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IDENTIFICATION OF *FUSARIUM* RESISTANCE TRAITS IN UK OAT VARIETIES

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Abstract

Previous studies identified that UK oats are routinely infected by *Fusarium langsethiae* and this infection can result in high levels of the fusarium mycotoxins, HT2 and T2 on harvested oat grains. The European Commission are currently considering legislative limits for the combined concentration of these mycotoxins (HT2+T2). An indicative limit of 1000 µg kg^{-1} recommended by the European Commission is exceeded, on average, in 18% of UK oat samples. The aim of this PhD project was to understand the variation in resistance of UK oat varieties to *F. langsethiae*. In previous studies conducted across the UK it was identified that all winter oat variety trials had higher levels of HT2+T2 mycotoxins compared to the spring variety trials but it was not known whether the difference observed was due to agronomic (i.e. sowing date) or genetic variations. To test the hypothesis that the differences observed were not due to agronomy, experiments were conducted with winter and spring varieties sown together in both autumn and spring sown experiments. Results indicated that some winter varieties had consistently higher HT2+T2 irrespective of sowing date, and are therefore genetically more susceptible to *F. langsethiae* infection. It was also previously observed that grains of naked oat varieties had lower levels of HT2+T2 at harvest but it was not clear how naked and conventional husked oat crops compared before harvest. Results from this study identified that some naked oat varieties have high HT2+T2 levels before harvest and as such these varieties have high susceptibility to *F. langsethiae*. In previous studies high concentrations of HT2+T2 were detected on grains of dwarf varieties compared to conventional height varieties. To test the hypothesis that the difference observed between dwarf and varieties of a conventional height was not due to morphological trait of crop height experiments were conducted with a dwarf and tall variety with height further manipulated by a

range of PGR doses. To test the same hypothesis of whether crop height is a susceptibility trait, experiments at the University of Aberystwyth with a mapping population developed from a cross between short and tall winter oat varieties were used for the identification of QTL for resistance and to determine genetic linkage with agronomic traits such as height. Results identified that height per se is a resistance factor, but is only one of many, or there is close genetic linkage between the dwarf gene and a susceptibility QTL. As currently our knowledge of factors facilitating *Fusarium* resistance in oat varieties is limited, experiments with artificial inoculation of the model cereal *Brachypodium* with *F. langsethiae* were performed as part of this project. Results indicated that *Brachypodium* is a host for *F. langsethiae* and produces typical head blight symptoms after infection. Results from this project have identified differences in *F. langsethiae* resistance within UK oat varieties and identified potential QTL for use in marker assisted breeding.

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For assisting with experiments I must thank Cath Edwards for help with milling part of the samples and Danielle Henderson-Holding and Samantha Mountain for help with milling and analysing for HT2+T2 part of the panicle samples collected. The linkage map used in this study for Chapter 6 as well as map of linkage groups 1, 29 and 30 was kindly supplied by Dr Catherine Howarth. I am also thankful for the *Brachypodium distachyon* seeds supplied by Luis Mur (University of Aberystwyth) and Antoine Peraldi (John Innes Centre).

Last but not least, I am very grateful to my family and my husband Daniel for all the love and support.

This thesis is dedicated to my parents Ivanka and Milivoje, brother Strahinja and sister Tamara as I will always be grateful for their constant care.

Table of contents

List of figures

List of tables

Chapter 1: General introduction

1.1. Literature review

1.1.1. Oats

1.1.1.1. Origin

Oat is a grain commonly consumed in Nordic countries although its origin is in Levant and West-Asia (Bjørnstad, 2012). Ancient Greeks and Romans were familiar with the oat crop but there is evidence suggesting that the crop was cultivated for the first time in China (Olson & Frey, 1987). Oats arrived into the European continent from Asia Minor as a weed of wheat and barley. Historic sources indicate that oats were grown at a reasonable scale only in the northern regions of Western Europe (Barker, 1985). The Roman conquest (43 AD) was when oats were established as a cultivated crop in Britain. Oats would thrive in the cool, wet climate and they were needed by the Roman cavalry for horse feed. Besides high energy feed, oat was valued for bedding and as a source of manure. Medieval farmers probably preferred what is known as naked oats as they did not need a mill to be processed into meal. In support of this is its price equivalent to wheat in the fourteenth century (Hallam & Thirska, 1988). The medieval intensification of agriculture powered by horses established oat as a major crop thanks to its high energy content. Hallam (1981) noted that the possession of horses resulted in an increased oat production due to the amount of feed needed. Having horses symbolized a prosperous economy (Hallam, 1981). From being primarily grown as feed, oats were an integral part of human diet until late Victorian times, being consumed as a principal cereal in Northern England and

Scotland. In the beginning of 20th century its consumption declined but recently with dietary recommendation to increase fibre intake, there is a renewed interest in oats (Moore-Colyer, 1995).

1.1.1.2. Oat evolution, genetics and breeding

Oats (*Avena spp. L.*) belong to the family *Gramineae*. Different oat species can differ in the number of chromosomes that can be either 14, 28 or 42 chromosomes. Hexaploid oats (2n = 6x = 42 chromosomes) are economically the most important with the common spring oat or white oat (*Avena sativa L.*) being the most important. The other commercially important oat species is the red-type oat known as winter oats. Similar to the cultivated oats, *A. fatua L.* is a hexaploid wild oat, considered to be a very persistent weed species while *A. sterilis L.* from the Mediterranean region was used by oat breeders as a source of germplasm for improving the cultivated oats especially with regards to protein content and disease resistance (Olson & Frey, 1987). Although the hexaploid oat genome has the same number of chromosomes as wheat (42 chromosomes in 3 genomes) it is smaller with 13 billion bp, but considered to be more variable than the wheat genome (Bjørnstad, 2012).

1.1.1.3. Biology and morphology

Oats are monocotyledons. The main axis called rachis gives rise to a panicles where flowering branches called spikelets are formed (Figure 1.1). Each spikelet has typically two or three florets each giving two to three seeds when pollinated. Anthesis follows shortly after the extrusion of panicles. Anthers can be extruded if florets open due to swelling in the floret base but as oats are self-fertilized, the opening is not essential. The glumes and the husk comprising lemma and palea enclose the florets. Glumes are easily removed during grain development but

lemma and palea are much harder and can only be removed during grain processing (White, 1995). The term naked oats is used to mark those varieties whose grain when harvested is not enclosed with lemma and palea therefore they are referred to as huskless. In conventional husked varieties, the percentage of husk is high, around 25% (w/w). It increases the fibre content and reduces its digestible carbohydrate content. The husk consists of lemma and palea that enclose the caryopsis called groat or kernel (White, 1995).

