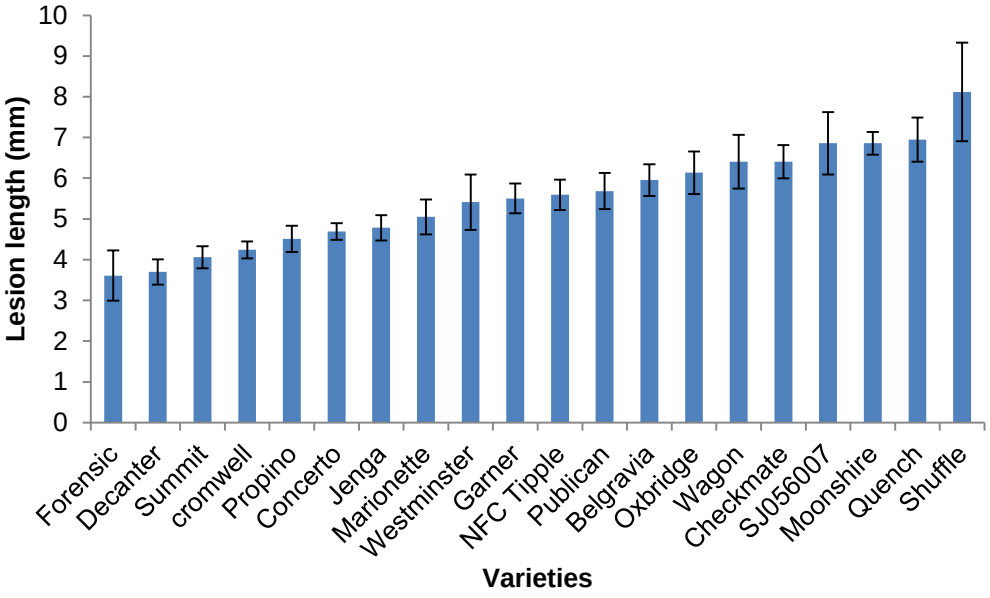


Figure 5.6. Lesion length on (a) winter barley ($P < 0.001$ and %CV = 33.2) and (b) spring barley ($P < 0.001$ and %CV = 29.2). Error bars represent standard error of means.

5.4. Discussion

Artificial wounds were created on leaf surfaces for this experiment because in previous work by Imathiu et al. (2009), *F. langsethiae* isolates were found to be pathogenic to wounded oat and wheat detached leaves but only on oat leaves in an unwounded leaf assay. The leaf surface serves as a physical barrier to fungal infection and therefore tougher cuticles may be harder for pathogenic fungi to penetrate into the underlying tissues. Creating artificial wounds on the leaf surface therefore eliminates this barrier and creates a uniform lesion for the initiation of infection.

All *F. langsethiae* isolates were pathogenic to wheat, barley and oats under the experimental conditions. Observed symptoms on the detached leaves seem to vary among the different cereals used. On oats, lesions were more necrotic, with necrosis starting from the point of inoculation. Lesions on wheat were characterized by a small chlorotic region around the point of inoculation with a well-defined necrotic boundary. The opposite was observed on barley where lesions had a small necrotic area around the point of inoculation with a rather larger chlorotic region surrounding it. The observed lesion characteristics on wheat and oats agree with that of Imathiu et al. (2009) who observed more necrosis on lesions developed on oats leaves than those developed on wheat. *Fusarium langsethiae* is not the only fungal species that has been reported to show variation in lesion characteristics on wheat and oats. Browne and Cooke (2004) reported that *Michrodochium majus* caused lesions on detached wheat leaves that differed from those seen on oats, with lesions on detached wheat leaves accompanied by chlorosis.

The ability of *F. langsethiae* to cause chlorosis and necrosis on wheat, barley and oat leaves is not surprising. This is because *F. langsethiae* is a prolific producer of HT-2+T-2, two of the most potent trichothecenes known (Edwards et al., 2009). HT-

2+T-2 are phytotoxic and have been reported to cause chlorosis and necrosis in plant tissues (Zonno and Vurro, 2002). They are known to inhibit RNA and DNA synthesis as well as triggering apoptosis; a process of programmed cell death in multicellular organisms (Uno et al., 1995, Yang et al., 2000). Although programmed cell death is essential for plants, necrotrophic pathogens are able to trigger programmed cell death in healthy plant tissues to cause disease (Coffeen and Wolpert, 2004).

Variability in the agressiveness of different *F. langsethiae* isolates has been reported by Imathiu et al. (2009). Five of the seven most aggressive isolates in the current experiment were isolated from oat grains. Three out of these five isolates were isolated in 2009 from oat grains harvested in 2008. This suggests three possibilities; their aggressiveness may be related to cereal species from which they were isolated; all the isolates may be from one population or their high agressiveness was due to the fact that they were isolated recently and had been stored only for a short period. *Fusarium* species, if not stored properly for a long period of time, like other fungal species are susceptible to cultural degeneration which can lead to a decrease in aggressiveness (Leslie and Summerell, 2006). Results from the regression analysis showed that the length of storage (age) or the host from which isolates were cultured from did not significantly affect the length of lesions formed on the leaves of the different cereals. This is in agreement with the work of Imathiu et al. (2009) who reported that, the aggressiveness of six (three isolates from wheat and three from oat) *F. langsethiae* isolates on detached leaf assay was not dependant on the source of isolation. This suggests that there is no host specialization within the different *F. langsethiae* isolates used in this studies.

Generally *F. langsethiae* isolates were more pathogenic on oats than barley and wheat in this assay, confirming the preference for oat by *F. langsethiae* observed in

the field (Chapter 4). There is very limited data on the susceptibility of different cereals towards *F. langsethiae* infection thus the need to study the resistance of different UK wheat, barley and oat varieties to *F. langsethiae* infection.

In assessing the resistance of different UK wheat, barley and oat varieties to *F. langsethiae* infection, lesions were formed on all varieties used in this experiment. The ability of *F. langsethiae* to show disease symptoms in the form of chlorosis and or water soaked regions on all three cereal species and varieties indicate that wheat, barley and oat have no complete resistance against *F. langsethiae* infection just like the *Fusarium* species responsible for FHB (Parry et al., 1995; Brown and Cooke, 2005). Lesions formed on oat varieties were the longest among varieties of all cereals studied. This further confirms already observed host preference reported for *F. langsethiae* (Chapter 4). The *in-vitro* leaf assay data agrees with data from the three year field sampling (Chapter 4) where winter oats had the highest level of *F. langsethiae* DNA and HT-2+T-2 levels. This was followed by winter wheat and spring barley respectively. It should be noted that although in the field survey winter wheat had the second highest *F. langsethiae* DNA and HT-2+T-2 levels (changing places with spring barley in the *in-vitro* assay) there was no significant difference between the means of lesion lengths formed on leaves of these two cereals. Using the length of lesions formed by *F. langsethiae* on the different cereal species, it was observed that lesion lengths overlapped and as such were no distinct sub-groups with distinct differences in resistance. This could be because as already stated there may not be cereal species-specific resistance against *F. langsethiae* infection. It could also be that the use of just one component of PDR was not effective enough to differentiate the different varieties into distinct groups. In the evaluation of resistance to FHB different authors have used either single or a number of PDR components in combination. Brown and Cooke (2005) in assessing components of PDR in wheat, barley and oats used the incubation period and latent period. Kumar

et al. (2011) used incubation period, latent period and lesion length and according to the authors the latent period and lesion length were effective in measuring FHB resistance components in barley. Imathiu et al. (2009), used lesion lengths to study the pathogenicity and aggressiveness of *F. langsethiae* towards wheat and oats.

The *F. langsethiae* isolate used was the most virulent among 20 other isolates. Fungal isolates that have the ability to cause disease rapidly, when used in detached leaf assays are not able to differentiate between cereal cultivars with intermediate resistance (Brown and Cooke, 2004). This could also be another reason why the *in-vitro* leaf assay could not differentiate the different varieties into distinct resistance or susceptible groups.

The results from this work has shown that an *in-vitro* leaf assay measuring lesion length could be a quick and easy method to screen oat varieties for resistance or susceptibility to *F. langsethiae* and subsequent HT-2+T-2 accumulation in grains. This is because comparing data from the *in-vitro* leaf assay (without that of dwarfed and naked varieties) with three years field data of HT-2+T-2 levels from UK oat variety trials (Edwards, 2012), recorded lesion lengths correlated positively with HT-2+T-2 levels in grains. Data for dwarfed and naked oat varieties were not included in the regression analysis because their lesion lengths did not correlate well with HT-2+T-2 data probably because of some resistance conferred on them as a result of their morphology which could not be accounted for by measuring lesion length alone on leaves. A number of components for FHB resistance have been proposed. These components include resistance to invasion (type I), resistance to spread (type II), resistance to kernel infection (type III), tolerance (type IV), resistance to toxin accumulation (type V) (Mesterhazy, 2002). Among these components, type I and II are the most common. Type I is normally assessed by point inoculation and II by spray inoculations (Schroeder and Christesen, 1963). For type I resistance plant

morphological characters such as plant height among others contribute to this resistance (Zhu et al., 1999; Bushnell et al., 2003). There are two schools of thought as to why plant height plays a role in FHB resistance; true resistance and disease escape. Studies have shown that a number of FHB resistance loci are associated with plant height QTLs (Steiner et al., 2004; Dreager et al., 2007). Dreager et al. (2007) showed that FHB susceptibility was associated with the dwarfing allele *RhtD1b*. A study comparing *RhtD-1b* and *RhtD-1a* (tall allele) revealed that *RhtD-1b* lines were more susceptible to initial infection but unaffected in resistance to spread (Srinivasachary et al. 2008). Srinivasachary et al. (2009) however, stated that not all height QTL are coincident with FHB QTL implying that the relationship between height and FHB is not simply a consequence of disease escape. The other school of thought propose that shorter cereal varieties are generally known to have less resistance to FHB compared to taller varieties simply because of the difference in micro-climate created around these varieties, with shorter varieties creating conditions which favour initial infection (Miedaner, 1997). Cooke (1981) also stated that plant height could confer disease escape and that such characters are independent of the PDR variables measured using a detached leaf assay. It therefore seems reasonable that measured lesion lengths did not correlate with HT-2+T-2 levels in dwarf winter oat varieties.

The inability of measured lesion length to correlate well with HT-2+T-2 concentration in naked oat varieties could be because of the 'naked' nature of the grain after harvest. Data from de-hulling experiments have shown that the process reduces HT-2+T-2 levels in oat grains and this reduction is typically 90% (Edwards, 2007; Scudamore et al., 2007). This has also been confirmed by data from this PhD research (Chapter 5). This suggests that a large proportion of these mycotoxins occur in the outer hulls (Edwards et al., 2009). In naked oat the hulls are removed during harvest unlike the husked varieties. This means that naked varieties may

have lower concentrations as a result of the absence of the hulls. Estimated HT-2+T-2 in naked varieties are unlikely to be a true reflection of what existed in the standing crop before harvest thus the poor correlation between the *in-vitro* leaf assay data with HT-2+T-2 data for naked oat varieties.

Field data on HT-2+T-2 levels in the different UK wheat and barley varieties is lacking and therefore it is not possible to determine whether the use of an *in-vitro* leaf assay measuring lesion length would be a good tool in predicting field occurrence of *F. langsethiae* and subsequent HT-2+T-2 concentration in heads of these cereal species.

5.5. Conclusions

While most of the cereals varieties used in this study had no total resistance to *F. langsethiae* infection, it is interesting to note that in oats, varieties with low HT-2+T-2 in heads under field conditions also had shorter lesion lengths *in-vitro* suggesting an intermediate resistance to *F. langsethiae* infection.

The difficulty in using lesion length to totally differentiate the cereal varieties into distinct resistant or susceptible groups may suggest that a single component of the PDR is not sufficient in discriminating among these cereal varieties when it comes to resistance to *F. langsethiae* infection. It is therefore suggested that future studies should also measure incubation and or latent period should. It is believed that data from *in-vitro* leaf assays can be a good predictor of HT-2+T-2 concentration in harvested grain if it is combined with knowledge of the impact of height and nakedness on fusarium mycotoxins at harvest.

While resistance measured in detached leaf assay may be important components of whole plant resistance to FHB and mycotoxins production and a rapid pre-screening tool in breeding (Cooke, 1981), its use should not lessen the need for whole plant

evaluation of *F. langsethiae* resistance under field conditions as this is needed to fully understand the resistance mechanism underlying *F. langsethiae* infection as well as the general understanding of *F. langsethiae*-cereal interaction. Since there is no effective method of artificially inoculating wheat, barley and oats with *F. langsethiae* for whole plant studies, there is the need to develop or optimize existing inoculation methods for other FHB pathogens so as to make whole plant studies of *F. langsethiae*-cereal interaction studies in the glasshouse and field a possibility.

Chapter Six

6. Study of *Fusarium langsethiae*-cereal relationship through artificial inoculations

6.1. Introduction

Disease establishment in plants consist of three general processes; inoculation, incubation and symptom development. Inoculation is the transfer of inoculum from a source to an infection site on a plant. It is the initial contact of a pathogen with a plant part where infection is possible (Dhingra and Sinclair, 1995; Agrios, 2005). Incubation involves all the complex plant-pathogen interactions that take place between the period of inoculation and the appearance of disease symptoms (Agrios, 2005). Symptoms are the visible signs of a disease occurrence. The inducement of symptoms is indicative of a successful infection by a pathogen (Dhingra and Sinclair, 1995; Agrios, 2005).

According to Dhingra and Sinclair (1995), there are a number of reasons why attempts are made by scientist to artificially establish a disease in a plant. These include (1) testing the pathogenicity of a potential causal agent (2) to evaluate cultivars, lines or selections for pathogen resistance and (3) to screen pathogen isolates for physiological specialization.

In order to artificially establish a disease in a host, it is important to select a suitable inoculation method and determine the quality and quantity of inoculum (Dhingra and Sinclair 1995). Spores and spore suspension in the form of sprays and/or point inoculations have been used by a number of authors to study resistance to FHB in wheat (Miedaner et al., 2003).

The growth stage of the host at the time of inoculation is important in disease development (Dhingra and Sinclair, 1995). Cereals are most susceptible to FHB

from head emergence through to the end of flowering (Xu, 2003; Edwards, 2004). Artificial inoculation of *F. langsethiae* on wheat, barley and oats was carried out by Imathiu (2008). However, the methods which included spraying of panicles with spore suspension, point inoculation with spore suspension and the use of inoculated grains as inoculum failed to cause typical head blight symptoms or infection. This was confirmed when high levels of *F. langsethiae* DNA were detected on inoculated plants, leaving room for speculation that traditional methods of inoculating FHB pathogens are not suitable for the study of *F. langsethiae*. However, since there are a number of inoculation methods that can be adapted and the possibility that *F. langsethiae* may produce symptoms that are different from traditional FHB symptoms, there was the need for further experiments to be carried out to establish the relationship between *F. langsethiae* infection and the host plant reaction through artificial inoculations.

The objective of these experiments was to develop artificial inoculation methods for *F. langsethiae* in cereals and to use these methods to study the pathogen-host relationship.

6.2. Materials and Methods

In all glasshouse experiments, unless otherwise stated, spring varieties of wheat (Belvoir), barley (Tipple) and oat (Firth) were used. Except in the seed infection assay, plants were sown in 1.2 L pots. Plants were treated against mildew infection using Attenzo® plus Opus® (BASF) and Aphox® (Syngenta) against Aphids at the maximum recommended field rates. Unless otherwise stated, DNA extraction and HT-2+T-2 analysis were carried out as detailed in Sections 2.2.3 – 2.3.2 of the general methods.

6.2.1. Seed Infection Assay

Seed samples of Gerald from the 2008 harvest with known HT-2+T-2 were supplied by Prof. S. Edwards. All oat seeds used in the experiment were untreated. Five seed lots known to have high HT-2+T-2 (> 500 µg kg^{-1}) content, one with low level (267 µg kg^{-1}) and four known to have undetectable levels of HT-2+T-2 (Table 6.1) were sown in bread trays (57 x 37 x 13 cm) containing John Innes Compost No. 2. Approximately 75 seeds were sown in each tray. Before sowing, an initial QPCR as detailed in Section 2.3.2 was carried out to determine whether the *F. langsethiae* DNA in each seed lot corresponded with their HT-2+T-2 levels. Two replicate trays were produced for each sample and these were laid out in a randomised arrangement in a polytunnel. Plants were drip irrigated and fertilized as required. At maturity plant heads were manually harvested, threshed and grain milled before mycotoxins and DNA analysis was carried out.

Table 6.1. Oat samples and their initial HT-2+T-2 concentrations used in the seed infection assay

Sample	HT-2+T-2 concentration (µg kg^{-1})
0/08/005	<10
0/08/015	<10
0/08/018	<10
0/08/022	<10
0/08/009	267
0/08/038	581
0/08/050	951
0/08/060	682
0/08/078	490
0/08/081	734

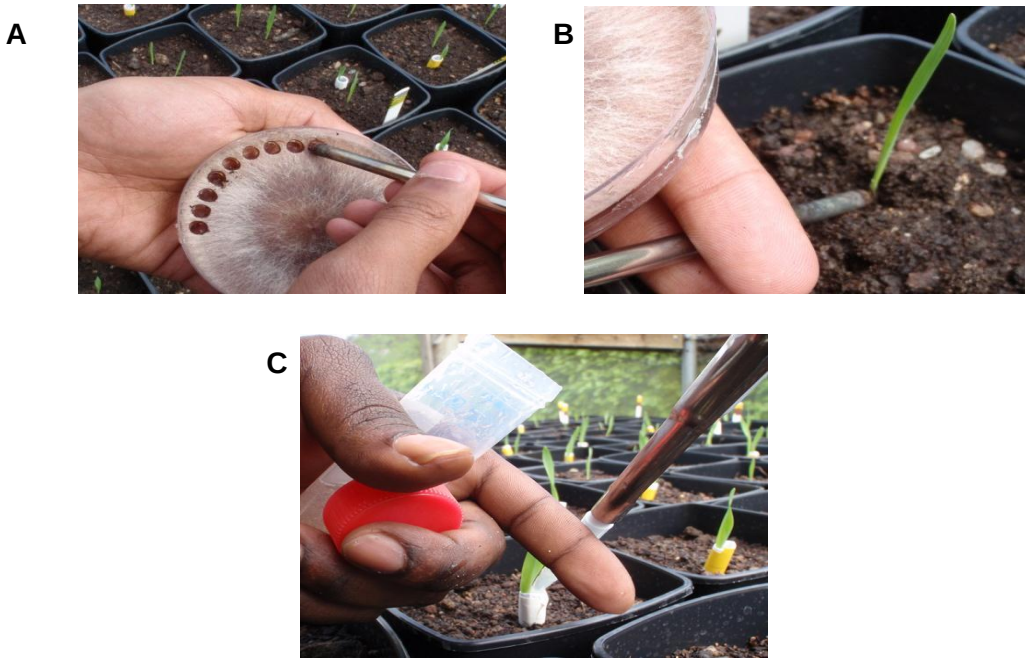
Source: Samples provided by Prof. S. Edwards. Mycotoxin analysis completed as part of HGCA project RD-2007-3332.

6.2.3. Stem base infection

Two seeds per cereal variety were sown in 10.5 x 11.5 cm pots filled with John Innes number 2 compost. At GS11 seedlings were thinned out to a single seed per pot after which stem base inoculations were done. The experiment was laid out in a complete randomized design with four replicates per treatment. Two stem base infection methods were carried out.

In the first method, a mycelial plug was cut from the margin of a 14 day old *Fusarium langsethiae* colony grown on PDA using a cork borer (radius 3 mm) (Figure 6.1A) and placed directly at the base of the seedling. The plug was then covered with a thin layer of soil to keep it in place and to prevent it from desiccating. Uninoculated PDA plugs and plugs from *F. graminearum* isolate were used as negative and positive controls respectively (Figure 6.1B).

For the second inoculation method, paper cylinders (ca.1.5 cm in diameter) were produced and placed around the base of wheat, barley and oat seedlings just after emergence. Cylinders were protected from water damage by covering them in adhesive tape. Inoculum was prepared by culturing and harvesting fungal spores as detailed in Section 2.1.6 with the exception that the spore suspension was made using 0.75% water agar. This was done by first preparing the 0.75% water agar and then cooling it down to about 25^{o}C. A concentrated spore (1x10^{9}) suspension was then added to the agar to achieve a concentration of 1x10^{6} spore ml^{-1} (working concentration). The agar-spore suspension was then mixed by shaking by hand in a 50 ml centrifuge tube after which 1 ml of spore suspension was pipetted into the paper cylinders around the seedlings (Figur 6.1. C).



Figuree 6.1. Illustration of stem base inoculation methods: A and B mycelium plug method and C agar spore suspension method.

Fourteen and 28 days after inoculation, destructive sampling was undertaken to assess for any stem base infection and there after plants were monitored for stem and head symptoms each week over the growing period. At maturity plants were uprooted and again assessed for stem base symptoms after which they were divided into stem and head sub-samples. Samples were then milled in order for DNA and mycotoxin analysis to be carried out.

6.2.2. Boot inoculation

Boot inoculation experiments were carried out in 2009 and 2010. In 2009 a field experiment was carried out on Blackbirch field at Harper Adams University College on a commercial crop of wheat (Oakley) and oats (Gerald). An area of 4m x 4m was selected for each cereal. Ten plants were selected randomly for each treatment application. In 2010 the field experiment was repeated but using winter and spring varieties of wheat, barley and oat. This was carried out at Harper Adams University College (Shropshire UK). In the same year a glasshouse

experiment was also carried out. This involved the use of ten plants each of spring varieties of wheat (Belvoir), barley (Tipple) and oat (Firth). Two seeds were sown per pot and thinned down to one plant per pot after seedling establishment. Inoculum was prepared as detailed in Section 2.1.6 of the general methods.

6.2.2.1 Factors under investigation and treatment application

In the first field experiment (2009) two factors were investigated; inoculation at two levels (inoculated and non-inoculated) and three growth stages (Zadoks growth stages (GS) 47, 49 and 62). In the second field experiment (2010) as well as the glasshouse experiment in 2010, plants were inoculated at GS45, 47 and 49 with spore solutions of four different concentrations (10^3-10^6 spores ml^{-1}). At GS45 and GS47, 0.25 ml of inoculum was 'injected' into boots using a hypodermic needle (Terumo 33G X 1") with dimensions 0.6 x 25 mm. At growth stage GS49, inoculum was applied using a 286-Atomizer (DeVilbliss®). This was to allow uniform spread of inoculum on the developing head within the boot. Ten sprays of spore solution (*ca.* 0.5 ml) were applied into each opening boot. Distilled water amended with Tween 20 (0.05%) was applied in the same manner to serve as negative control whilst a *F. graminearum* isolate was used as a positive control. The experiments were laid out as a complete randomized design with four replicates per treatment. The plants were examined for any disease or symptom development two days post inoculation (DPI) and every other day until GS70 and thereafter on a weekly basis. Twenty-one days after inoculation, the percentage cereal head emergence was estimated by counting the number of emerged heads (half to full head emergence) and expressed as a percentage of the total number of each cereal species per treatment. Plant heads were manually harvested at maturity (GS92), air dried and milled before DNA and mycotoxin analysis was carried out.

Figure 6.2. Boot inoculation. A injecting inoculum into a boot through a hypodermic needle (red arrow) and B introducing inoculum into an opening boot using a 286-Atomizer

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A dose response analysis was carried out to determine the effect of spore concentration and the time of inoculation (GS) on the cereal head length and *F. langsethiae* DNA in heads at maturity. A simple linear regression of spore concentration used for inoculation against *F. langsethiae* recovered from heads at harvest grouped by stage of inoculation was done to see the effect of these factors on *F. langsethiae* accumulation in cereal heads at harvest. On the basis of the results from the regression analysis ANOVA was carried out. Where statistical differences were observed a Tukey test was used to determine statistical differences between the means. Statistical analysis was carried out using Genstat (V.13 VSN International Ltd.). Analyses of data for boot inoculated plants were restricted to only glasshouse experiments. This is because of loss of field samples before harvesting.

6.2.4. Spray inoculation

Spore suspension (inoculum) was prepared as detailed in Section 2.1.6. At GS59, selected heads of wheat, barley and oat were sprayed with the prepared inoculum with a plastic mist spray bottle (Focus®, Cheshire, UK). About 5 ml of inoculum was applied to each head. Heads were then bagged with two types of polythene bags; unperforated clear (50 x 38 cm) and perforated cryovac (Cryovac® Bake-Ready®) (45 x 30 cm) (Figure 6.3). One pot per treatment was selected randomly and a data logger (placed in the pot to measure temperature and relative humidity. In pots where heads had been bagged, the data logger was placed within the bag. Bags were kept on heads for periods of one week, two weeks and until harvest. During these periods heads were assessed for any disease symptoms. Cereal heads of control pots were sprayed with SDW. The experiment was laid out in a complete randomized design with four replicates per treatment. At maturity heads were harvested and milled before DNA and mycotoxin analysis was carried out as detailed previously.

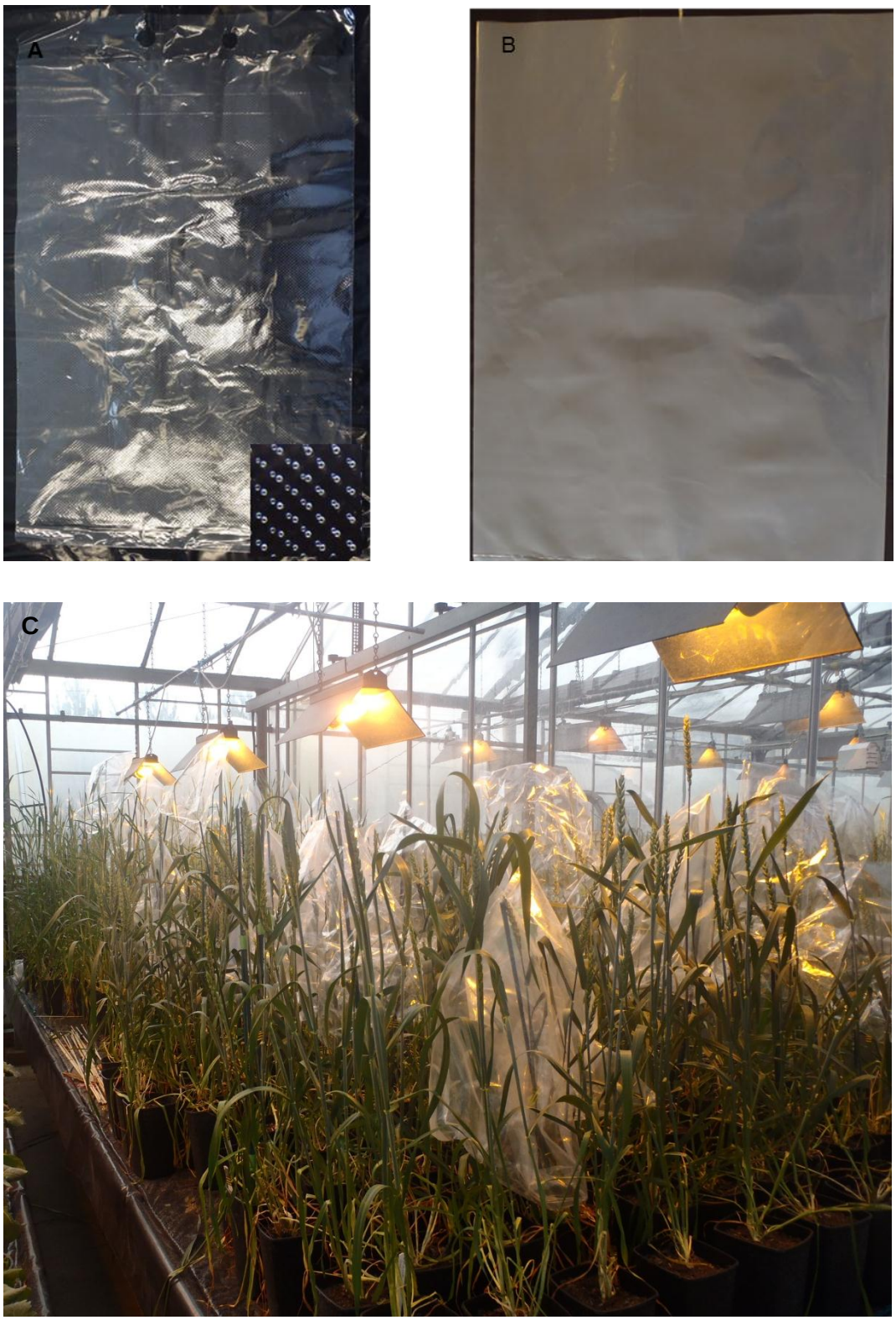

Figure 6.3. Bag types for used for experiments; A perforated cryovac bag (the insert shows a closer image of the perforation), B normal clear bag and C section of experimental set up with inoculated heads bagged

6.3. Results

6.3.1. Seed infection assay

The initial HT-2+T-2 levels in all samples corresponded with the initial *F. langsethiae* DNA in seeds before sowing (Table 6.1 and 6.2). With the exception of three samples, all oat samples at harvest had HT-2+T-2 levels below 10 µg kg^{-1}. There was no detectable *F. langsethiae* DNA (<LoD) in the harvested grains of the eight oat samples. Two samples had detectable non-quantifiable levels (<LoQ) of *F. langsethiae* DNA (Table. 6.2.).

Table 6.2. Initial *F. langsethiae* DNA and final HT-2+T-2 and *F. langsethiae* DNA in oat samples used in the seed infection assay

Sample	Initial *F. langsethiae* DNA (pg ng^{-1})	Final HT-2+T-2 (µg kg^{-1})	Final *F. langsethiae* DNA (pg ng^{-1})
0/08/005	0.002	18	<LoQ
0/08/015	0.004	<10	<LoD
0/08/018	0.003	<10	<LoD
0/08/022	0.004	<10	<LoD
0/08/009	0.786	16	<LoD
0/08/038	0.119	<10	<LoD
0/08/050	0.395	<10	<LoD
0/08/060	0.339	24	<LoD
0/08/078	0.970	<10	<LoD
0/08/081	0.755	<10	<LoQ

<LoD means no signal and <LoQ means signal below the lowest standard 10^{-4} pg ng^{-1}

6.3.2. Stem base infection

Fourteen days after inoculation, the *F. graminearum* inoculated plants showed signs of stem based browning. This was in the form of brown lesions which were mostly restricted to the outer leaf sheath. This was more pronounced in wheat and least pronounced in oat. *F. langsethiae* and PDA (control) inoculated plants did not show any stem base symptoms (Figure 6.4).

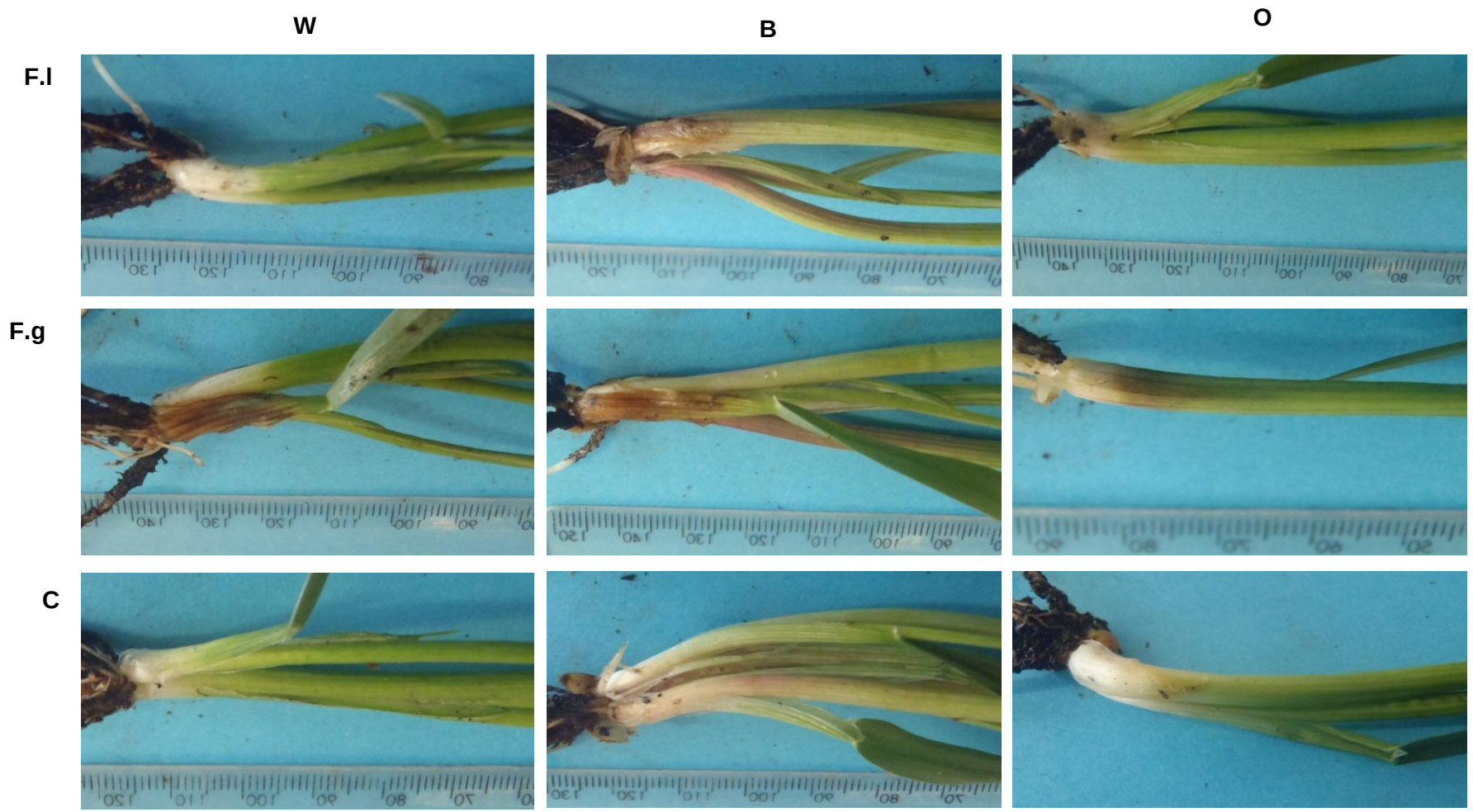

Figure 6.4. Myceliium-plug inoculated wheat (W), barley (B) and oat (O).F. l = *F. langsethiae*, F g = *F. graminearum* and C = PDA plug inoculated seedlings 14 DPI.

The situation was different in spore-agar inoculated plants where no stem base infection was observed in any of the plants (Figure 6.5).