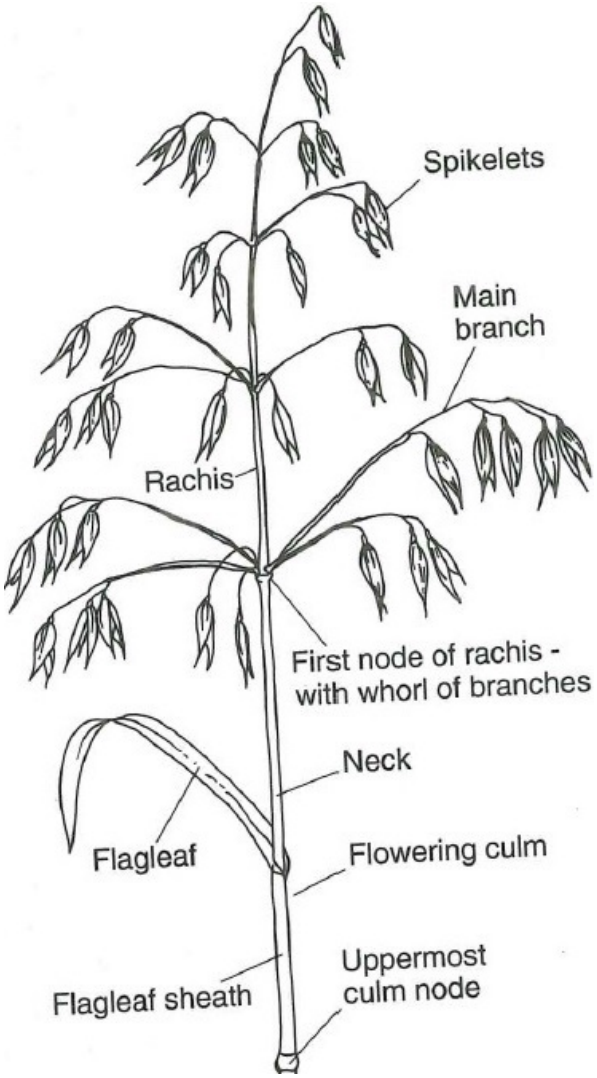

Figure 1.1 Structure of an oat panicle (source: White, 1995).

1.1.1.4. Production and agronomy

Worldwide oat is grown as forage and as a grain crop. It is recognized for its low nitrogen needs and good competitiveness with weeds which makes it suitable for organic production (Marshall *et al.*, 2013). It is often used as a break crop as it breaks the lifecycle of *Gaeumannomyces graminis var. tritici*, the 'take-all' root rot disease of cereals, due to avenacins produced in the roots (Crombie *et al.*, 1986). Oats are known to thrive in poorer soils and cooler climates whilst tolerating a range of pH values (Bjørnstad, 2012).

The main genetic differences of oat diversity are associated with differences of spring vs. winter varieties (Tinker *et al.*, 2009). Oats can be sown in spring or winter but winter varieties are limited in northern climates due to lacking frost hardiness as oats are considered to be prone to winter damage. In the UK the majority of oats are grown as winter crops due to higher yields and better rooting system (Marshall *et al.*, 2013). As winter sown oats are considered to benefit the soil by preventing erosion and can be harvested earlier with higher yields, there are attempts to develop winter varieties that could be grown in Nordic countries (Chawade *et al.*, 2012).

Oats are grown on all continents with production in Europe being the largest with 64.3 %, followed by the Americas with 24.8 % of the world production (Figure 1.2).

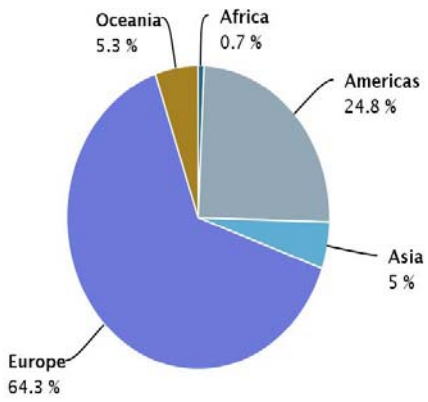

Figure 1.2 Production share of oats by regions (source: FAOSTAT).

Russia is the biggest oat producer followed by Canada, USA and Poland which is the biggest oat producer in Europe (Figure 1.3).

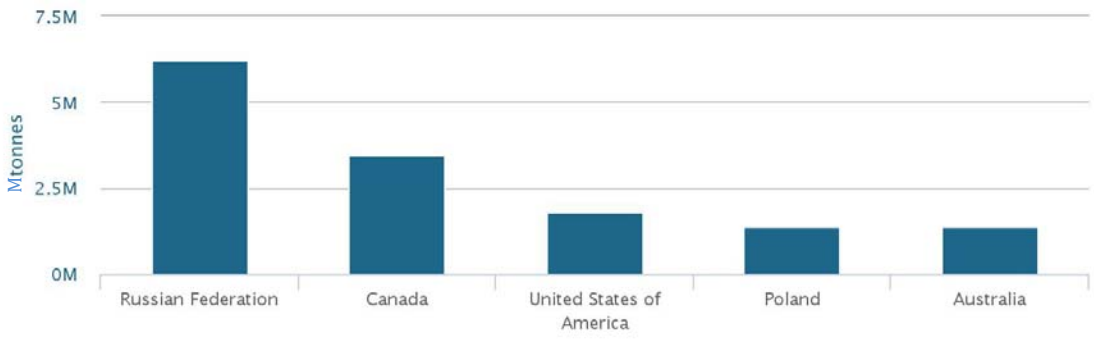

Figure 1.3 Top five oat producing countries (source: FAOSTAT).

When comparing Scandinavian countries where oat has been a traditional grain (Bjørnstad, 2012) with the biggest producers in the world (Canada and Russia), with Poland as the biggest European oat producer and the UK; there is a large disparity in an area harvested and yield. Russia is harvesting the biggest area in hectares (Figure 1.4) but its yield is the lowest when compared with Canada, Norway, Poland, Sweden and the UK (Figure 1.5). A similar trend is true for

Canada and Poland whereas Scandinavian countries with a lower area harvested have much higher yields. Whilst the area harvested in the UK is amongst the smallest, the yields are the highest.