Twenty-eight days after inoculation, *F. langsethiae* inoculated wheat and oats showed no signs of stem base infection in both mycelium-plug and spore-agar inoculated plants. However, barley plants showed signs of stem base infection but these were not as distinct as in *F. graminearum* inoculated plants (Figure 6.6 and 6.7)

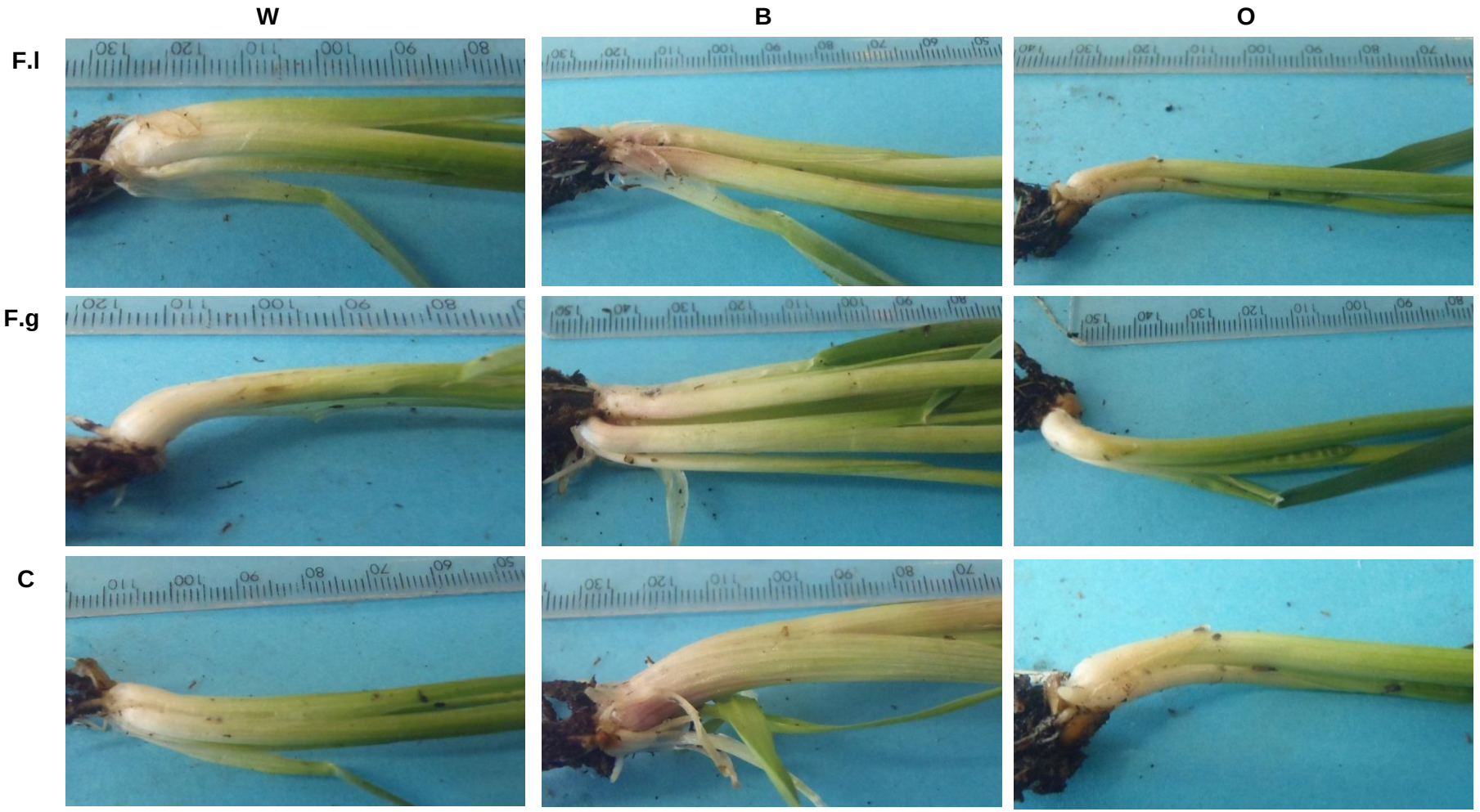

Figure 6.5. Spore-agar inoculated wheat (W), barley (B) and oat (O) F. l = *F. langsethiae*, F. g = *F. graminearum* and C = water-agar inoculated seedlings 14DPI.

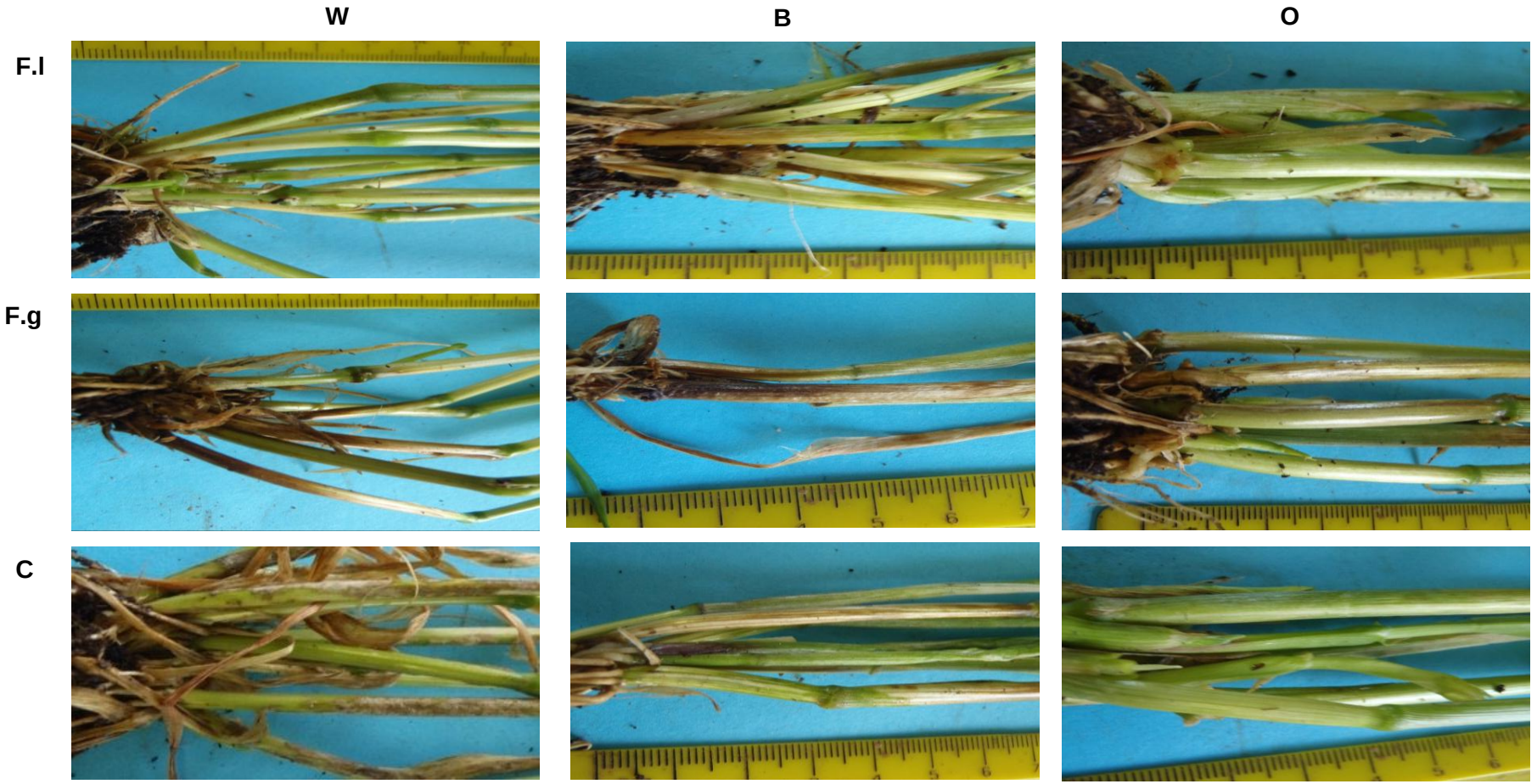

Figure 6.6. Mycelium-plug inoculated wheat (W), barley (B) and oat (O) (28 DPI).F. l = *F. langsethiae*, F g = *F. graminearum* and C = PDA plug inoculated seedling.

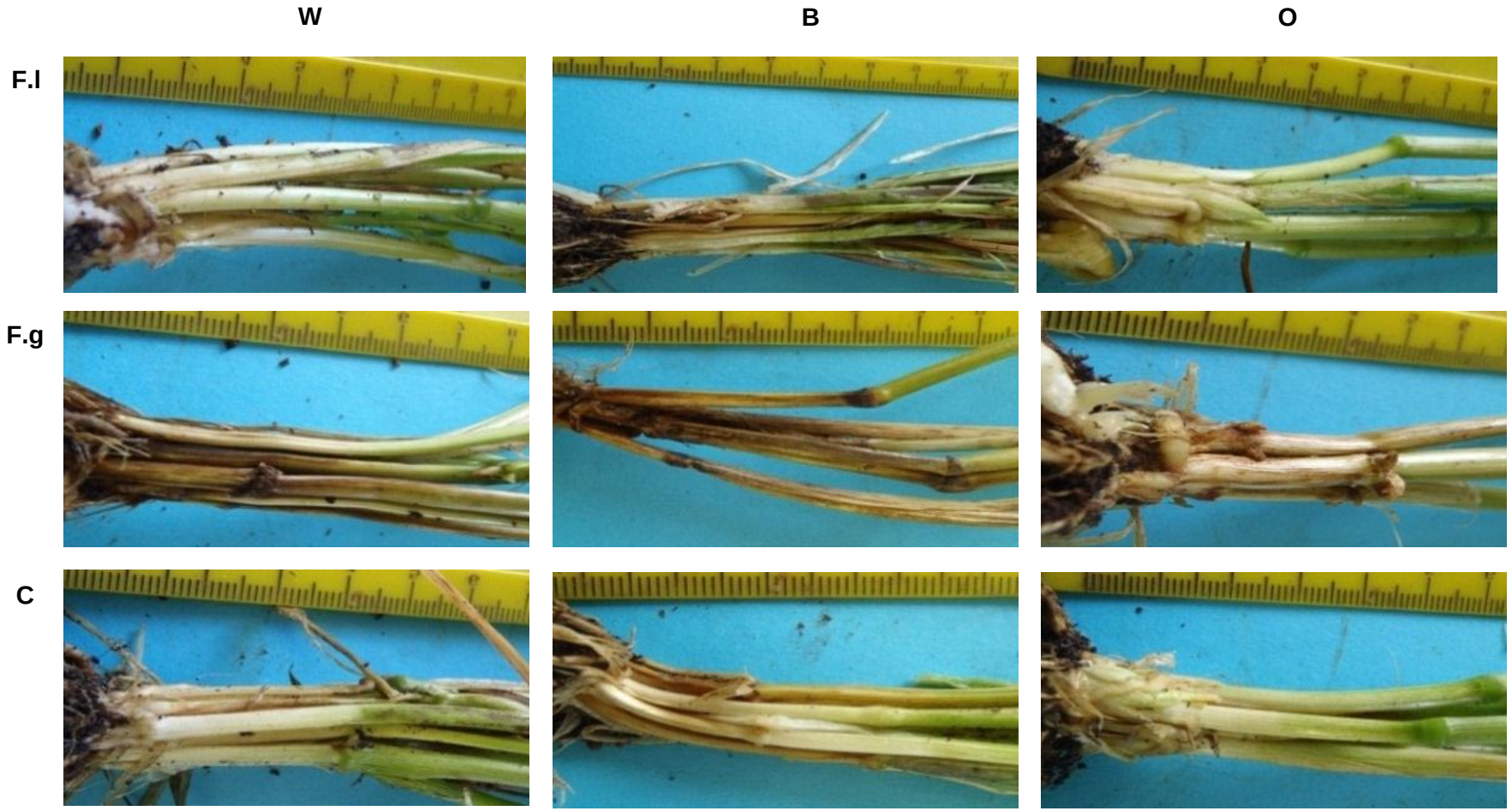

Figure 6.7. Spore-agar inoculated wheat (W), barley (B) and oat (O) (28 DPI). F. l = *F. langsethiae*, F g = *F. graminearum* and C = water-agar inoculated seedlings.

At harvest there were clear signs of stem base infection in plants inoculated with *F. graminearum* using both inoculation methods. This was in the form of dark brown to black necrosis with a loss of tissue of the lower stem base especially in wheat and barley. In barley about 75% of plants lost their roots on uprooting. Stem base infection was more severe on plants inoculated with spore-agar suspension. Masses of perithecia were also observed on the lower stem base of wheat, barley and oat but were more evident on wheat and barley (Figure 6.8 and 6.9).

Quantitative PCR data from lower, upper stem and head samples at harvest showed that a higher percentage incidence of *F. langsethiae* in cereal heads had occurred when plants were inoculated using the mycelium-plug method (Table 6.3a and b). However, a higher *F. langsethiae* DNA concentration was detected in cereal heads of wheat and barley when they were inoculated using the agar-spore method with barley having the highest *F. langsethiae* DNA concentration. *F. langsethiae* DNA in barley heads inoculated using the agar-spore method ranged between 0.004 to 0.007 pg ng^{-1} with a mean of 0.005 pg ng^{-1}. *F. langsethiae* DNA was not detected in oat heads using the same inoculation method (Table 6.3b).

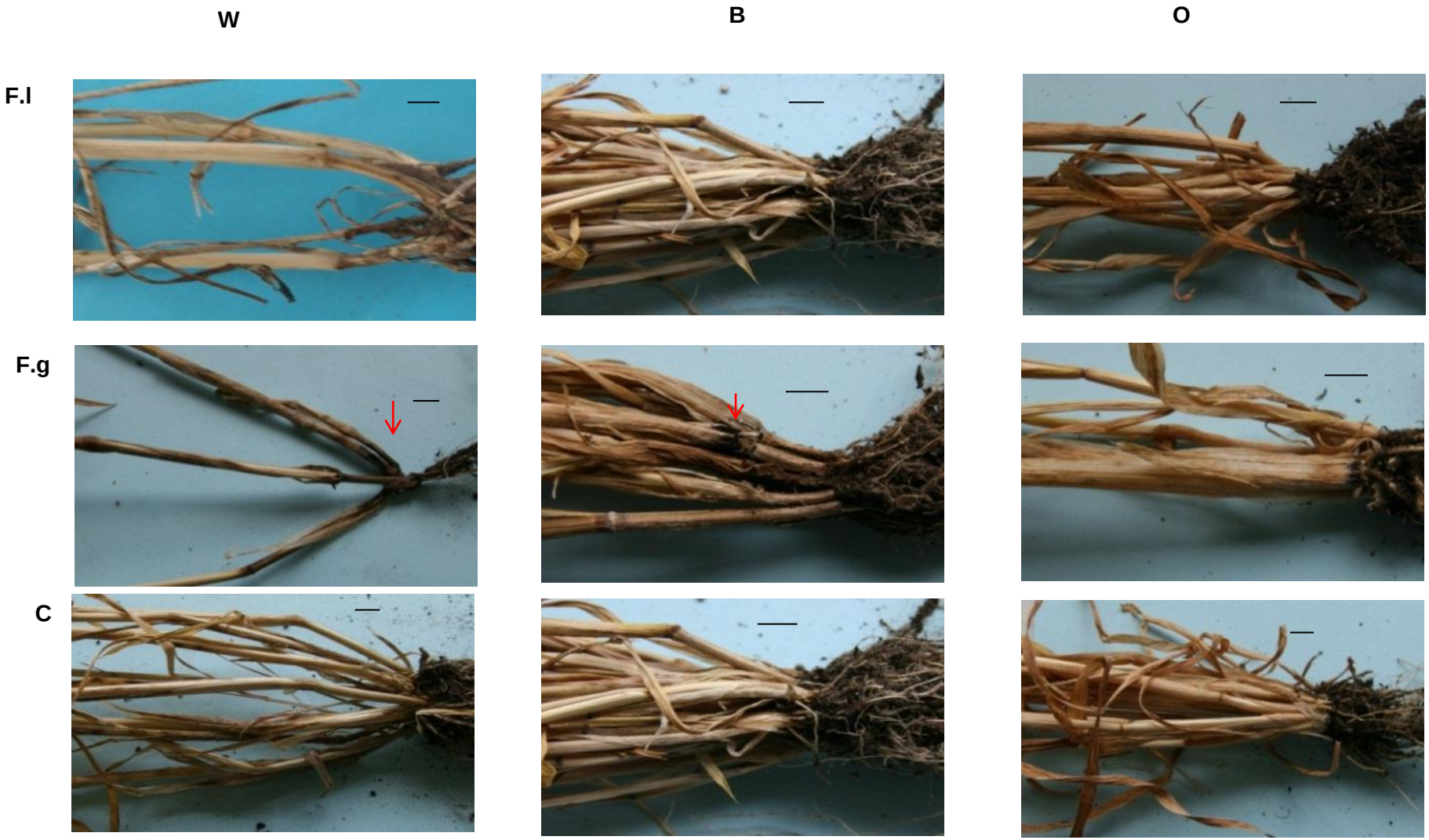

Figure 6.8. Mycelium-plug inoculated wheat (W), barley (B) and oat (O). A, B and C = *F. langsethiae, F. graminearum* and PDA plug inoculated plants as seen at harvest. Bar =1 cm. Red arrow indicates position of perithecia

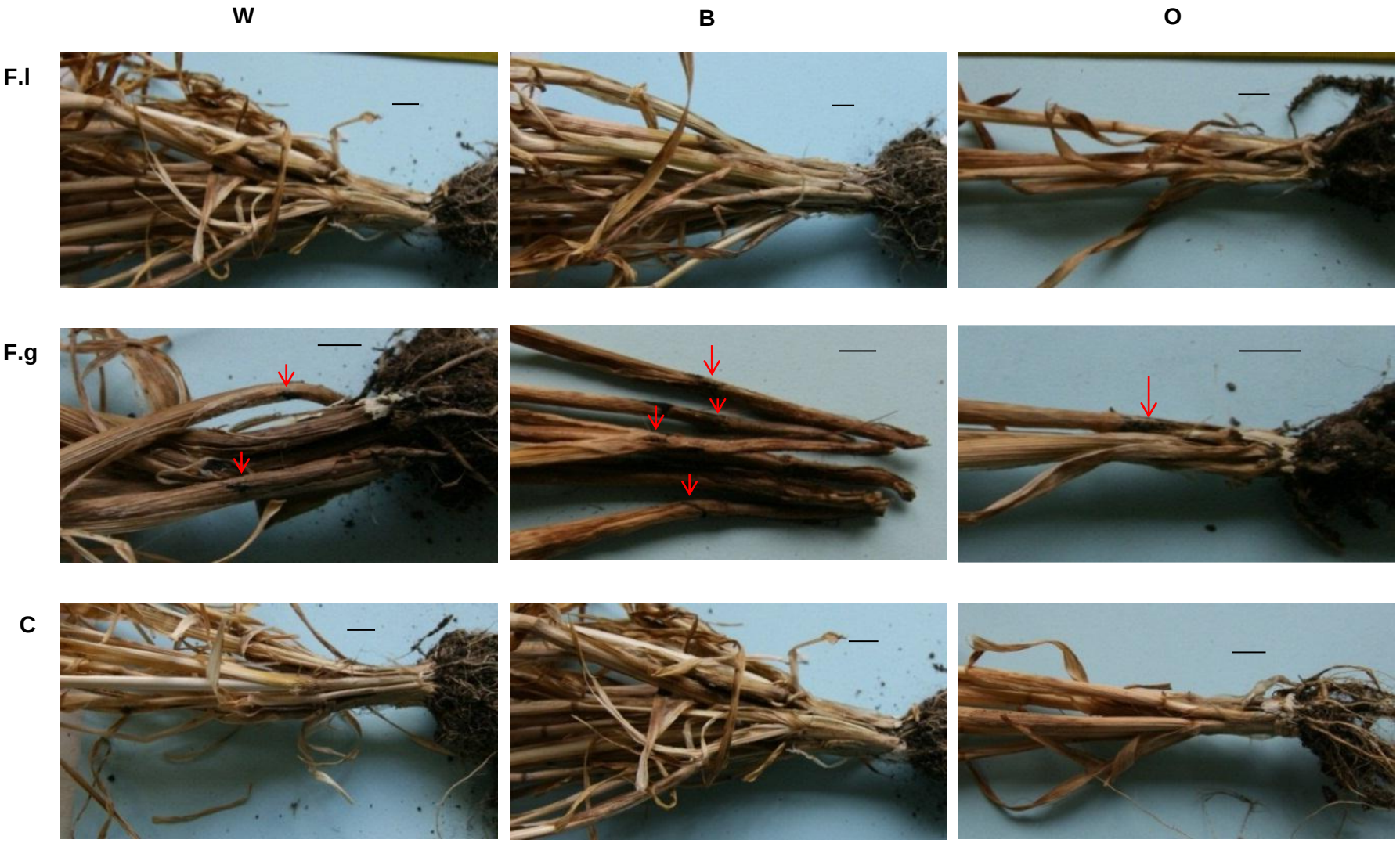

Figure 6.9. Spore-agar inoculated wheat (W), barley (B) and oat (O). A, B and C = *F. langsethiae, F. graminearum* and water agar inoculated plants as seen at harvest. Bar = 1 cm. Red arrow indicates position of perithecia**.**

Table 6.3. Incidence of *F. langsethiae* as determined by QPCR in different cereal parts when inoculated using mycelium-plug method (a) and agar-spore method (b).

a

	Lower stem			Upper stem			Head		
Cereal	< LoQ	> LoQ	Incidence	< LoQ	> LoQ	Incidence	< LoQ	> LoQ	Incidence
Wheat	25%	0%	25%	75%	25%	100%	75%	25%	100%
Barley	25%	50%	75%	25%	50%	75%	50%	50%	100%
Oat	25%	0%	25%	25%	0%	25%	25%	25%	50%
Wheat control	0%	0%	0%	25%	0%	25%	25%	0%	25%
Barley control	25%	0%	25%	0%	0%	0%	50%	25%	75%
Oat control	25%	0%	25%	9%	0%	0%	50%	0%	50%

b.