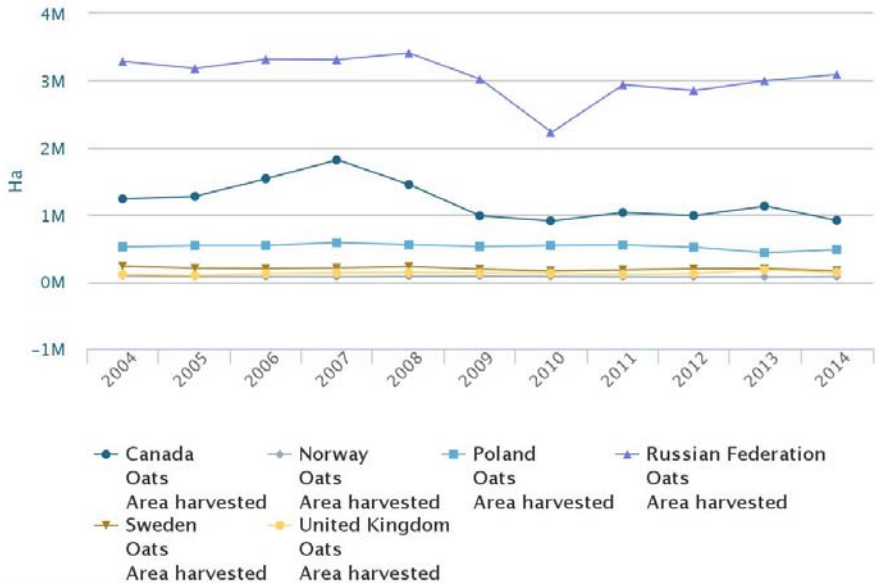

Figure 1.4 Area of oats harvested in Russia, Canada, Norway, Sweden, Poland and the UK between 2004 - 2014 (source: FAOSTAT)

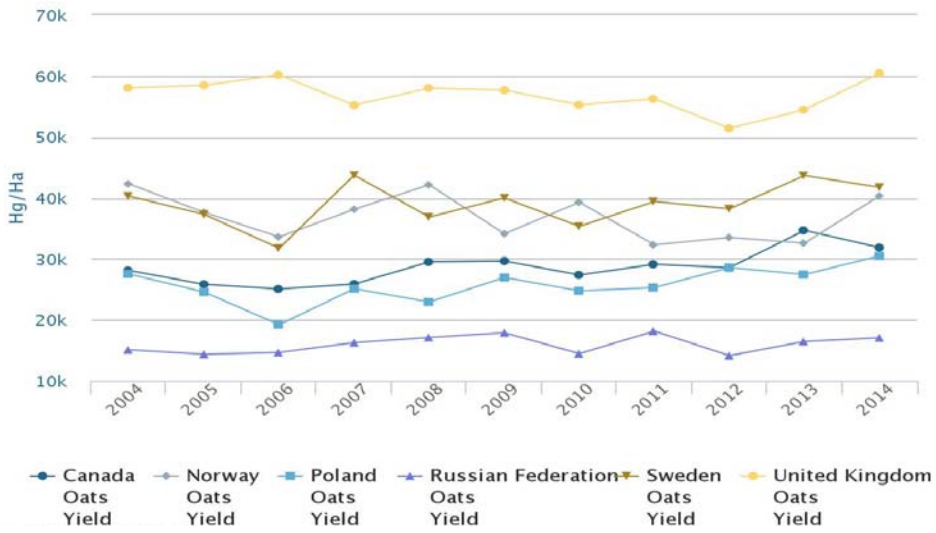

Figure 1.5 Oat yields in Russia, Canada, Norway, Sweden, Poland and the UK between 2004 - 2014 (source: FAOSTAT).

Despite recognized benefits of oat as a break crop and a grain with health benefits its production in the UK is much lower compared with other cereals. The area under oats (Figure 1.6) is much less than wheat and barley and the yield (Figure 1.7) is much less than wheat. Despite this, oat yield in the UK is among the highest in Europe.

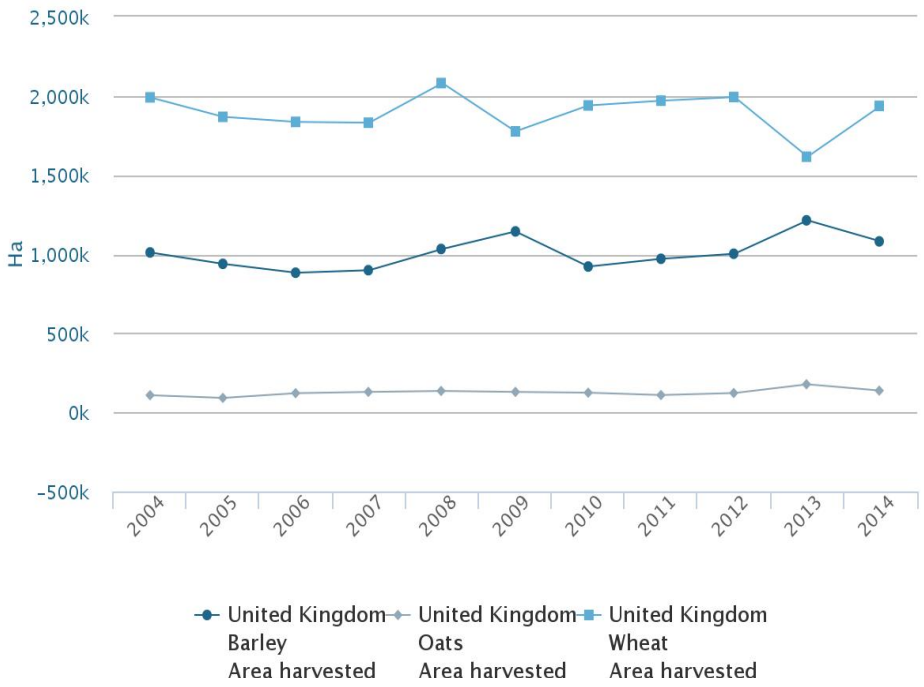

Figure 1.6 Area harvested under barley, oats and wheat in the UK between 2004 - 2014 (source: FAOSTAT)

Oat production in the UK in 2014 was 820 thousand tonnes and in 2015 it was 779 thousand tonnes (Figure 1.8). As a consequence of this reduction in area planted, there was a small reduction in total yield.

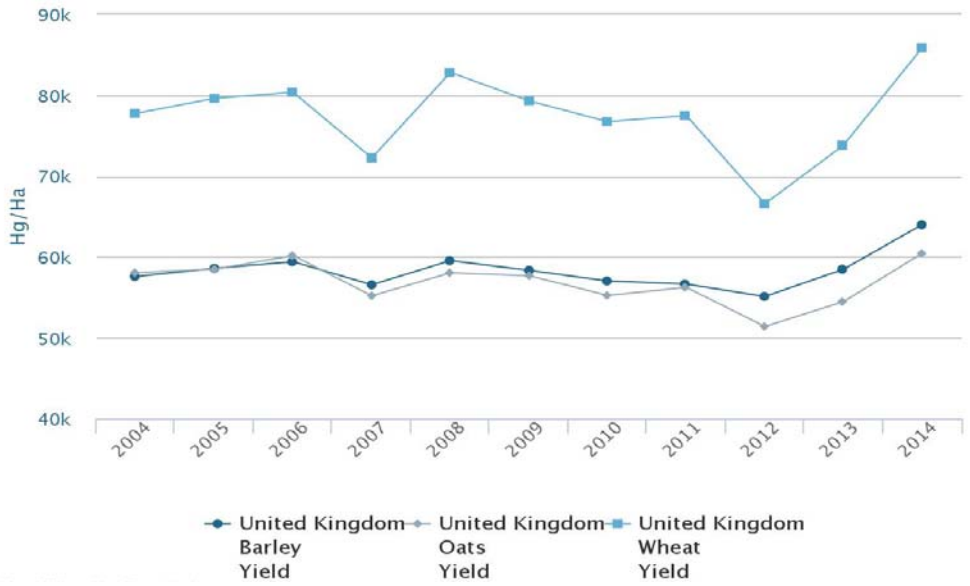

Figure 1.7 Yield of barley, oats and wheat in the UK between 2004 - 2014 (source: FAOSTAT).

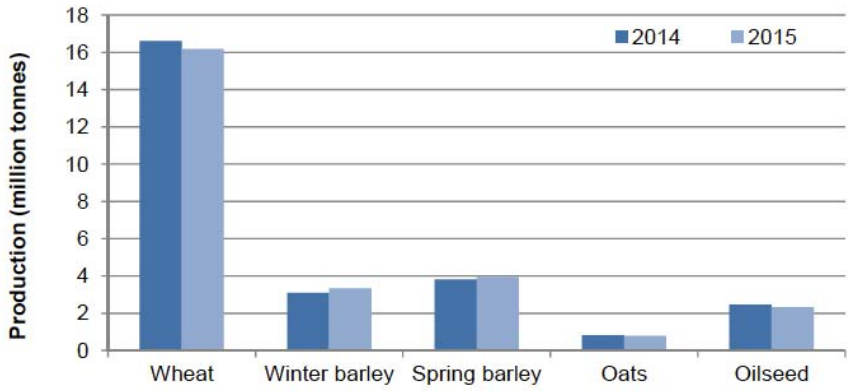

Figure 1.8 Crop production in the UK between 2014 and 2015 (source: DEFRA Farming Statistics. Provisional 2015 cereal and oilseed rape production estimates United Kingdom).

Oat yields are currently ca. 6 tonnes/ha and are closely following barley yields (Figure 1.9).

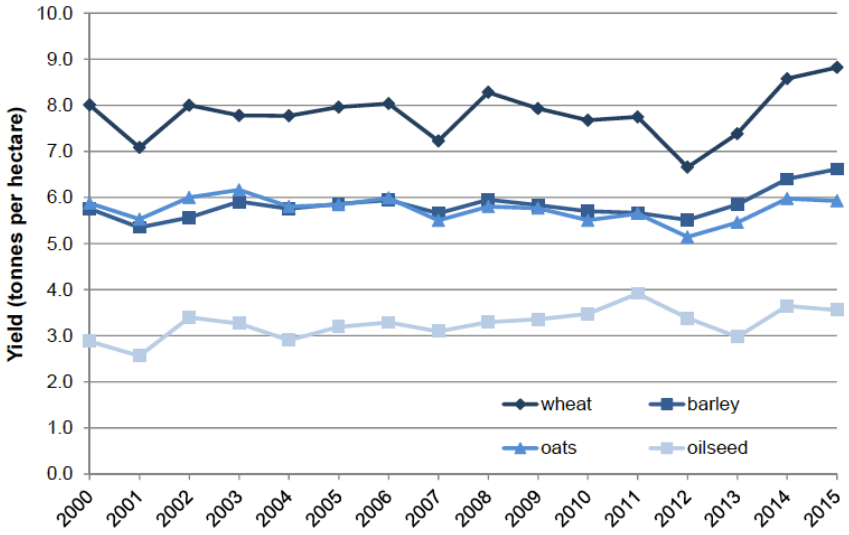

Figure 1.9 UK cereal crop yields between 2000 and 2015 (source: DEFRA Farming Statistics. Provisional 2015 cereal and oilseed rape production estimates United Kingdom)

1.1.1.5. Importance and consumption

Oats are rich in β-glucan (dietary fibre), antioxidants and proteins. Milk from cows fed with oats makes softer and more spreadable butter as oats are rich in unsaturated fatty acids (Ekern *et al.*, 2003). There have been numerous attempts to breed oats with higher concentration of β-glucan, the major endospermic cell wall polysaccharide, as certain health benefits are suggested. This has led to an increase in the promotion and consumption of oat products (Marshall *et al.*, 2013). In 2010, the European Safety Authority panel in charge of health claims has agreed that there is a link between daily consumption of oat β-glucan and improving or lowering blood cholesterol thus reducing the risk of heart diseases (EFSA NDA Panel (EFSA Panel on Dietetic Products Nutrition and Allergies), 2010).

Oat has a diverse utilisation as food but it has also use in the feed industry while straw can be used for bedding for animals. Oat groat is considered to have a high amount and good quality of protein, but the protein concentration can be influenced by the environment. Embryonic axis, scutellum, bran and endosperm have the highest protein concentration. The groat is rated high with regards to the amino acid composition and the composition of essential amino acids that humans cannot synthesize. The major carbohydrate is starch. There are a limited amount of free sugars such as sucrose, fructose and glucose for example and they are in lower quantities than in wheat (Olson & Frey, 1987). Being a low input cereal with health benefits, oats has received much attention in breeding for improved nutritional qualities and agronomic traits (Marshall *et al.*, 2013).

1.1.2. Fusarium genus

The *Fusarium* genus was identified in 1809 by Johann H. F. Link, the director of the Berlin Botanic Garden (Desjardins, 2006). It belongs to the Ascomycota phylum of the Fungi Kingdom (Goswami & Kistler, 2004). Species belonging to this genus are important plant pathogens including those infecting wheat, barley, oats, rye, triticale and different members of the grass family (Parry *et al.*, 1995). Other economically important *Fusarium* species include those causing wilt on fruits and vegetables such as different *Fusarium oxysporum* f.sp (Agrios, 2005a).