	Lower stem			Upper stem			Head		
Cereal	< LoQ	> LoQ	Incidence	< LoQ	> LoQ	Incidence	< LoQ	> LoQ	Incidence
Wheat	25%	0%	25%	25%	75%	100%	0%	50%	50%
Barley	50%	50%	100%	25%	50%	75%	0%	100%	100%
Oat	0%	0%	0%	50%	0%	50%	0%	0%	0%
Wheat control	0%	0%	0%	0%	0%	0%	25%	25%	50%
Barley control	0%	0%	0%	0%	0%	0%	50%	0%	50%
Oat control	0%	0%	0%	0%	0%	0%	50%	0%	50%

n = 4 for each cereal and part.

6.3.3. Boot inoculation

No symptoms developed on negative control plants. Four to six days after inoculation disease symptoms in the form of lesions developed on *F. langsethiae* and *F. graminearum* inoculated plants just above the uppermost culm node. The symptoms were similar for all inoculated growth stages, at all concentrations (although lesions were bigger and darker at concentrations of 10^5 and 10^6 spores ml^{-1}), on all cereals and in both glasshouse and field experiments. In wheat however, lesions were closer to the uppermost culm node than in barley and oat. Lesions started as light brown patches developing into dark brown with well-

defined boundaries (Figure 6.10). *F. graminearum* inoculated plants showed similar symptoms but with a more intense browning.

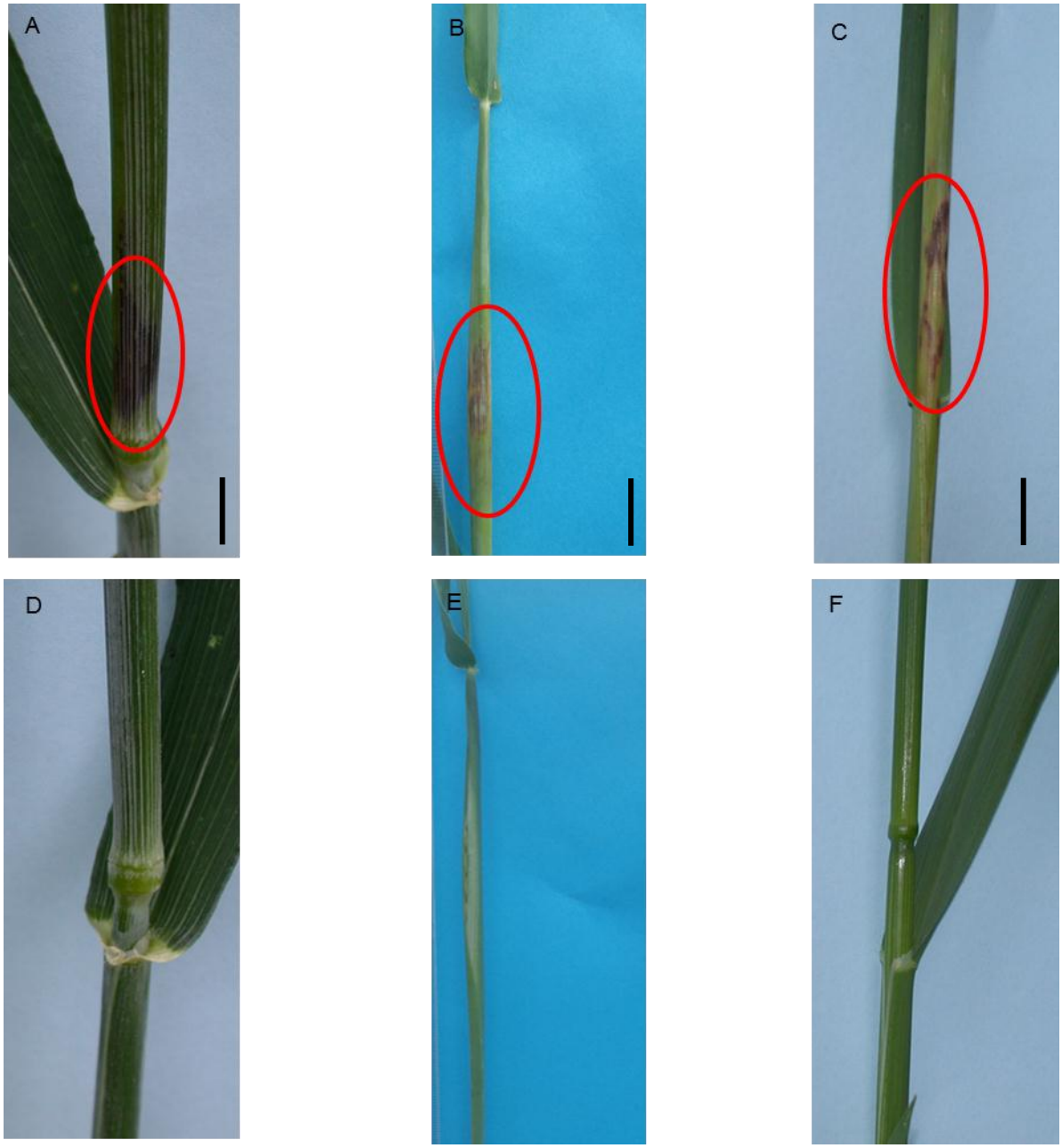

Figure 6.10. *F. langsethiae* boot inoculated wheat, barley and oat (A, B and C respectively) (inoculation at GS47 with 10^5 spores ml^{-1}) and the corresponding water inoculated plants (D, E and F respectively) in the glasshouse. Pictures were taken 14 DPI. Bar =1.8 cm on A, 1.6 cm on B and 2 cm on D.

Head emergence in inoculated plants was delayed in inoculated plants and at 21 days after inoculation there were still a number of plants whose heads had not fully

emerged. This was more pronounced when inoculations were done at GS45 and more evident in barley (Table 6.4). In *F. graminearum* inoculated plants, emerged heads were bleached in all three cereals. This was not the case in *F. langsethiae* inoculated plants (Figure 6.11). *F. langsethiae* inoculated plants had mild bleaching on single wheat spikelets and on barley there were signs of developing lesions on single spikelets.

Table 6.4. Percentage head emergence of wheat, barley and oat heads 21 DPI after boot inoculation with different concentrations of *F. langsethiae* spore suspension in the glasshouse

Spore concentration (spores ml^{-1})	Percentage head emergence (%)								
	Wheat			Barley			Oat		
	GS45	GS47	GS49	GS45	GS47	GS49	GS45	GS47	GS49
10^3	100	100	100	100	60	100	80	100	100
10^4	100	100	100	40	80	100	60	100	100
10^5	80	100	100	60	80	80	80	100	100
10^6	40	100	100	60	60	60	60	100	100
water	100	100	100	100	100	100	100	100	100

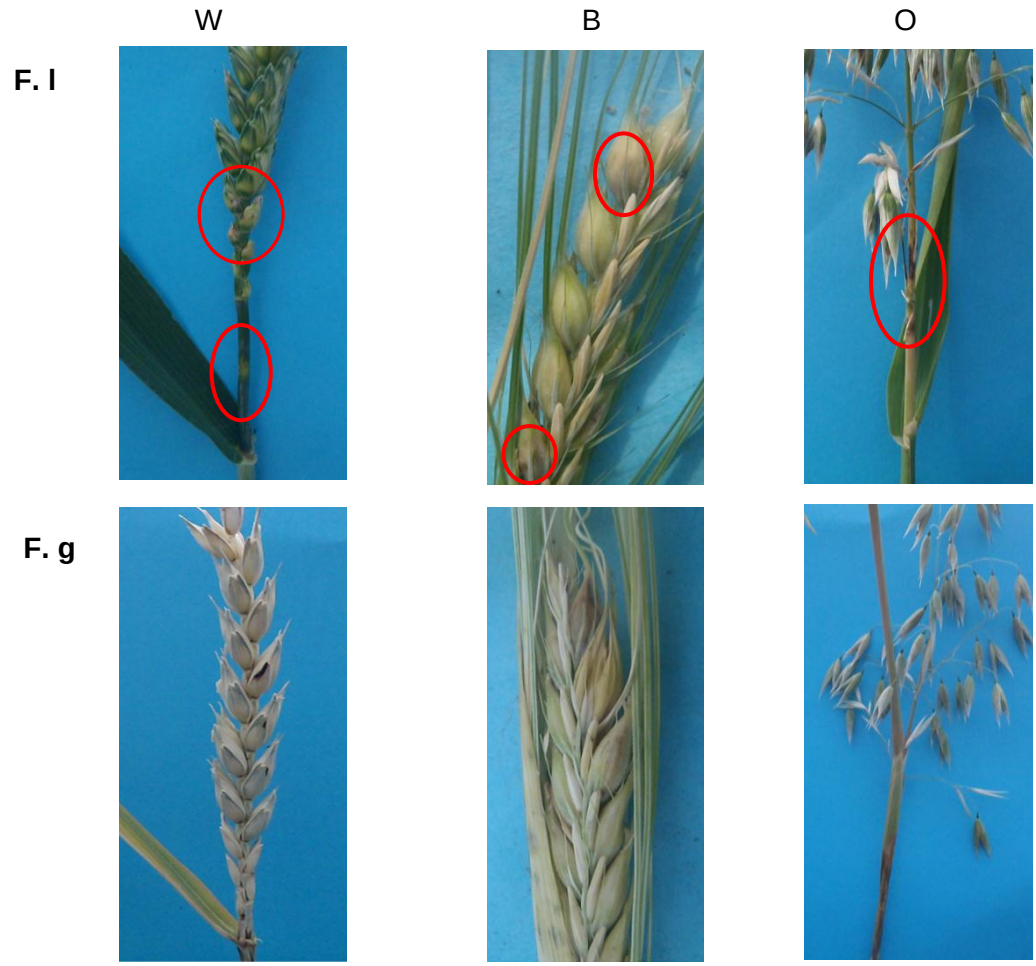

Figure 6.11. Boot inoculated wheat (W), barley (B) and oat (O) (inoculation at GS47 with 10^5 spores ml^{-1}) in the glasshouse. Pictures were taken 21 DPI. F. l = *F. langsethiae* and F. g = *F. garminearum* inoculated plants. Red circles show lesions on panicles and grain**.**

At harvest emerged heads of *F. langsethiae* inoculated plants were generally shorter than their corresponding water inoculated plants. A dose response of head length and *F. langsethiae* spore concentration used for inoculation and SDW as zero dose showed that in wheat, dose effect was significant ($P<0.001$) at all growth stages and indicated a significant quadratic relationship at GS45 ($P = 0.009$), at GS47 ($P = 0.003$) and at GS49 ($P = 0.002$) (Figure 6.12).

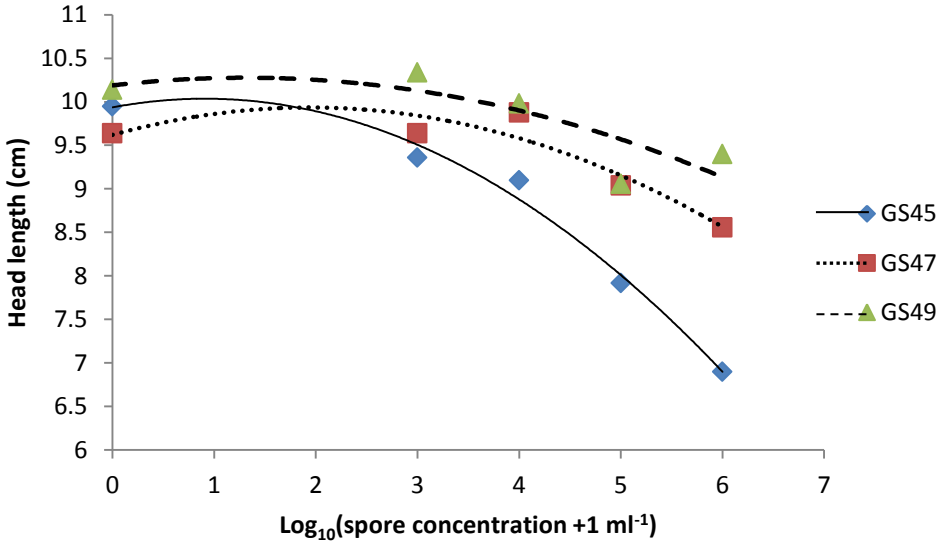

Figure 6.12. Dose response curve showing relationship between *F. langsethiae* spore concentrations of 10^3 to 10^6 spores ml^{-1}on head length at harvest of wheat plants inoculated at GS45, 47 and 49. Lines represent predicted functions calculated by fitting the following models:

Inoculation at GS45 $y = -0.12x^2 + 0.22x + 9.94$ [$R^2 = 0.99$, CV% = 8.7]

Inoculation at GS47 $y = -0.08x^2 + 0.32x + 9.62$ [$R^2 = 0.88$, CV% = 5.0]

Inoculation at GS49 $y = -0.52x^2 + 0.14x + 10.19$ [$R^2 = 0.66$, CV% = 2.8]

In barley, a similar analysis showed significant ($P<0.001$) dose effect at all growth stages with significant quadratic relationship at GS45 ($P<0.001$), at GS47 ($P = 0.045$) and at GS49 ($P = 0.050$) (Figure 6.13).

In oat, dose response analysis showed dose effect to be significant at all growth stages ($P<0.001$). A significant linear relationship ($P<0.001$) was observed at GS45 and GS49. At GS47 however, a significant quadratic relationship ($P = 0.03$) between *F. langsethiae* spore concentration and head length at harvest was observed (Figure 6.14).

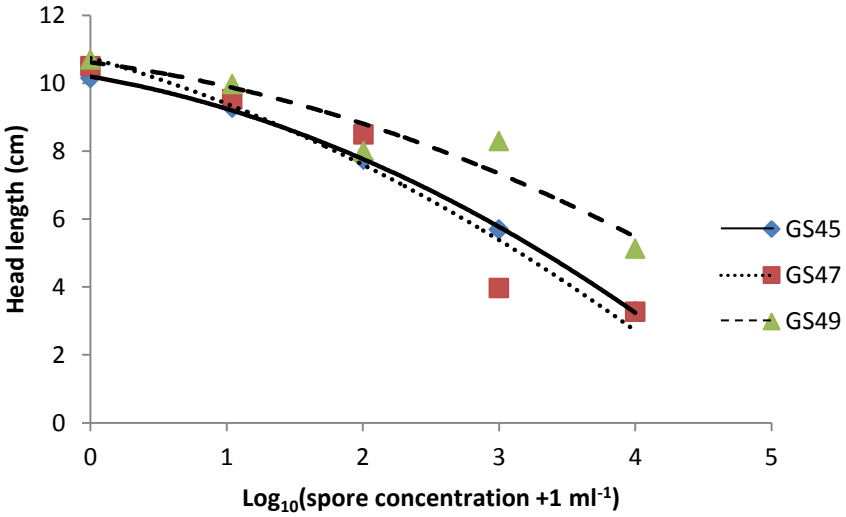

Figure 6.13. Dose response curve showing relationship between F. langsethiae spore concentrations 10^3 to 10^6 on head length at harvest of barley plants inoculated at GS45, 47 and 49. Lines represent predicted functions calculated by fitting the following models:

Inoculation at GS45 $y = -0.26x^2 + -0.69x + 10.20$ [$R^2 = 0.99$, CV% = 6.9]

Inoculation at GS47 $y = -0.21x^2 + -1.13x + 10.75$ [$R^2 = 0.93$, CV% = 11.7]

Inoculation at GS49 $y = -0.19x^2 + -0.52x + 10.62$ [R2 = 0.91, CV% = 9.1]

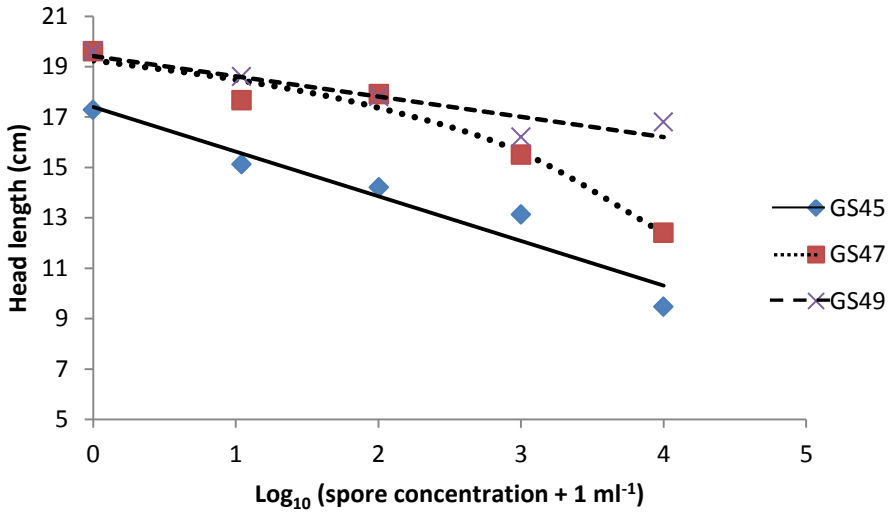

Figure 6.14. Dose response curve showing relationship between *F. langsethiae* spore concentrations 10^3 to 10^6 on head length at harvest of oat plants inoculated at GS45, 47 and 49. Lines represent predicted functions calculated by fitting the following models:

Inoculation at GS45 $y = -1.77x + 17.40$ [$R^2 = 0.94$, CV% = 6.9]

Inoculation at GS47 $y = -0.35x^2 - 0.25x + 19.24$ [$R^2 = 0.95$, CV% = 7.9]

Inoculation at GS49 $y = -0.8032x + 19.414$ [$R^2 = 0.86$, CV% = 4.8]

For *F. langsethiae* inoculated plants, apart from the reduced head length no other visible symptoms were observed on wheat at harvest. On barley there were dark brown patches at the base of the grain (Figure 6.15 A). These patches were observed on some grains of barley that were inoculated at all growth stages with spore concentrations of 10^5 and 10^6 spores ml^{-1}. In oat there were a number of necrotic blind spikelets. These mostly occurred on the lower whorls (Figure 6.15 B. i.). Dark brown necrotic patches that were observed at earlier growth stages on panicles were still visible at harvest (Figure 6.15 B ii).

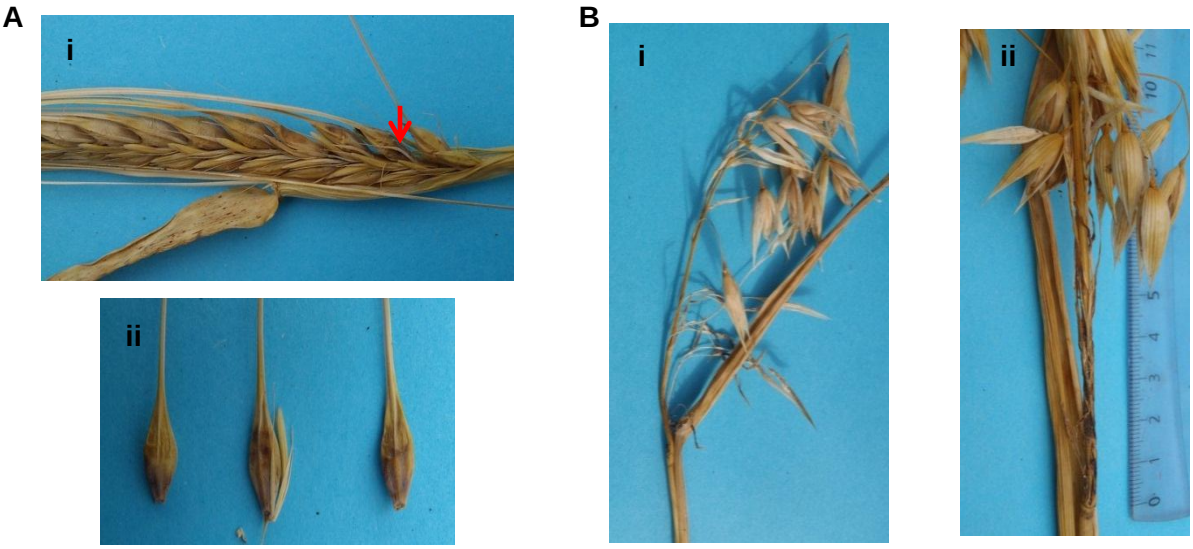

Figure 6.15. Symptoms observed on A: barley with (i) dark brown necrotic patches at base of grain (red arrowed) and (ii) a close-up on single barley grains with browning at the base of the grain. B: oats with (i) dark brown blind spikelets on the lower whorls and and (ii) necrotic dark-brown to black lesions on panicles of harvested plants

Regression analysis of Log_{10}(spore concentration+1) against Log_{10} (*F. langsethiae* DNA) in cereal heads at harvest grouped by the growth stage at which inoculation was done showed that the growth stage of inoculation had no influence on *F. langsethiae* DNA in cereal heads at harvest (P = 0.283 for wheat, P = 0.653 for barley and P = 0.532 for oat). However, the spore concentration used for

inoculation had a significant (P<0.001) influence on *F. langsethiae* DNA in all the cereal heads at harvest. ANOVA carried out based on these results showed that *F. langsethiae* DNA in cereal heads at harvest increased with increasing the spore concentration used for inoculation (Table 6.5).

Table 6.5. Log_{10} *Fusarium langsethiae* DNA recovered from wheat, barley and oat heads at maturity after boot inoculations with four levels (10^3-10^6) of *F. langsethiae* spore concentrations

Mean Log_{10} *F. langsethiae* DNA in cereal heads at harvest (pg ng^{-1})			
Spore concentration (spores ml^{-1})	Wheat	Barley	Oat
0	-3.30 (0.0005)[a]	-3.24 (0.0006)[a]	-3.28 (0.0005)[a]
10^3	-3.14 (0.0007)[a]	-2.23 (0.0059)[b]	-2.63 (0.0023)[b]
10^4	-2.45 (0.0035)[ab]	-1.30 (0.0498)[c]	-2.36 (0.0044)[bc]
10^5	-2.18 (0.0066)[b]	-1.26 (0.0555)[c]	-2.21 (0.0062)[bc]
10^6	-2.13 (0.0074)[b]	-0.86 (0.1377)[c]	-1.95 (0.0113)[c]
P value	0.001	< 0.001	< 0.001
%CV	24.4	23.1	19.3

Values with same letter in the same column are not statistically different from each other according to Tukey multiple range test. Back transformed values are presented in parentheses.

A dose response ANOVA of *F. langsethiae* DNA in cereal heads at harvest and *F. langsethiae* spore concentration used for inoculation and SDW as zero dose showed that in wheat, dose effect was significant at all growth stages (P = 0.025 at GS45 and P = 0.035 at GS47 and P = 0.01 at GS49). A significant linear relationship (P = 0.003 at GS45, P = 0.03 at GS47 and P = 0.009 at GS49) between *F. langsethiae* DNA in cereal heads at harvest and spore concentration used for inoculations was also observed (Figure 6.16).

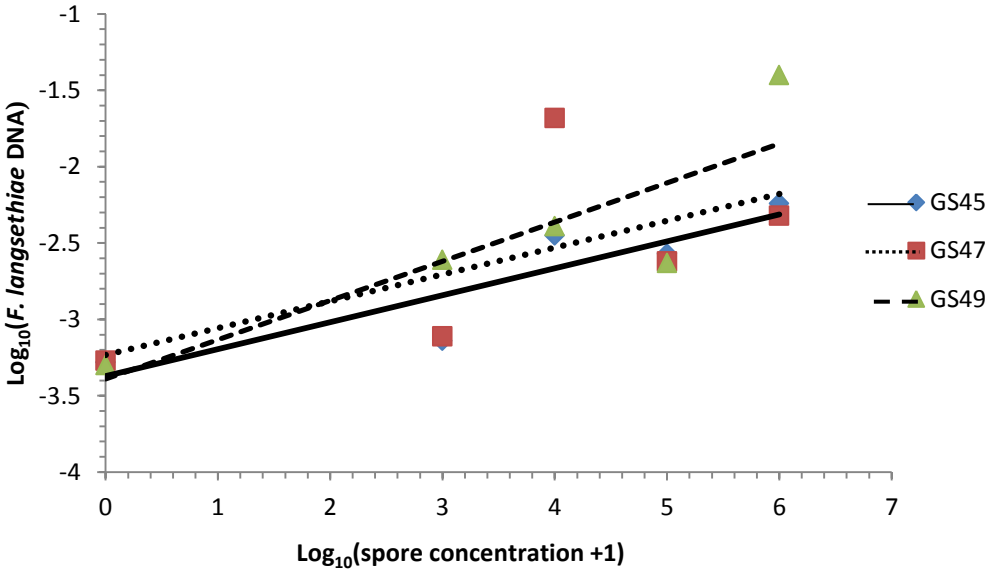

Figure 6.16. Dose response curve showing the relationship between *F. langsethiae* spore concentrations used for inoculations and *F. langsethiae* DNA detected in wheat heads at harvest when plants were inoculated at GS45, GS47 and GS49. Lines represent predicted functions calculated by fitting the following models:
Inoculation at GS45 y = 0.18x – 3.37 [R^2 = 0.81, CV% = 27.4]
Inoculation at GS47 y = 0.18x – 3.23 [R^2 = 0.40, CV% = 21.3]
Inoculation at GS49 y = 0.25x – 3.39 [R2 = 074, CV% = 20.0]

In barley, dose response ANOVA showed that dose effect was significant (P<0.001) at GS45 and GS47 as well GS49 (P = 0.001). A significant linear relationship (P<0.001) was observed between doses of *F. langsethiae* spore concentration used for inoculation at the different growth stages and the *F. langsethiae* DNA recovered from the barley heads at harvest. However, it was observed that some form of cross contamination occurred at the latter two inoculation timings as indicated by the high *F. langsethiae* DNA in SDW inoculated samples (Figure 6.17).

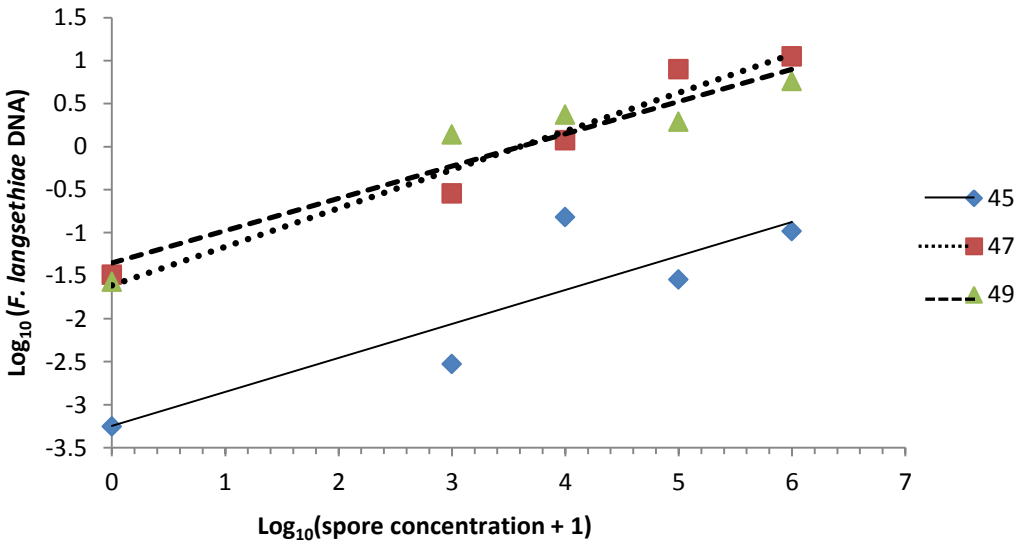

Figure 6.17. Dose response curve showing the relationship between *F. langsethiae* spore concentrations used for boot inoculations and *F. langsethiae* DNA detected in barley heads at harvest when plants were inoculated at GS45, GS47 and GS49. Lines represent predicted functions calculated by fitting the following models:
Inoculation at GS45 y = 0.40x – 3.24 [R^2 = 0.76, CV% = 20.0]
Inoculation at GS47 y = 0.45x – 1.61 [R^2 = 0.96, CV% = 19.6]
Inoculation at GS49 y = 0.38x – 1.35 [R^2 = 0.91, CV% = 21.9]

A similar analysis for oats showed a significant dose effect (P = 0.01 at GS45, P = 0.05 at GS47 and P<0.001 at GS47). A linear relationship between doses of *F. langsethiae* spore concentration used for inoculation at the different growth stages and *F. langsethiae* DNA in oat heads at harvest was observed (P = 0.02 at GS45, P = 0.01 at GS47 and P<0.001 at GS49) (Figure 6.18).

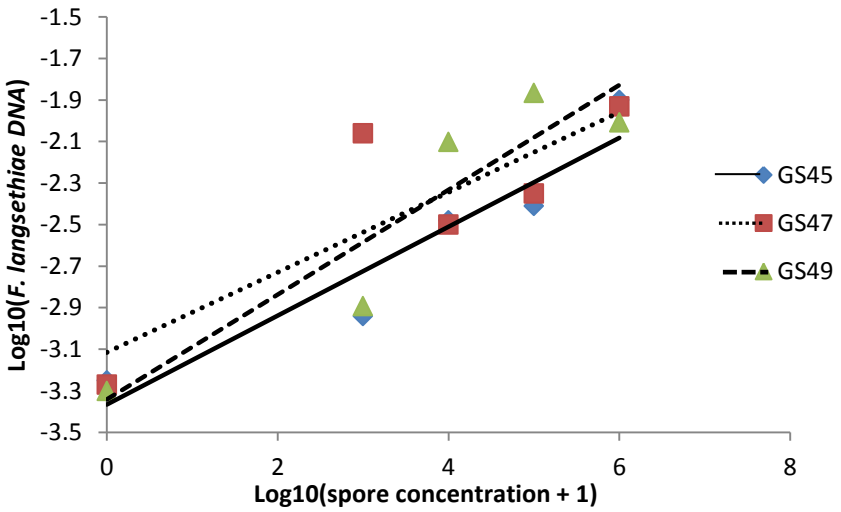

Figure 6.18. . Dose response curve showing the relationship between *F. langsethiae* spore concentrations used for boot inoculations and *F. langsethiae* DNA detected in oat heads at harvest when plants were inoculated at GS45, GS47 and GS49. Lines represent predicted functions calculated by fitting the following models:
Inoculation at GS45 y = 0.21x – 3.37 [R^2 = 0.90, CV% = 22.9]
Inoculation at GS47 y = 0.19x – 3.12 [R^2 = 0.71, CV% = 20.0]
Inoculation at GS49 y = 0.25x – 0.91 [R^2 = 0.86, CV% = 13.0]

Regression analysis of *F. langsethiae* DNA against HT-2+T-2 in heads at harvest grouped by cereal species showed a significant regression (P<0.001, r^2 = 0.56) (Figure 6.19). Although a significant interaction was observed between *F. langsethiae* DNA recovered from cereal heads at harvest and the cereal species (P = 0.01), cereal species accounted for only an additional 2.2% of the observed variance.

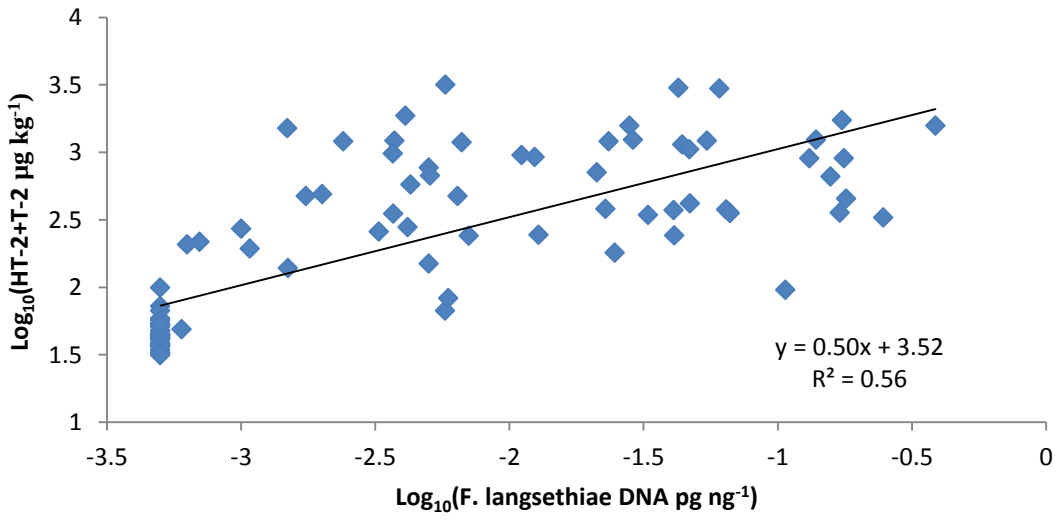

Figure 6.19. The relationship between *F. langsethiae* DNA and HT-2+T-2 in heads of wheat, barley and oat after boot inoculation with a *F. langsethiae* spore suspension.

6.3.4. Spray inoculation

Temperature and relative humidity data showed that average temperatures around cereal heads were highest when heads were bagged with the normal clear bags followed by those bagged with the perforated cryovac bags with that around un-bagged heads having the least (Table 6.6. A, B and C). Relative humidity around cereal heads bagged with the normal clear bags was highest followed by un-bagged heads with that around the perforated cryovac bags having the lowest (Table 6.6A, B and C). The periods with high humidity (>90and 95%) were much greater for heads covered with normal bags and there was little difference between heads covered by cryovac bags and un-bagged heads.