Fusarium species are plant pathogens but some of them can be opportunistic human pathogens in immunocompromised patients (Nucci & Anaissie, 2007). Between 2005-2006, there have been reports of *Fusarium* keratitis in the USA, (Chang *et al.*, 2006), Singapore (Khor *et al.*, 2008) and France (Gaujoux *et al.*,

2008) due to the use of a particular contact lens solution which was consequently withdrawn from the market. Many factors might be involved in outbreaks (Ahearn *et al.*, 2008) but it is shown that the solution in question had weaker biocidal activity (Levy *et al.*, 2006). In India, at the beginning of 2015 there has been the first report of human keratitis due to *Fusarium langsethiae* (Vasantha *et al.*, 2015).

Another *Fusarium* species, *Fusarium venenatum* strain that does not produce toxins is used in QUORN™, a product marketed as a vegetarian source of proteins or mycoproteins (Wiebe, 2002).

1.1.2.1. Fusarium head blight

Fusarium head blight (FHB) is a disease complex of different *Fusarium* species on cereals. Commonly found species are *Fusarium graminearum* and *Fusarium culmorum*, amongst others. Some of the species involved in a FHB complex are producers of potent mycotoxins. Besides head blight, the species involved in the disease complex can cause seedling blight and foot rot and are found across the world where cereals are grown (Parry *et al.*, 1995).

Fusarium induced diseases are of great concern in food production. Yield can be reduced, grains shrivelled with reduced milling and baking quality thus making the grain not suitable for human or animal consumption. Besides a reduction in quality, importance lies in contamination of grains with mycotoxins that occur in the field before harvesting (Edwards, 2004; Kazan *et al.*, 2012). Also, *Fusarium* contaminated seeds of wheat or oats have shown a reduced germination rate (Murray *et al.*, 1998; Tekle *et al.*, 2012b) and therefore are unsuitable as seed stocks.

Small grains and corn are the staple crops in much of Northern America and FHB outbreaks were often reported. During the 1996 wheat harvest, with the average price in that year and losses of 30% due to FHB, it is estimated that the loss came up to $23 million (Shaner, 2003). Based on calculations of Johnson *et al.* (2003) between 1991 and 1997, losses from FHB on wheat in nine states of USA were $1.3 billion where North Dakota and Minnesota accounted for 68% of the total. If the same calculation were extrapolated to more states, total losses would come to $4.8 billion. Another report calculated that between 1993 and 2001, in FHB affected states of the USA, the losses from FHB on wheat and barley were estimated at $7.7 billion (Nganje *et al.*, 2004). FHB was also reported as a devastating disease in many parts of China in several years (Bai *et al.*, 2003).

In the UK surveys before 1932, FHB was not recorded as a widespread disease but rather sporadic in a few wheat fields. Most common species were *Fusarium culmorum* and *Fusarium avenaceum*, recorded almost every year. *Fusarium culmorum* was the most commonly isolated species in Northwest Europe and it was considered that *Fusarium* species' geographical distribution is related to climate needs (Parry *et al.*, 1995). In 1989 and 1990 *Fusarium poae* was most often isolated from the ears of winter wheat in the UK (Polley & Turner, 1995).

The more recent data from a national survey collected in 2014 showed that 82% of DEFRA winter wheat survey samples showed symptoms of fusarium blight disease complex. Of the samples analysed, non-toxigenic *Microdochium* species were found in 83% of the samples and *Fusarium poae* in 52% of the samples. *Fusarium graminearum* was present in 18% of the samples and *Fusarium culmorum* in 4% of the samples. It should be noted there is no national disease survey of oats (see www.cropmonitor.co.uk).

Harvesting and sowing contaminated seeds plays a role as a source of inoculum as *Fusarium* species can be seed-borne (Murray *et al.*, 1998). Due to *Fusarium* saprophytic capabilities, debris in the soil can be colonized and used as a reservoir of inoculum (Figure 1.10). This is particularly important during the wet season around flowering time as spores can be splashed and carried upwards in the canopy (Graham *et al.*, 2008) or wind-blown in some species (Murray *et al.*, 1998). *Fusarium* species with a known sexual stage (such as *Fusarium graminearum*) release wind dispersed ascospores from perithecia that can infect cereals at flowering if the weather conditions are favourable (Kazan *et al.*, 2012). Flowering has been shown to be the most susceptible stage in cereal development and with extended humid weather at that stage, infection can be severe (Murray *et al.*, 1998). Asexual spores (conidia) are associated with rain dispersal (Agrios, 2005b).

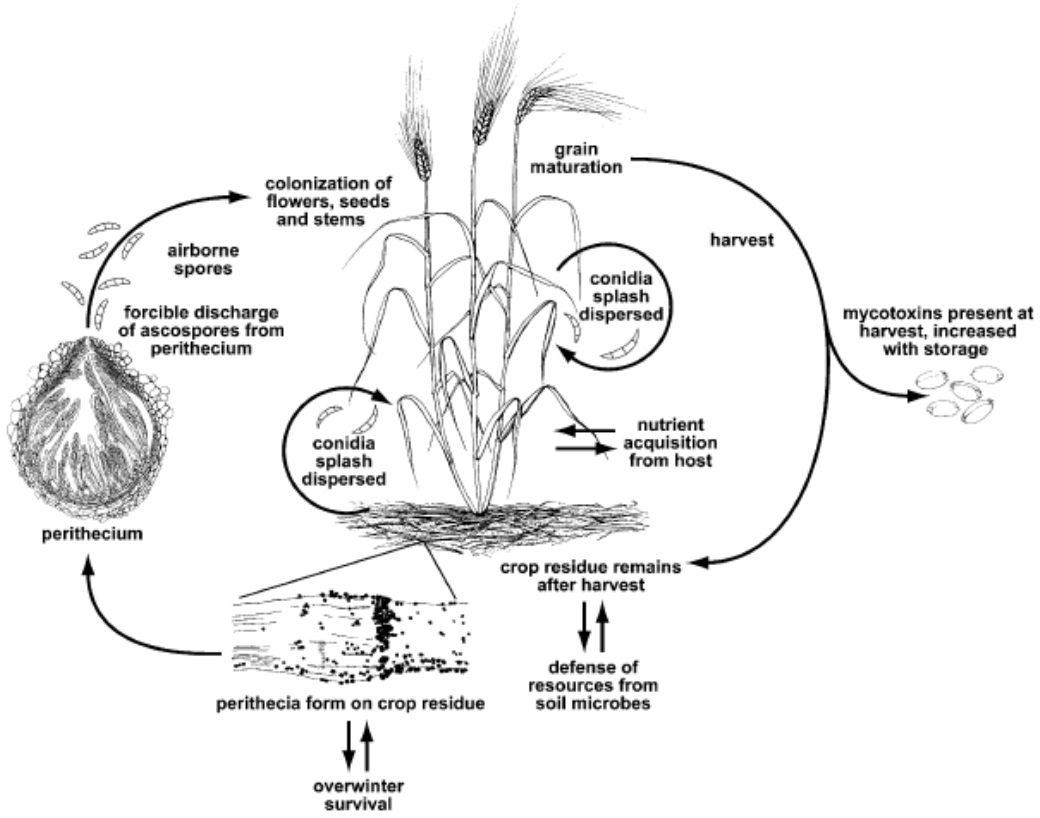

Figure 1.10 *Fusarium graminearum* life cycle (source: Trail, 2009).

The most studied *Fusarium* species on cereals is *Fusarium graminearum* on wheat. Pritsch *et al.* (2000) studied inoculated wheat spikes within the first 48 h to 76 h. After entering through glume stomata and growing along stomatal rows, the colonisation happens. Earlier expression of defence response genes (accumulating PR-4 and PR-5) was observed in a resistant cultivar and no direct penetration of the glume was seen. Similar studies that included a range of cultivars with different levels of resistance from different sources would aid our understanding better.

Guenther & Trail (2005) showed that colonization progresses from wheat heads downwards to stems, throughout culms while seeds are developing and slows down at senescence. In 25% of plants the infection did not progress from head to

stem and in those cases occlusions were found in vessels of the rachis (Guenther & Trail, 2005).

Symptoms develop a few days after infection. The most common symptom of head or ear blight is premature bleaching of a cereal head where tissue is colonized by the fungus (Trail, 2009). In some cases pink or orange coloured *Fusarium* mycelium with masses of macroconidia develop and can be visible on wheat heads (Murray *et al.*, 1998). Spikelets infected with *Fusarium* species will often be sterile or with shrivelled and discoloured grain whereas other grains may look healthy but can be contaminated with mycotoxins (Bockus *et al.*, 2010).

1.1.2.2. Fusarium head blight on oats

Oats are considered to be more resistant to pests and diseases compared with other cereals. The significant exemption are the *Fusarium* species due to their production of harmful mycotoxins (Marshall *et al.*, 2013). Previously it was reported that only four *Fusarium* species were involved in the *Fusarium* disease complex in oats (Clifford, 1995). In 1995 the following species were considered to be a problem on oats: *Microdochium nivale* (formerly *Fusarium nivale*), *Fusarium culmorum*, *F. avenaceum* and *F. graminearum*.

The most dominant *Fusarium* species on cereals are *Fusarium graminearum* (present mainly in warmer climates) and *F. culmorum* (present mainly in colder climates). Both cause significant problems to maize and wheat production worldwide. They mainly produce the mycotoxins deoxynivalenol (DON) and zearalenone (ZON) and humid weather at flowering was shown to be conducive for infection (Parry *et al.*, 1995; Bottalico & Perrone, 2002).

Microdochium nivale (formerly *Fusarium nivale*) is not known for mycotoxin production (Chełkowski et al., 1991). It is often isolated from barley, oats and wheat and causes pre-emergence death with diseased seedlings showing brown lesions. If infection occurs at later growth stage, seeds are shrivelled (Millar & Colhoun, 1969). It is not unusual in the northern hemisphere during wet and cool autumn, when snow falls on unfrozen soil and stays during the winter (Murray *et al.*, 1998).

Fusarium avenaceum besides being found on some cereals can infect various perennial plants and legumes and is a soil saprophyte as well. It is common in temperate parts of the world (Leslie & Summerell, 2006) and known for production of moniliformin and beauvericin mycotoxins (Bottalico & Perrone, 2002).

Fusarium poae and *F. sporotrichioides* are widespread and frequently isolated from grains in temperate regions (Leslie & Summerell, 2006). *Fusarium poae* mainly produces nivalenol (NIV) and diacetoxyscirpenol (DAS) mycotoxins. While not all isolates of *F. poae* can produce HT2 and T2, most *Fusarium sporotrichioides* isolates produce these toxins (Thrane *et al.*, 2004).

Reviewing *Fusarium* species, producers of toxins in Europe, Bottalico & Perrone (2002) did not mention *F. langsethiae* as it was not fully described (Torp & Nirenberg, 2004) before their review was published.

1.1.2.3. Fusarium langsethiae

In 1999, Torp and Langseth reported isolates of *Fusarium* which produced globular microconidia that resembled *F. poae* but which produced high quantities of T2 toxin which is not typical of *F. poae*. These isolates were first referred to as 'powdery *Fusarium poae*' due to less aerial mycelium and powdery appearance when microconida were produced on Czapek-Dox Iprodione Dichloran (CZID) agar (Torp & Langseth, 1999). In 2004, the 'powdery *Fusarium poae*' was described as a new *Fusarium* species, named *Fusarium langsethiae* after Dr Wenche Langseth. It was observed that microconidia of *F. langsethiae* are formed on bent phialids whereas microconidia of *F. poae* are formed on straight monophialids (Torp & Nirenberg, 2004).

Wheat, barley and oats are suitable hosts from which *F. langsethiae* is isolated and reported. It has been isolated from cereals in the UK, France, Norway, Denmark, Germany, Austria, Czech Republic, Poland, Italy, Serbia, Finland and Sweden (Yli-Mattila *et al.*, 2004; Torp & Nirenberg, 2004; Bocarov-Stancic *et al.*, 2008; Lukanowski *et al.*, 2008; Edwards *et al.*, 2009; Fredlund *et al.*, 2010). For the first time it was reported on durum wheat in Italy by Infantino *et al.* (2007). It is not clear whether conditions on other continents are not favourable for *F. langsethiae* or that this species occurs on other continents but has not yet been isolated, correctly identified and/or reported.

Nielsen et al. (2011) recently reported analysing archived samples from 1957 onwards; they showed that *F. langsethiae* was present in high amounts in Danish wheat and barley from 1977 to 1980. From analysing archived wheat samples it was reported that *F. langsethiae* was present in the UK since 1998, although it was not stated how old were the oldest material analysed within this study (Wilson

et al., 2004). It would be interesting to test archived samples of wheat held at Rothamsted Research station. Their Broadbalk experiment of continuous wheat production started in 1843 and samples of wheat grains have been collected and archived since then.