Table 6.6. Average temperatures and relative humidity around cereal heads bagged with the different bags over the different bagging periods. A = normal clear bags, B = perforated cryovac bags and C = un-bagged. Averages are from three data loggers for each bag and two from un-bagged heads

A

	Number of days of bagging		
	7	14	56
Average Max. Temperature	19.6	20.7	21.5
Average Min. Temperature	17.9	18.6	19.5
Average Max. Humidity	85.1	75.7	61.6
Average Min. Humidity	80	68.4	54.9
Hours Humidity > 90	67	148	347
Hours Humidity > 95	35	71	145

B

	Number of days of bagging		
	7	14	56
Average Max. Temperature	18.3	18.1	20.9
Average Min. Temperature	16.3	16.1	18.9
Average Max. Humidity	63.9	66.6	58.8
Average Min. Humidity	57.7	58.8	52.1
Hours Humidity > 90	3	7	59
Hours Humidity > 95	0	0	12

C

	Number of days of bagging		
	7 Days	14 Days	56 Days
Average Max. Temperature	16.1	16.3	19.8
Average Min. Temperature	14.3	14.5	17.9
Average Max. Humidity	71.7	71.9	60.8
Average Min. Humidity	60.7	61.8	53.7
Hours Humidity > 90	3	7	50
Hours Humidity > 95	0	0	0

Fourteen days after spray inoculations, disease symptoms were seen on heads of wheat, barley and oats that were bagged (normal and perforated bags). This ranged from bleaching of single spiklets to whole cereal heads. Bleaching of cereal heads was more severe on heads that were bagged with the normal plastic bags.

In wheat, the bleaching covered about three quarters of the total head with visible signs of sooty moulds on anthers of spray inoculated and uninoculated heads (Figure 6.20. A).

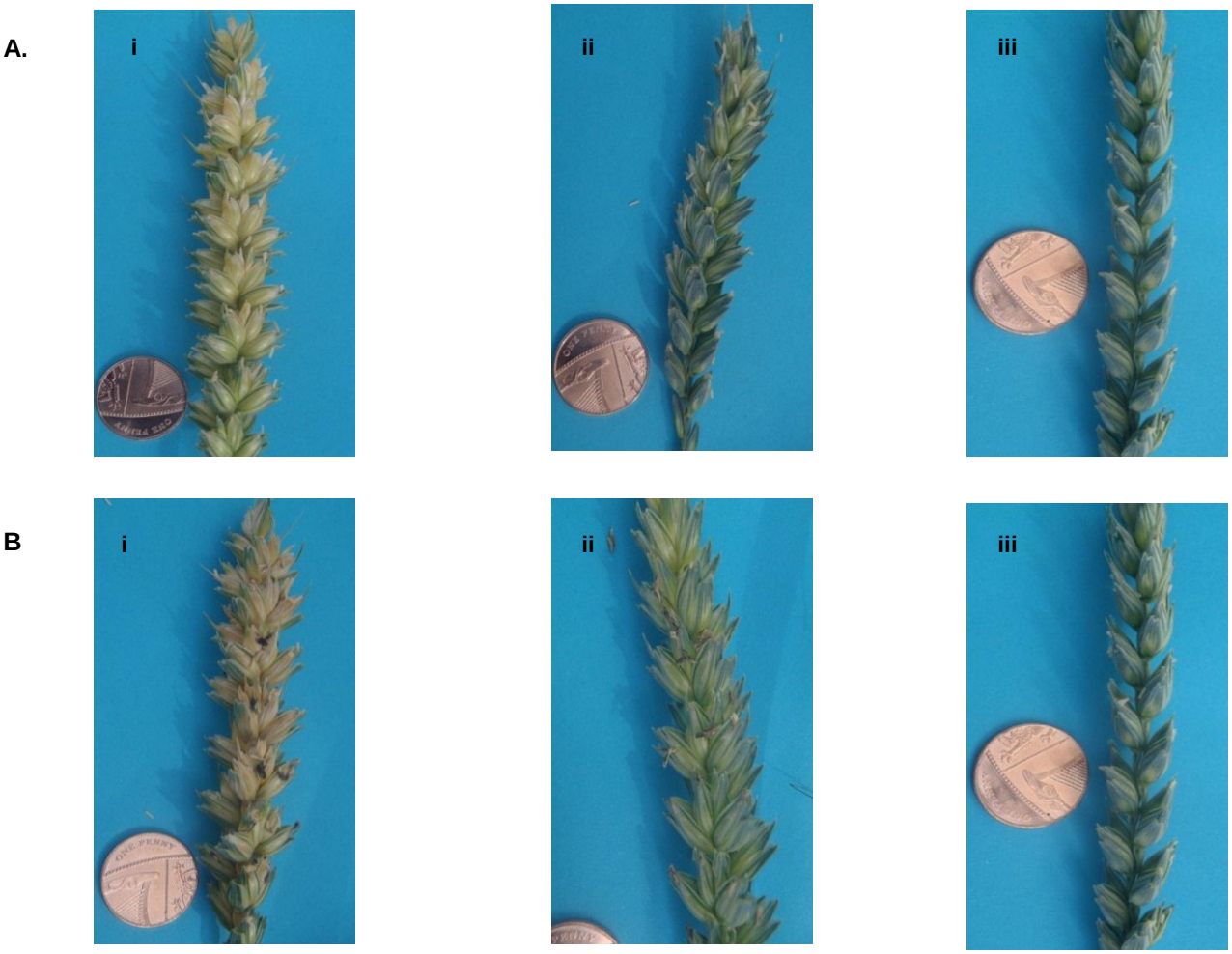

Figure 6.20. Spray inoculated wheat heads; A and B are heads bagged with perforated and normal bags respectively (14 DPI). (i), (ii) and (iii) are inoculated and bagged, bagged but uninoculated and inoculated without bagging of heads respectively.

In barley, whole cereal heads were bleached especially when heads were bagged with the normal bags (Figure 6.21)

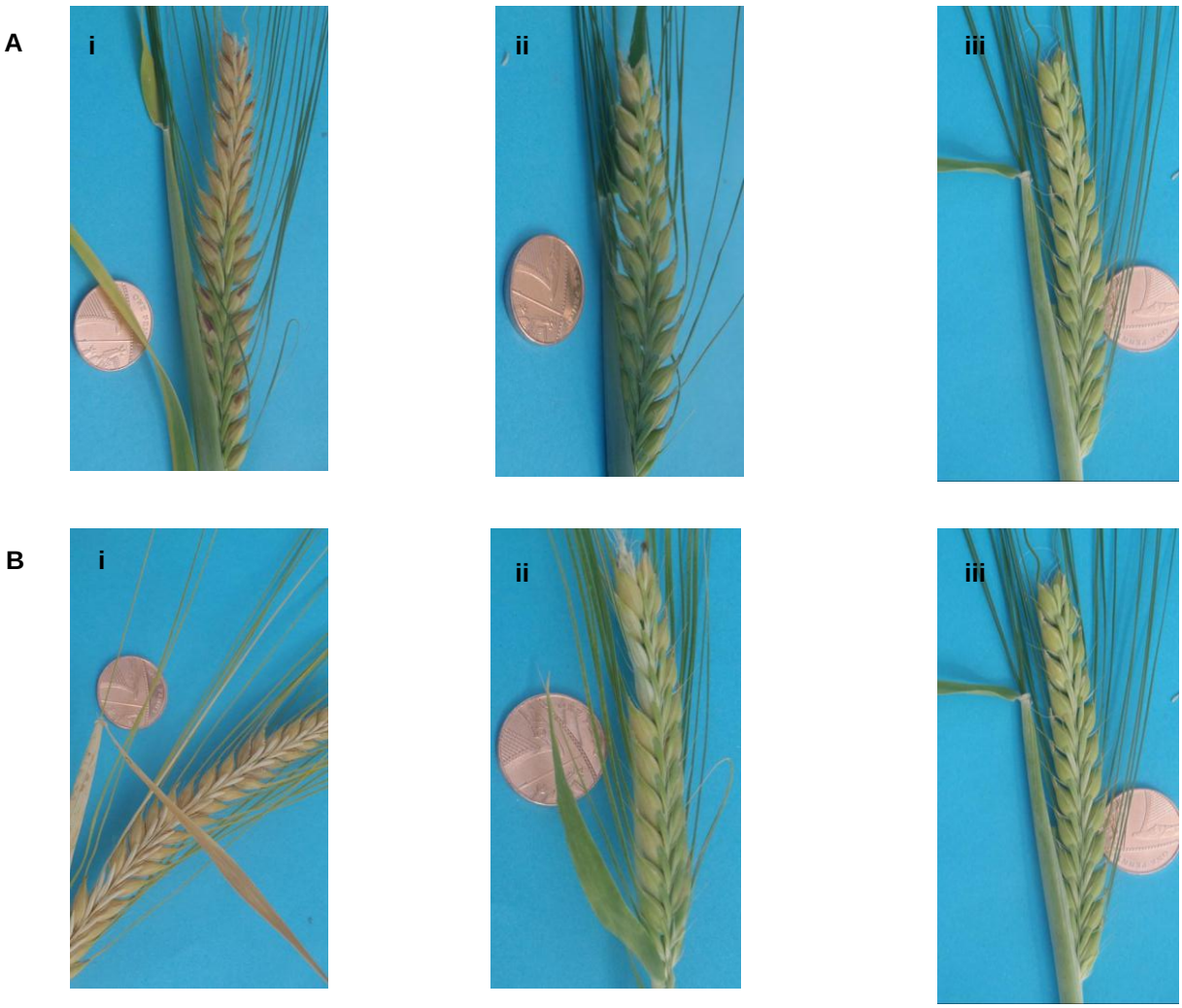

Figure 6.21. Spray inoculated barley heads; A and B are heads bagged with perforated and normal bags respectively (14 DPI). i, ii and iii are inoculated and bagged, bagged but uninoculated and inoculated without bagging of heads respectively.

Bleaching occurred in oat when heads were bagged with perforated bags, with bleaching covering about half of the total cereal head whilst bleaching covered almost the whole head when bagging was done with the normal plastic bags (Figure 6. 22).

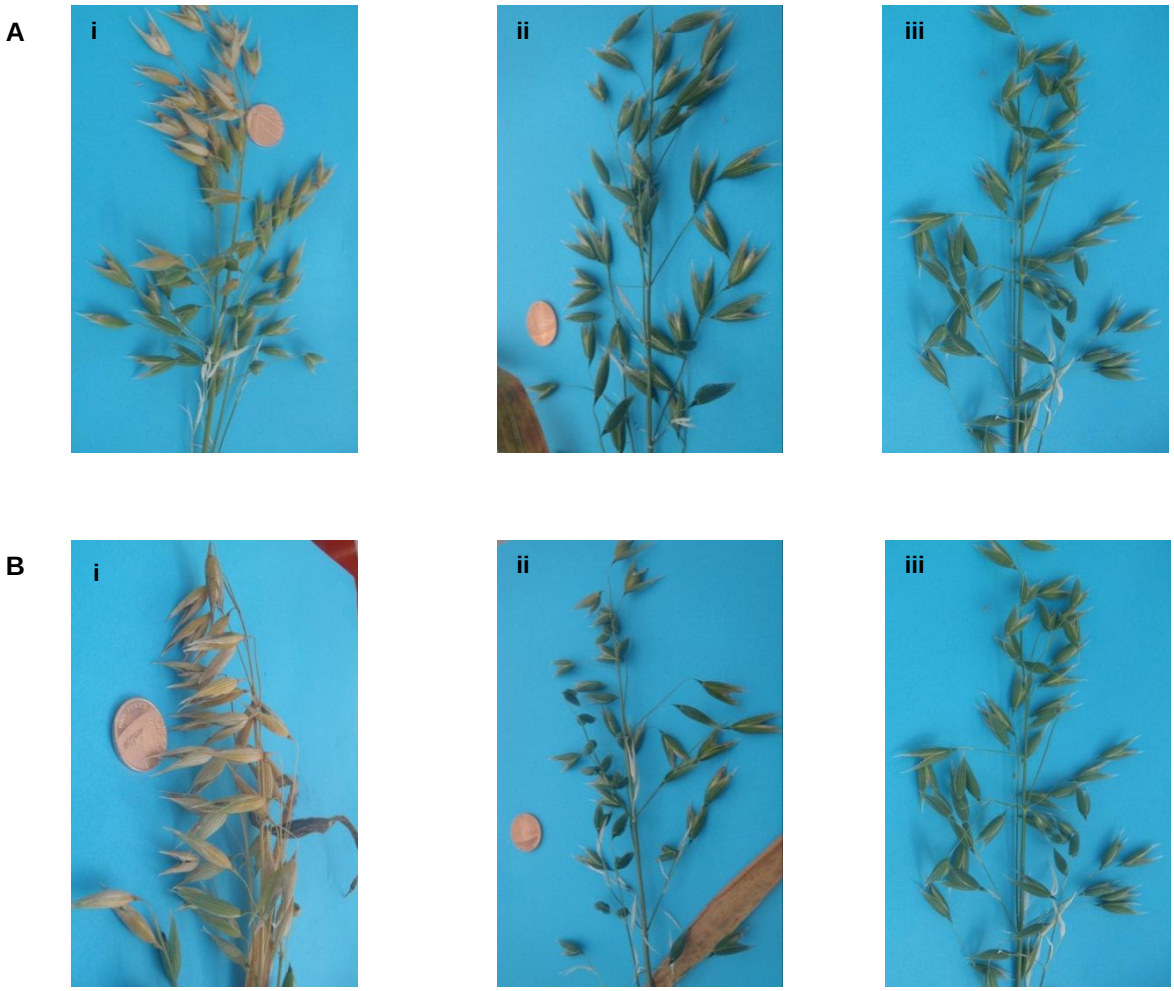

Figure 6.22. Spray inoculated oat heads; A and B are heads bagged with perforated and normal bags respectively (14 DPI). i, ii and iii are inoculated and bagged, bagged but uninoculated and inoculated without bagging of heads respectively.

At harvest there were no observable disease symptoms on any of the treated wheat heads. In barley there were disease symptoms on heads that were inoculated and bagged for two weeks as well as those bagged until harvest. These were in the form of shrivelled grains with dark brown to black patches at the base of the grain and at the upper part and the under-side of the grain with signs of mycelium growth. These symptoms were more severe on heads that were bagged until harvest (Figure 6.23).

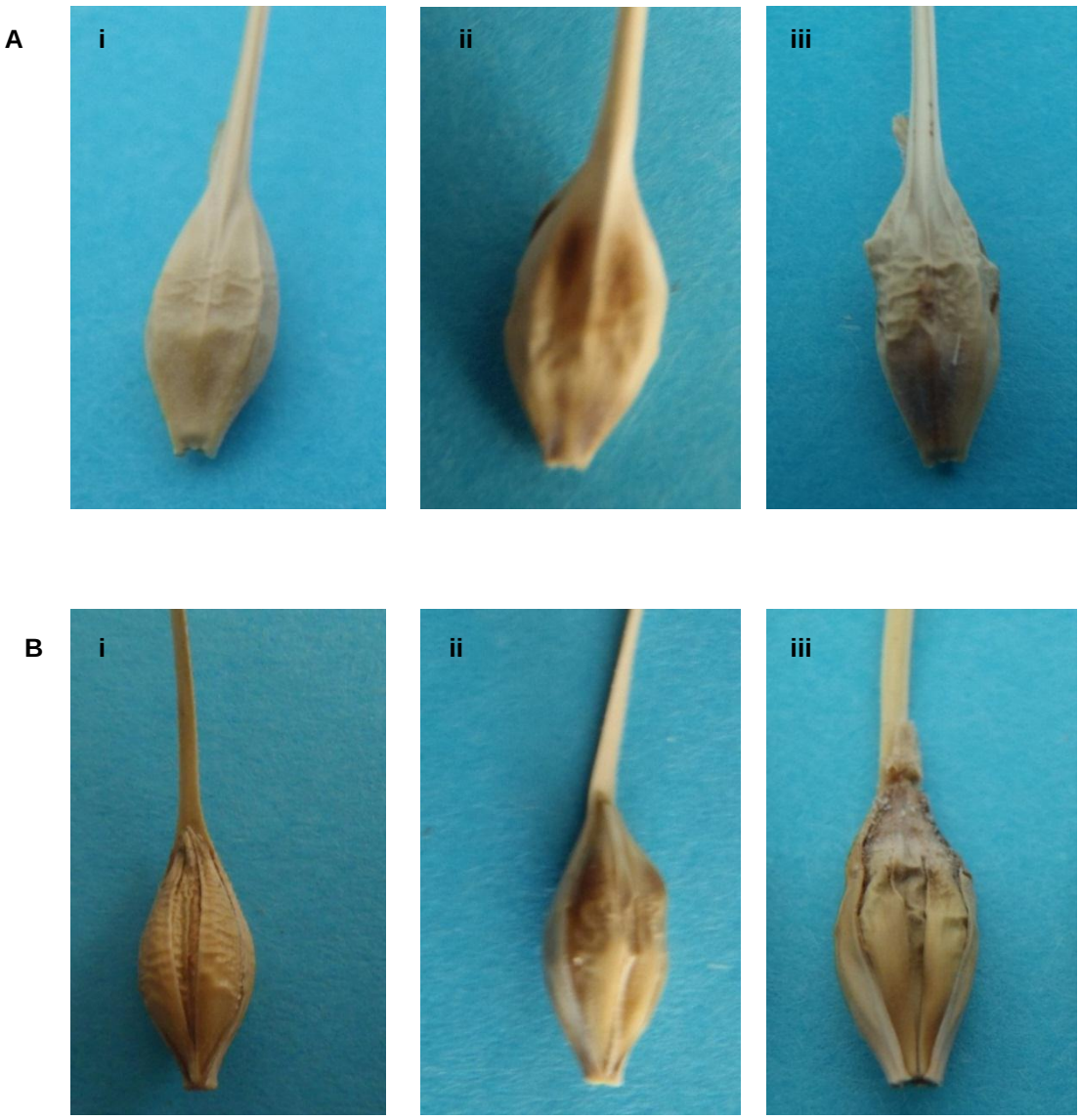

Figure 6.23. Spray inoculated barley grains at harvest A the upper surface and B the crease side of the same grain. Showing the extent of darkening (lesion) on the grain. i = un-bagged, ii = bagging for two weeks and iii = bagging till harvest

On oats, there was mycelial growth on spiklets. The mycelium proved to be that of *F. langsethiae* upon plating on PDA.