As *F. langsethiae* exhibits slower growing rate than other similar species, it is not easy to isolate and that may be the reason why it is was not described earlier (Torp & Nirenberg, 2004). Contrary to similar species, *Fusarium langsethiae* exhibits reduced growth rate at 25°C on potato sucrose agar (PSA) and potato dextrose agar (PDA) with its optimum below 20°C (Imathiu, 2008). As described by Torp and Nirenberg (2004) mycelium of *F. langsethiae* is less aerial than *F. poae* with colour ranging from whitish and yellowish to pale red with orange tone or violet centre. Margins were reported to be complete or lobed. As detailed by the same authors, the most noticeable character is a powdery appearance of colonies with sporulation starting within a week. *Fusarium langsethiae* has not been reported to have a sexual stage with airborne ascospores. Colouration can be explained by production of aurofusarin (AUF), a red pigment present in many *Fusarium* species but is produced only by a few *F. langsethiae* strains (Thrane *et al.*, 2004). It is not known what triggers the production of AUF and what its role is.

In a study by Thrane *et al.* (2004) it was shown that all strains of *F. langsethiae* and *F. sporotrichioides* can produce T2 toxin and its derivatives (HT2 and neosolaniol) where just some *F. poae* strains can produce this toxin in lower amounts. Based on this, it is possible to speculate that some strains previously identified as *F. poae* could have been *F. langsethiae* (Thrane *et al.*, 2004; Torp & Nirenberg, 2004). Genomic similarity of *F. sporotrichioides* and *F. langsethiae* is reported by several authors (Wilson *et al.*, 2004; Knutsen *et al.*, 2004; Niessen *et*

al., 2004; Thrane *et al.*, 2004). Among different mycotoxins produced by *F. langsethiae*, T2 and HT2 (HT2+T2) are produced in the highest amounts (Thrane *et al.*, 2004). These two mycotoxins are considered to be very potent but there is a need for more research about their toxicity and the agronomy influencing their accumulation in susceptible host plants (Contam-Efsa, 2011).

In a UK survey from 2002-2004, *F. langsethiae* DNA was detected in 99% of oat samples (Edwards *et al.*, 2012). A highly significant relationship between the level of HT2+T2 and *F. langsethiae* DNA concentrations in UK cereal samples was detected indicating that this species is the predominant producer of these toxins in the UK (Opoku *et al.*, 2013). Although isolated from different cereals, oat is reported to be the preferred host for *F. langsethiae* and it accumulates more HT2+T2 than other cereal species per unit of fungal biomass (Opoku, 2012).

One major difficulty in studying *F. langsethiae* is the inability to successfully artificially inoculate with this fungus. Divon *et al.* (2012) reported that spray inoculation of oat panicles at early dough stage gave the most visible symptoms in comparison to inoculation at other growth stages. Although symptoms were not easily distinguishable, this is the first report of successful inoculation of oat panicles with *F. langsethiae* in controlled conditions. Up to date there are no reports of successful inoculation in field conditions. Another major issue in studying *F. langsethiae* is the lack of disease symptoms in the field or on harvested grain (Opoku *et al.*, 2013).

As screening for *F. langsethiae* resistance of oats in field and glasshouse is currently not possible due to symptomless infection and no reliable method of artificial inoculation, *in vitro* leaf assays are suggested to be of potential use for

quicker and easier pre-screening. In the study comparing wounded and unwounded leaves of different cereals species, it was shown that different oat cultivars can develop lesions of different sizes when a conidial suspension of *F. langsethiae* was positioned on detached leaves (Imathiu *et al.*, 2008). This might be correlated to the field resistance of different cultivars. By using *in vitro* detached leaves assay, it was shown that *F. langsethiae* is pathogenic to wounded leaves of several cereals but only to unwounded leaves of oats, identifying it as a more vulnerable host (Imathiu *et al.*, 2008). Using the same assay, it was identified that oats are the most susceptible cereal species to the infection of *F. langsethiae* although this study included only one variety of each cereal tested (Opoku *et al.*, 2011).

Fusarium langsethiae is observed to be one of the earliest detected *Fusarium* species after panicles emergence (Parikka *et al.*, 2007). The high quantities can be detected as early as full panicles emergence (Zadoks growth stage [GS] 59, Zadoks, Chang, & Konzak, 1974) with another peak at harvest ripe (GS 92) (Opoku *et al.*, 2013). This indicates that infection of cereals with *F. langsethiae* might occur before flowering. Not being able to detect *F. langsethiae* in the roots and seedlings, suggested that it is not a seedling pathogen (Opoku *et al.*, 2013). It has been reported that *F. langsethiae* exhibits strong affinity towards panicles (Divon *et al.*, 2012). It may be the same case as reported for *F. graminearum*, where the main point of infection is the floret mouth (Tekle *et al.*, 2012a). Lack of symptoms in the field, raises the question whether *Fusarium langsethiae* should be considered as an endophyte. Divon *et al.* (2012) suggested that it should rather be considered as a weak pathogen with a slow growth as they could not detect any seed to head transmission that is a characteristic of endophytes. However, neither is there much evidence that *F. langsethiae* is pathogenic to its

hosts. No symptoms were observed in the field (Opoku *et al.*, 2013) and no impact on yield has been identified (Edwards, 2012a).

For the quantification of trichothecene A producing *Fusarium* species, most researchers rely on quantification of the mycotoxins, HT2+T2 and/or quantification of fungal biomass as measured by DNA using quantitative PCR. Chromatographic analyses are thought to be the most precise for the analyses of mycotoxins. Recent studies comparing chromatographic analyses with immunoassays based on antibodies found a good correlation (Edwards *et al.*, 2012). The *Tri5* gene codes for trichodiene synthase and is preserved and common to all trichothecene producing *Fusarium* species and as a result this can be used for distinguishing those species from non-trichothecene producers (Edwards *et al.*, 2001). Sequencing a fragment from the *Tri5* gene and aligning sequences showed similarity of 98.7% between *F. langsethiae* and *F. sporotrichioides* and the two species could not be distinguished phylogenetically. A *Tri5* gene specific forward primer combined with a taxon-specific reverse primer for *Fusarium langsethiae* amplified *F. sporotrichioides* as well (Niessen *et al.*, 2004). In agreement with these results, Wilson *et al*. (2004) also could not distinguish between *F. langsethiae* and *F. sporotrichioides* when using the *Tri5* region. However, using RAPD (Random Amplification of Polymorphic DNA) they were able to find an amplicon common to *F. sporotrichioides* isolates and not found in *F. langsethiae* isolates. Based on the polymorphism between the two species identified in this amplicon, species-specific forward primers for *F. langsethiae* and *F. sporotrichioides* and one common reverse primer for both species were designed (Wilson *et al.*, 2004). Using the *F. langsethiae* specific primers designed by Wilson *et al*. (FlanF3 and LanspoR1), a real time PCR assay was designed with SYBR Green (Edwards *et al.*, 2012). Similar but slightly less sensitive assays with

SYBR Green and Taqman methods using the same primers were developed by Fredlund *et al.* (2010). Polymorphic regions of the *EF-1α* (elongation factor 1α) gene can be used for species identification within *Fusarium* genus; another set of primers was designed and quantitative PCR assay developed for *F. langsethiae* using this region (Knutsen *et al.*, 2004; Nicolaisen *et al.*, 2009).