ANOVA showed that among the cereal species studied, the highest amount of *F. langsethiae* DNA was recovered from oats and this was significantly different from that recovered from wheat which had the lowest amount of *F. langsethiae* DNA (P

= 0.003). The type of bag used for bagging also had a significant effect on *F. langsethiae* DNA recovered from the cereal heads at harvest (P<0.001). The period of bagging was not statistically significant (P = 0.154) and there was no significant interaction between any of the factors studied (Table 6.7).

Table 6.7. Effect of bag type and period of bagging on Log_{10} transformed *F. langsethiae* DNA in heads of wheat, barley and oat at harvest when spray inoculated at GS59.

F. langsethiae DNA (pg ng^{-1})								
	Normal bag			Cryovac bag			No bag	Cereal mean
Bagging period (weeks)	1	2	8	1	2	8		
Cereal								
Wheat	-2.838	-2.755	-2.102	-3.301	-3.301	-3.301	-3.077	-2.954[a]
	(0.0014)	(0.0018)	(0.0079)	(0.0005)	(0.0005)	(0.0005)	(0.0008)	(0.0011)
Barley	-2.163	-2.635	-2.082	-3.197	-3.301	-2.555	-2.996	-2.693[ab]
	(0.0069)	(0.0023)	(0.0082)	(0.0006)	(0.0005)	(0.0028)	(0.001)	(0.0020)
Oats	-1.872	-1.711	-1.425	-3.188	-2.586	-2.574	-2.876	-2.319[b]
	(0.0134)	(0.0194)	(0.0376)	(0.0006)	(0.0026)	(0.0027)	(0.0013)	(0.0048)
Bag type mean	-2.176[b] (0.0067)			-3.03[a] (0.001)			2.983[a](0.001)	
P- Cereal	0.003							
P- bag type	< 0.001							
P-value Bagging period	0.154							
%CV	36.7							
s.e	0.97							

Means with same letter are not statistically different from each other according to Tukey multiple range test. Back transformed values in parentheses.

Based on the results indicating that *F. langsethiae* infection was more severe on cereal heads when bagged with the normal clear bag and that *F. langsethiae* DNA

was highest in oat heads among the three cereals, ANOVA was done taking into consideration these parameters and the number of weeks of bagging for both *F. langsethiae* DNA and HT-2+T-2 (Table 6.8).

Table 6.8. The effect of bagging period when using normal bags on the accumulation *of F. langsethiae* DNA in the heads of oat at harvest following spray inoculation with F. langsethiae spore suspension at GS59**.**

Period of bagging (weeks)	*F. langsethiae* DNA (pg ng^{-1})	HT-2+T-2 (µg kg^{-1})
0	-2.89 (0.001)[a]	2.98 (966.)[a]
1	-0.44 (0.361)[b]	3.20 (1600)[a]
2	-0.16 (0.696)[b]	3.49 (3097)[ab]
8	0.48 (3.025)[c]	3.96 (9078)[b]
P value	< 0.001	0.002
CV%	22.7	7.1

Means with same letter are not statistically different from each other according to Tukey's multiple range test. Back transformed values in parentheses

A simple linear regression of *F. langsethiae* DNA against HT-2+T-2 concentrations in all the cereal heads at harvest showed a highly significant regression ($P<0.001$, $r^2 = 0.69$) (Figure 6.24).

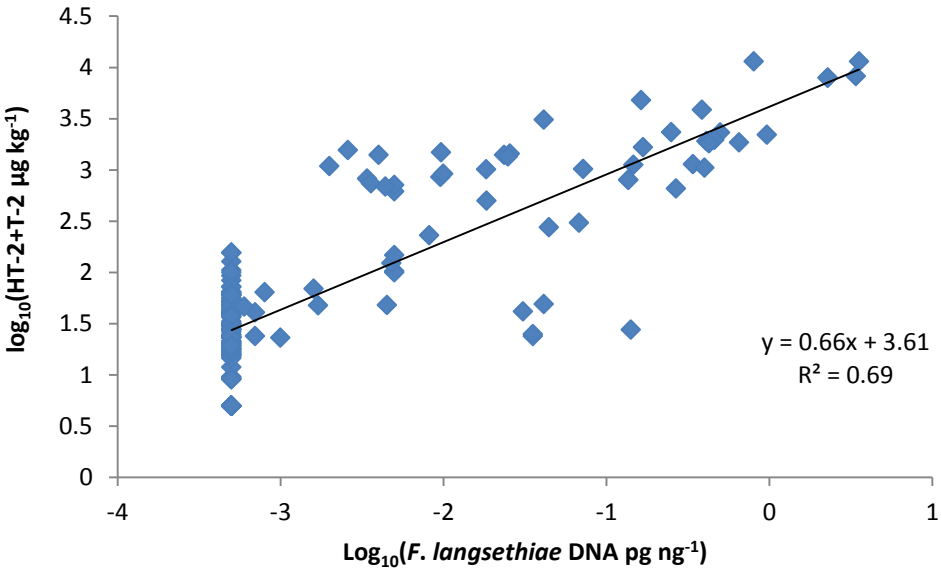

Figure 6.24. The relationship between *F. langsethiae* DNA and HT-2+T-2 in heads of wheat, barley and oat sprayed with *F. langsethiae* spore suspension (10^6 spores ml^{-1}) at GS59 under the different bagging conditions

6.4 Discussion

The seed infection assay was aimed at determining if *F. langsethiae* had the ability to infect cereal heads when infected seeds were used as planting material; that is systemic seed transmission. Plant pathogenic fungi can be seed borne and or seed transmitted. Although these two terms have sometimes been used interchangeably, they are distinct and have different implications in plant pathology. According to Neergeard (1979), a plant pathogenic fungus is said to be seed borne when it is carried in or with the seed. This could result when the spores of the fungi simply adheres to the seed surface or mixes with a seed lot. Transference of a seed-borne fungus and establishment of infection in a plant directly from the seed is referred to as seed transmission.

A number of steps in disease cycle of a pathogen could result in systemic seed infection in plants. Intraembryal infection followed by systemic infection occurs when a pathogen or a propagule of the pathogen resides in the seed embryo and becomes active as a result of the germination of the seed. As the plant grows the

pathogen penetrates through the stem of the plant or follows closely behind the growing point of the plant such as in *Colletotrichum dematium* in soyabean. Another stage in a disease cycle which could result in systemic seed infection is when there is extraembryal infection followed by systemic infection. In this case the infection is outside the embryo (could be in the endosperm, seed coat or pericarp), but as the seedling germinates, the pathogen grows into the young plant and penetrates further up the plant during the growth of the plant; example is in *Drechslera graminea* in barley (Neergaard, 1979). The seed may also be contaminated (outside the seed coat) with the pathogen or its propagule and as the seed germinates the pathogen penetrates into the seedling and proceeds upwards towards the growing points (Neergaard, 1997).

A number of plant pathogenic *Fusarium* species infecting cereals are known to be seed-borne. These include *F. avenaceum, F. culmorum, F. graminearum* and *F. poae* among others (Neergeard, 1979). However, seed transmission has been reported for only a few. *F. moniliforme*, causing ear rot in corn and the production of high levels of fumonisins even in symptomless corn heads has been reported to be a seed transmitted pathogen and according to Munkvold and Carlton (1997) seed transmitted infections could result in stalk rot, ear rot and symptomless infection in corn plants. Evidence of transmission from seed to stems has also been demonstrated for *F. graminearum* in winter wheat (Duthie and Hall, 1987). Xi et al. (2008) reported that even when systemic seed infection by *Fusarium* species occurs in wheat and barley, it is restricted to the lower stem base not passing the third node and does not result in head blight symptoms. Within the limitations of this experiment, results do not show any evidence of systemic seed infection of *F. langsethiae* in oat.

Although the seed infection assay showed no evidence of systemic infection, it does not necessarily mean that other types of infection at the early stages of the plants life-cycle could not result in a systemic infection. Some *Fusarium* species responsible for FHB are known to cause stem base infection, which could subsequently result in head blight infection (Parry et al., 1995). In the preparation of the stem base infection experiment, attention was given to keeping the inoculum in close contact with the stem base of the plant. The use of 0.75% agar allowed for the agar-spore suspension to solidify around the stem of the seedling within seconds after pipetting. This ensured that the fungal spores stayed in close contact with the stem base. Again, by placing the mycelium plug in direct contact with the stem base and keeping it in place and covered with a thin layer of soil helped achieve the same purpose and helped keep it moist.

Many of the species of the fusarium head blight complex have the ability to cause seedling blight in cereals world-wide (Grey and Mature, 1988). Imathiu et al. (2010) showed that *F. langsethiae* did not cause seedling blight in wheat and oats. However, foot rot is the subsequent disease to occur after seedling blight in the cereal growing season. In the UK, *M. nivale* has been reported to be the most important pathogen responsible for foot rot, whilst the *Fusarium* species *F. avenacium, F. culmorum, F. graminearum* and *F. poae* contribute to a lesser extent to this disease (Parry, 1990). Foot rot symptoms start as brown lesions on the stem base which is often initially restricted to the outer leaf sheath. Infection then spreads up the leaf sheath causing long dark brown streaks. As the plant grows older symptoms of foot rot become darker and more obvious. This is the stage when the stem base of the cereal becomes brown to dark brown and rotten. When seedlings of wheat, barley and oats were inoculated with both the agar-spore suspension and the mycelium plug, *F. graminearum* inoculated cereals all showed similar symptoms as described above although to a lesser extent in oats. Although

it is known that cases of foot rot could result in white head symptoms (especially under drought stress) (HGCA; BASF, not dated) no such symptoms were observed in cereal heads in this study. *F. langsethiae* inoculated plants however, showed no foot rot symptoms suggesting that even in close contact and at high inoculum load, *F. langsethiae* does not cause any visible stem base infection. The inability of *F. langsethiae* to show visible stem base infection does not mean infection did not take place. This is because infection could be latent or symptoms could be internal. *Fusarium graminearum* and *F. culmorum* have been re-isolated from symptomless infected winter wheat (Clement and Parry, 1998). In this work re-isolation of fungi was not carried out. However *F. langsethiae* DNA was recovered (although at low concentrations) from the upper sections of the stem and in cereal heads. It is not clear if this was as a result of systemic infection or infection from air-borne spores. This is because a small amount of *F. langsethiae* DNA was also recovered from uninoculated plants. Again because plants were watered manually using a watering hose with a rose head it could be that there was splashing of *F. langsethiae* spores from the soil or lower stem base to the other plant parts. Dispersal of spores of *Fusarium* species through rain splash has been suggested to be one of the most important factors in FHB infection in cereals (Parry et al., 1995). Due to the low levels of *F. langsethiae* DNA recovered from cereals after stem base inoculations mycotoxin analysis was not carried out.

The first two experiments (seed infection assay and stem base infection experiments) where *F. langsethiae* inoculum was present at early stages of the cereals life-cycle, as already discussed, did not result in *F. langsethiae* infection in cereal heads. This was evident by the low levels of *F. langsethiae* DNA as well as HT-2+T-2 which were not comparable to levels observed in commercial field samples (Chapter.3).

Boot inoculation where the inoculum was directly injected into the boots of cereals between GS45 and GS49 allowed for the direct contact of the inoculum with the developing floral parts of the cereal. Boot inoculations have been used extensively in the study of Karnel bunt disease in wheat (Royer and Rytter, 1985; Warhamm et al., 1986). Results from the boot inoculations for the first time showed that *F. langsethiae* could cause some symptoms in cereals. These symptoms were in the form of lesions on the stem just above the uppermost culm node. It is not clear if these lesions were as a result of resistance or susceptibility of the cereal species to *F. langsethiae* infection. This is because HT-2+T-2 are known to trigger apoptosis (programmed cell death). In plant-disease response, localized programmed cell death is a characteristic phenotype which is a response to both susceptible and resistance plant-pathogen interactions (Khurana et al., 2005). In the susceptible disease response, plant cells are killed indirectly (e.g. through the inhibition of metabolic activities as a result of secretion of virulent factors) or directly by the toxins produced by the pathogen (in this case probably HT-2+T-2) (Greenberg, 1997). In incompatible plant-pathogen interactions (resistance), the process is characterized by hypersensitive response-linked programmed cell death (Khurana et al., 2005). Hypersensitive response-linked cell death is a rapid localized plant cell death that occurs at the site of infection of an avirulent pathogen characterized by distinct dry lesions (Heath, 2000). After initial lesion formation, symptoms were able to spread to the other parts of the cereal such as the panicle and the grains of boot-inoculated plants. It is therefore logical to assume that they were as a result of compatible cereal-*F. langsethiae* interaction.

Whilst *F. graminearum* inoculated plants developed clear symptoms of head blight, the symptoms were less clear in *F. langsethiae* inoculated plants. In barley however, single seed infections/symptoms appeared similar to the symptoms described for *F. poae* (HGCA; BASF, not dated). The severity of these symptoms

increased with increasing spore concentration. Similar symptoms have also been reported elsewhere (Fournier, 2012). Another interesting observation with the boot inoculated plants was the inability of heads to emerge. Furthermore, when they did emerge there was a marked reduction in the length of the emerged heads. A dose response analysis showed that as spore concentration for inoculation increased stunting effect on heads increased with this effect decreasing with increasing growth stage at which the inoculation was undertaken. This is in agreement with work done by Divon et al. (2012) in oats. Stunting in cereal heads is not a known symptom of any of the *Fusarium* species responsible for head blight in cereals (Divon et al., 2012). However, *F. graminearum* boot-inoculated plants also showed stunting (data not shown), which was not as severe as that observed in *F. langsethiae* boot-inoculated plants. *F. graminearum* produces high levels of DON whilst *F. langsethiae* is known to produce high levels of HT-2 and T-2. HT-2 and T-2 are more potent than DON (EC, 2002). It is therefore possible that the observed stunting was as a result of the production of these trichothecenes at early head development and the difference in stunting due to the difference in potency of DON and HT-2 and T-2. Linear regression and ANOVA results showed that increasing spore concentration used for inoculation also increased *F. langsethiae* DNA and subsequent HT-2+T-2 in cereal heads. In *Arabidopsis*, T-2 toxin has been demonstrated to have a dwarfing effect (Masuda et al., 2007).

Generally, in the study of Type 1 FHB resistance in cereals, spray inoculation of spores and increased humidity through mist irrigation or bagging is used (Mesterhazy, 1995). Inoculations are normally done when the plants are flowering through to the soft dough stage; the period when cereals are most susceptible to FHB (Xu, 2003; Edwards, 2004). In a previous study where *F. langsethiae* spores were applied to oat heads at different flowering stages using spray inoculation and the heads were bagged for two days, a low infection frequency was observed

(Imathiu, 2008). In this study, however, application of inoculum through spraying was done before flowering (around GS59). This was because two years field data had shown the presence of *F. langsethiae* DNA in cereal heads right from head emergence and before flowering, peaking at GS59. This indicated that *F. langsethiae* cereal head infection starts before flowering (Chapter. 3). Moisture is an important factor in the infection process of *Fusarium* species in cereals. Therefore, in this study the cereal heads were bagged with two types of plastic bag and for longer periods of time than that undertaken in the work of Imathiu (2008). The findings of this spray inoculation study are in contrast to the study conducted by Divon et al. (2012) where symptoms on heads occurred close to grain maturity. In this study, cereal head symptoms (in the form of bleaching) occurred 14 days post spray inoculation and towards maturity as heads senesced. Heads that showed initial symptoms could not be differentiated from uninoculated or symptomless heads at harvest (GS92) except in barley where there were additional symptoms in the form of dark brown patches on the grains. Bleaching (premature ripening) of single spikelets or whole heads is a known characteristic of FHB infection (HGCA; BASF, not dated). It is not clear how *F. langsethiae* infection leads to bleaching of the cereal heads, but it could be speculated that it is as a result of the production and presence of HT-2 and T-2 in cereal heads. When trichothecene non-producing *F. graminearum* obtained by disrupting the *Tri5* gene which is responsible for encoding trichodiene synthase catalyzing the first step in the trichothecene biosynthetic pathway was injected into florets of wheat, colonization of wheat heads occurred but the infected heads showed less disease symptoms compared to the wild type. This included a reduction in the bleaching of heads (Desjardins et al., 1996). This suggests that trichothecene production contributes to bleaching symptoms and thus the production of high HT-2 and T-2 by *F. langsethiae* in cereal heads could have contributed to the observed bleaching

effect in spray inoculated cereal head. Bleaching as already stated was observed on heads that were bagged and more evident on normal bagged heads. The relative humidity data in the micro-environment of the cereal heads bagged with the normal bag was higher than those in perforated bags, highlighting the importance of relative humidity in the infection of *F. langsethiae* and the subsequent production of HT-2 and T-2 in cereals.

Contrary to work done by Divon et al., (2012) *F. langsethiae* DNA in oat heads were higher when heads were spray inoculated than when boot inoculations were done. Observed *F. langsethiae* DNA and HT-2+T-2 concentration in spray inoculated oat heads were comparable to that observed in three years of a field survey (Chapter 3) when heads were bagged with the normal plastic bags.

6.5. Conclusions.

This chapter has provided novel information on the *F. langsethiae*-cereal relationship and most importantly on the pathogens life-cycle. Within the limits of this research, this study has established that although *F. langsethiae* is a seed borne pathogen it is not seed transmitted. Again *F. langsethiae* does not cause stem base infections in cereals. Although the boot inoculations resulted in disease symptoms in wheat, barley and oats, this method may not mimic what happens in nature and in agreement with Divon et al. (2012) is unlikely to be the natural infection route for *F. langsethiae*.

The ability of spray inoculated and bagged oat heads to produce *F. langsethiae* DNA with subsequent HT-2+T-2 levels in harvested oat heads is comparable to what has been observed under natural infections in commercial fields, suggesting that the infection route for *F. langsethiae* especially in oats may not be that different from the other fusarium head blight pathogens.

CHAPTER SEVEN

General Discussion.

Cereals are very important crops world-wide contributing significantly to both human and animal feed. Eight cereals grains, wheat, maize, rice, barley, sorghum, oats, rye and millet provide 56% of the food energy and 50% of the protein consumed on earth and thus, humanity has become dependent upon cereals (Stoskopf, 1985).

Like any other crop, cereal production is saddled with plant disease problems among which are fusarium diseases. All the important cereals including wheat, barley and oat are susceptible to one or more fusarium diseases although oat seems to have a better resistance to *Fusarium* infection than wheat and barley (Tekauz, 2004). *Fusarium* infection in cereal may result in a number of different diseases including seedling blight, foot rot and head blight. These diseases may result in reduced crop yield and grain quality due to shrivelled grains and more importantly mycotoxin contamination (Osborne and Stein, 2007).

Fusarium mycotoxins are secondary metabolites and thus are not directly involved in the normal growth, development and reproduction of the fungus (Desjardins, 2006). The reasons for the production of mycotoxins in cereals grains by some *Fusarium* species is not fully understood, but the danger of its contamination in cereals and cereal based products is a health concern (Desjardins, 2006). Among the group of mycotoxins produced by *Fusarium* species in cereal grains trichothecenes are among the most important and have been the toxins most strongly associated with chronic and fatal toxicosis of humans and animals (Desjardins, 2006). HT-2 and T-2 are two of the most potent trichothecene

mycotoxins known (SFC, 2002). In January 2012, discussion limits for HT-2 and T-2 were proposed by the EU (Anon. 2012b).

Fusarium langsethiae has been identified as the main species responsible for high levels of HT-2+T-2 concentrations in cereal grains in the EU and most importantly in UK oats. *F. langsethiae* is a newly identified species. It was first described in 1999 and classified as a species in 2004 (Torp and Nirenberg, 2004). There is very little information on its biology and epidemiology making the development of control strategies difficult. Current traditional methods such as fungicide application used for the control of other important *Fusarium* species such as FHB complex does not appear to be effective against *F. langsethiae*.

The studies of *F. langsethiae*-cereal interaction over the years have been hampered by the lack of symptoms on cereal heads in the field and the lack of effective artificial inoculation methods that can result in high HT-2+T-2 concentrations in cereal heads especially in oats as observed in the field.

To study the mode of infection of *F. langsethiae* in cereals, an initial seed infection experiment was carried out to determine if *F. langsethiae* which had been shown to be seed borne (Torp and Adler, 2004; Torp and Nirenberg, 2004; Imathiu, 2008) could be systemically transmitted through the seed to the other parts of oats (Chapter Five). The results from the experiment showed that although *F. langsethiae* was isolated from the oat seeds with high *F. langsethiae* DNA and high HT-2+T-2 concentration, at harvest *F. langsethiae* DNA was not quantified in the resulting heads and HT-2+T-2 levels were mostly below the limit of quantification. This demonstrated that *F. langsethiae* was not likely to be a systemically seed transmitted pathogen. This does not however, indicate that seeds may not play any role in the infection process of *F. langsethiae* in cereals. Systemic seed disease transmission is normally dependent on the way in which the pathogen is carried

with the seed and on how the pathogen develops during the growth of the plant from the infected or contaminated seed (Neergaard, 1979).

Infection from seed to the other parts of the plant is possible but may not necessarily be systemic through the plant. For example, the pathogen may be outside the embryo and during germination may be carried either passively on the cotyledons or in the seed coat. In this situation the pathogen may be transmitted to the young plant by air currents, rain splashes, insects etc. In such a situation infection does not occur systemically through the seedling to other parts of the plant. It could also be that the seed may be contaminated with the pathogen living saprophytically or at a resting stage in the soil or in plant residue and later infects the host at a later time (Neergaard, 1979). Rain splash is known to be an important means of disease transmission in FHB disease (Parry et al., 1995). It is therefore possible that in the field, infected cereal seeds may be important source of inoculum for *F. langsethiae* infection but the infection may be as a result of the fungus being outside the embryo and most likely in the outer seed coat and carried passively on the seed or may live as a saprophyte and infect the cereal at a later stage of the cereal life cycle with the help of rain splash and or air currents. In the seed infection experiment however, plants were drip-irrigated and thus splashing of water was avoided. This could be the reason why infection did not occur.

Different artificial inoculation methods were trialled (Chapter Six) to determine if an effective method could be identified or optimized for the inoculation of wheat, barley and oats with *F. langsethiae*. Among the methods trialled, spray inoculation was the most effective resulting in high *F. langsethiae* DNA with subsequent high levels of HT-2+T-2 in cereal heads at harvest which were comparable to levels observed in the field. This suggests that the infection route for *F. langsethiae* in cereals may not be that different from other *Fusarium species* responsible for FHB

in cereals and in agreement with work done by Divon et al. (2012). The experiment also showed moisture to influence *F. langsethiae* infection and subsequent HT-2+T-2 concentrations in cereal heads. Spray inoculation with *F. langsethiae* spore suspension resulted in the development of symptoms in the form of bleaching 14 days after inoculation in all cereal heads with bagging again influencing the severity of the observed symptoms. Although the boot inoculation method resulted in disease symptoms in the form of lesions on the boot and stunted cereal heads at harvest with stunting increasing with increasing spore concentration for inoculation may not be the natural infection route and thus may not be a good method to mimic natural infection. This is because the method involved the 'injection' of inoculum directly into the developing boot. However, there is ample evidence to show that fusarium infection in cereals takes place at anthesis (Xu, 2003; Edwards, 2004). Results from the three year field survey (Chapter Three) showed that infection of cereal heads by *F. langsethiae*, tended to occur at full head emergence, but before flowering. It is therefore reasonable to support Divon et al. (2012) that the boot inoculation method may not mimic natural infection.

Generally, this study has shown that among the cereals studied, oat has the least resistance to *F. langsethiae* infection. The three year field survey showed that in all three years *F. langsethiae* DNA and subsequent HT-2+T-2 concentrations in cereal heads was highest in oats (Chapter Three). A follow up experiment (Chapter Four) showed that even when wheat, barley and oats were cultivated under identical field and agronomic conditions, oats still had the highest concentration of *F. langsethiae* DNA in cereal heads. This study also determined that the concentration of HT-2+T-2 per unit of *F. langsethiae* biomass in oats compared to wheat and barley was significantly higher. Artificial inoculations also resulted in higher *F. langsethiae* DNA and HT-2+T-2 concentrations in oats confirming the preference of *F. langsethiae* for oats (Chapter Four). An *in-vitro* leaf assay experiment showed lesions that

developed on wounded oat leaves to be longer than those that developed on wheat and barley leaves (Chapter Five). In the said study, although no total resistance to *F. langsethiae* was observed in any of the cereal varieties, oats were found to be the most susceptible to *F. langsethiae* in comparison to wheat and barley. The reasons for *F. langsethiae* preference for oats and thus the resistance factors underlying *F. langsethiae*-oat interaction are poorly understood, but results from Chapter Four indicate they are as a result of genetic control rather than agronomy.

The prominence that *F. langsethiae* has generated over the years due to its production of HT-2+T-2 in cereals, especially in oats in the UK with recorded concentration levels in some field samples exceeding the discussion limits (Edwards, 2012) requires the need for a fast and efficient resistance screening method for *F. langsethiae* in the different cereal species. Results from the *in-vitro* leaf assay (Chapter five) showed that it could be an easy and a fast screening method. When available recommended UK wheat, barley and oat winter and spring varieties were screened using detached wounded leaf assay, none of the cereal varieties had total resistance to *F. langsethiae* infection. Lesion lengths formed on oat leaves correlated well with HT-2+T-2 field data from Edwards et al. (2012). It was however observed that lesion length formed on naked varieties did not correlate well with their corresponding HT-2+T-2 field data and this may be due to the high *F. langsethiae* DNA in the outer hulls of oat grains which in naked varieties tend to threshed off the grain during harvesting (Edwards et al., 2009).

This study has provided novel information on the life cycle of *F. langsethiae* in wheat, barley, oats and triticale. In all three years of the field survey (Chapter Three), no stem base infection was observed on any of the samples with high *F. langsethiae* DNA and HT-2+T-2 concentrations. This suggests that *F. langsethiae* may not cause stem base infections. When two stem base infections experiments

were carried out in Chapter Six, it was observed that whilst *F. graminearum* inoculated plants showed symptoms of foot rot from 14 days after inoculation, *F. langsethiae* inoculated plants showed no such symptoms and at harvest there was no difference between the stems of control and *F. langsethiae* inoculated plants confirming the inability of *F. langsethiae* to cause stem base infection even when inoculum is in close contact with the host plant. The field survey data showed that if *F. langsethiae* head infection in wheat, barley, oats and triticale occurs, it is at full head emergence but before flowering. This information is very important in the development of a control programme for *F. langsethiae* in cereals in the UK. This is because the current chemical spraying regime against *Fusarium* infection in cereals does not seem to be effective against *F. langsethiae* in the field (Edwards and Anderson, 2011) although under laboratory conditions some active fungicides seem to have antifungal activity on *F. langsethiae*. Mateo et al. (2011) showed that prochloraz, tebuconazole and fenpropimorph all decreased the growth of *F. langsethiae* and accumulation of HT-2+T-2 *in-vitro* although prochloraz and tebuconazole were more effective than fenpropimorph. Chemical spraying against FHB in wheat, barley and oat is done close to the infection period, that is early anthesis (GS61-65). Field experiments of FHB control in wheat have shown that fungicide timing is critical and is only effective in a narrow window (Pirgozliev et al., 2008). However, data generated from this study (Chapter Three) has shown that at anthesis *F. langsethiae,* if present, would have already infected the cereal head. This is evident by the presence of *F. langsethiae* biomass in cereal heads at head emergence at relatively high levels (Chapter Three). This may explain why these known fungicides, although effective against FHB species are not effective against *F. langsethiae*. This suggests that if the spraying time is optimized through experimentation, a chemical control could be effective against *F. langsethiae*.

Based on all the information gathered from this research it can be postulated that the generalised life cycle of *F. langsethiae* deviates from that of the known FHB pathogens. These deviations include its inability to cause stem base infection and its earlier head infection in cereals (Figure 7.1). Such information is critical to the development of effective control measures to reduce the infection *F. langsethiae* in cereals and the subsequent contamination of cereals with HT-2+T-2.

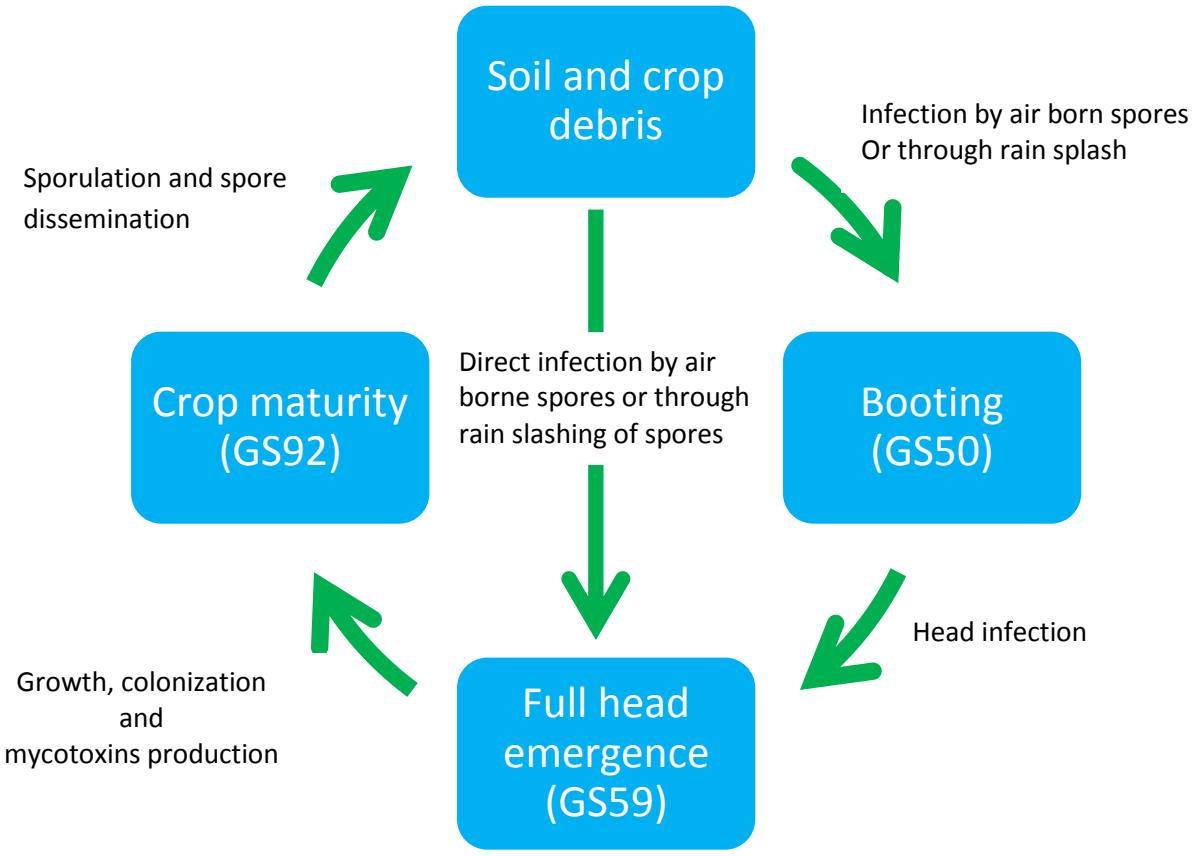

Figure 7.1. Postulated life-cycle of *F. langsethiae* in cereals based on a three year field survey, artificial inoculations and the generalized life-cycle of *Fusarium* species.

7.2. Recommendations for future work

This research has provided novel information on the life-cycle and the epidemiology of *F. langsethiae* in UK wheat, barley and oats. However, there are a number of unanswered questions to be addressed so as to fully understand the *F. langsethiae*-cereal relationship and thus there is a need for further investigations.

- This study could not identify how *F. langsethiae* overwinters. It was also not able to identify resting structures for *F. langsethiae.* It should be mentioned here that some sporodochia-like structures were identified on one year old PDA plates (Figure 7.2).

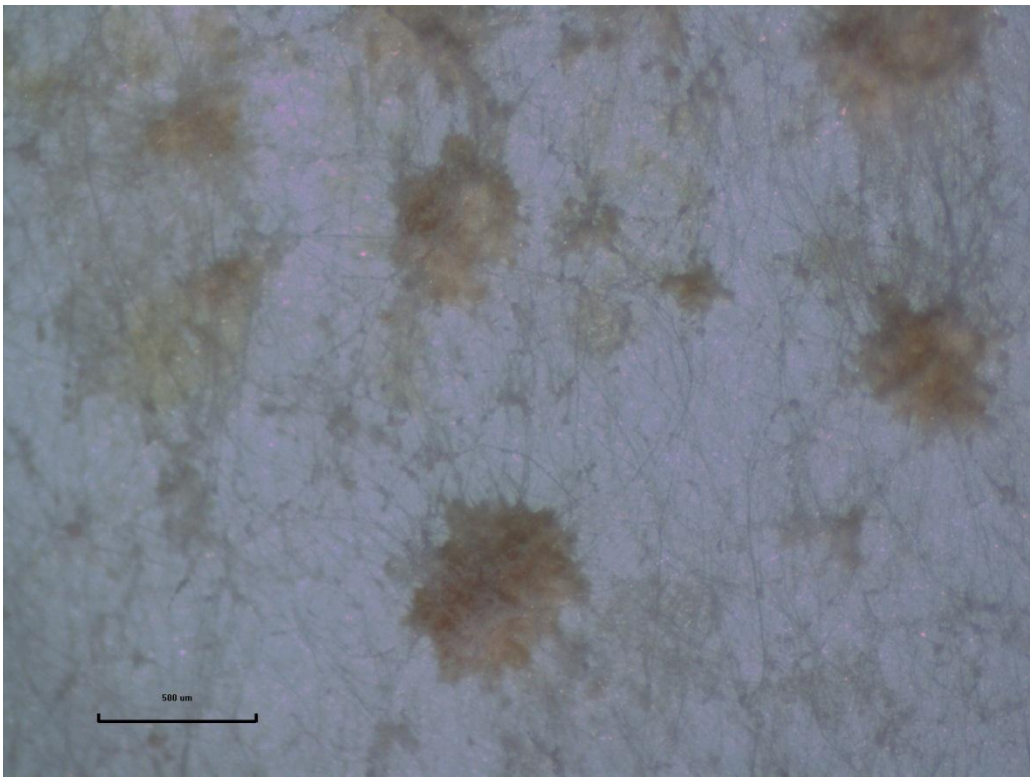

Figure 7.2. Sporodochia-like *F. langsethiae* structure on PDA plate left standing for one year Scale = 500 µm.

These structures, upon crushing in SDW were found to contain a large number of microconidia. Morphological studies and PCR proved that spores were that of *F. langsethiae*. It will therefore be of interest if further studies

are carried out to confirm or otherwise what these structures are and to properly describe them. These experiments could include culturing *F, langsethiae* on different low nutrient agars and other culturing methods under natural conditions over time.

- The field survey (Chapter Three) showed that *F. langsethiae* infection occurred at full head emergence. It was however not ascertained how the spores were transmitted onto the heads (although some assumptions have been made) and the conditions that stimulate the spores to be released. A spore trap experiment could be performed to monitor *F. langsethiae* dynamics in air and to determine if it could be related to specific weather conditions and crop growth stages.
- In Chapter Four, it was observed that under identical field and agronomic conditions, *F. langsethiae* infection was still highest in oats compared to wheat and barley. This relationship is poorly understood. There is therefore the need to carry out further studies to understand the factors leading to this observed preference of *F. langsethiae* for oats and most importantly the resistance mechanism and the genes that influence this relationship.
- Although this study has provided some information on the infection process of *F. langsethiae* it is still not clear how the actual head or grain infection takes place and the type of infection structures that are employed by this fungal species during infection. There is therefore the need to carry out more artificial inoculation studies coupled with microscopy studies to determine the mechanism of infection and the fungal and plant structures involved in this process. The use of GFP labelled genetically modified isolates of *F. langsethiae* would greatly facilitate such studies.

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