Studies on the relationship between the level of trichothecene mycotoxins and fungal biomass of producers in harvested grain have shown positive correlations. An increase in DON content on inoculated wheat seedlings with *F. culmorum* was correlated with *Tri5* expression but it was not correlated to fungal biomass (Doohan *et al.*, 1999). However, a good correlation between DON content and biomass of its *Fusarium* producers is seen where there was no correlation between the former and visual assessment of *Fusarium* head blight symptoms (Edwards *et al.*, 2001; Nicolaisen *et al.*, 2009). Similar to the previous studies, a correlation between *F. langsethiae* biomass and the level of HT2+T2 was observed in oat samples (Fredlund *et al.*, 2010; Opoku, 2012; Edwards *et al.*, 2012). Besides quantifying fungal DNA, quantification of ergosterol has been used for estimation of fungal biomass. A good correlation between DON and ergosterol was found (Miedaner *et al.*, 2002). The limitation of using ergosterol is that it is a component of every fungal cell wall so it quantifies all fungal species present. However, it can be used in experiments with controlled conditions and artificial inoculation.

Few *Fusarium* species have been sequenced such as plant pathogenic *F. graminearum* (Cuomo *et al.*, 2007) and *F. avenaceum* (Lysøe *et al.*, 2014). Recently, a draft genome of *F. langsethiae* was published (Lysøe *et al.*, 2016) which will make research focusing on genes involved in pathogenicity easier,

although it is hard to determine if a gene is important for pathogenicity or for the normal cell functioning of a pathogen (Trail, 2009).

1.1.3. Mycotoxins

Fungal species can infect cereals before or after harvest resulting in the contamination of harvested grains with toxic chemicals known as mycotoxins. They are toxic to both humans and animals and several countries have set legislative limits for a number of mycotoxins (Graham *et al.*, 2008). A range of mycotoxins (Table 1.1) can be produced by FHB species as reviewed by Bottalico & Perrone (2002).

Table 1.1 Fusarium mycotoxins isolated from wheat in Europe (source: Bottalico & Perrone, 2002).

Species	Geographical incidence		Mycotoxin
	North/Centre	South	
F. graminearum	+++	+++	**DON**, **NIV**, **ZEN**, AcDON, FUS
F. avenaceum	+++	++	**MON**, BEA, ENS
F. culmorum	+++	++	**DON**, **ZEN**, ZOH,NIV
F. poae	++	+	**NIV**, **BEA**, DAS, FUS, ENS
F. equiseti	++	+	**DAS**, ZEN, ZOH
F. tricinctum	+	+	**MON**
F. cerealis	+	±	**NIV**, **FUS**, **ZEN**, ZOH
F. sporotrichioides	+	±	**T2**, **HT2**, **T2ol**, NEO
F. acuminatum	±	±	**T2**, NEO
F. subglutinans	±	—	**MON**
F. solani	±	—	—
F. oxysporum	±	—	—

AcDON = Monoacetyl-deoxynivalenols (3-AcDON, 15-AcDON); BEA = Beauvericin; DAS = Diacetoxyscirpenol; DON = Deoxynivalenol (Vomitoxin); ENS = Enniatins; FUS = Fusarenone-X (4-Acetyl-NIV); HT2 = HT-2 toxin; MON = Moniliformin; NEO = Neosolaniol; NIV = Nivalenol; T2 = T-2 toxin; T2ol = T-2 tetraol; ZEN = Zearalenone; ZOH = zearalenols (α and β isomers).

Fungal species producing mycotoxins have evolved mechanisms of protection so that the toxins they produce are not harmful to them (Kimura *et al.*, 1998; Alexander *et al.*, 1999). Lemmens *et al.* (2005) reported that the major QTL for FHB resistance in wheat, Qfhs.ndsu-3BS, either encodes or regulate the expression of DON-glucosyltransferase that detoxifies DON. Similarly, Schweiger *et al.* (2010) reported that barley contains genes that can detoxify DON.

1.1.3.1. Trichothecenes

Fusarium species can infect cereal crops resulting in a disease, fusarium head blight (FHB) and the contamination of cereal grains with fusarium mycotoxins among which are several trichothecenes. Trichothecenes are divided into two groups (type A and type B) based on their chemical structure. The type A trichothecenes include the closely related mycotoxins, HT2 and T2. These mycotoxins have been quantified at high levels in UK oat grains at harvest (Edwards, 2009). These toxins are produced primarily by *F. langsethiae* in UK oats (Imathiu, 2008). Type B trichothecenes include DON and NIV (nivalenol) produced by *F. graminearum* and *F. culmorum* (Kimura *et al.*, 2001) and are commonly detected in wheat.

The first trichothecene was isolated from *Trichothecium roseum* in 1948 by Freeman & Morrison (1948) and the first *Fusarium* trichothecene was isolated from *F. scirpi* in 1961 (Desjardins, 2006). Trichothecenes inhibit protein synthesis in eukaryotes (Kimura *et al.*, 2001). These toxins are associated with fusarium diseases in cereals and are problematic especially when the harvest is delayed. It is considered that grain moisture at about 20-25% and a wide range of temperatures from 0°C to 30°C are favourable for toxin production. As mycotoxin

formation occurs in the field they are not consider as a problem at storage. If produced, trichothecenes can be found in all by-products of a contaminated grain and are heat stable so cannot be destroyed by cooking nor baking (Bockus *et al.*, 2010; Schwake-Anduschus *et al.*, 2010).

Farnesyl pyrophosphate to trichodiene is the start of the trichothecenes pathway in *Fusarium* species (Figure 1.11). A series of oxygenations and isomerizations occur and the final product will depend on the genetic background (e.g. *Tri* genes) of the individual *Fusarium* species and isolates that have different chemotypes. The reactions need to happen in a certain order and it seems that for T-2 production around 15 reactions are needed and that the*Tri7* and *Tri8* genes are not necessary for the production of DON but needed for the production of T-2 toxin (Kimura *et al.*, 2001; Brown *et al.*, 2001).

Kang & Buchenauer (1999) showed that *Fusarium* toxins DON and 3-ADON and 15-ADON are associated with pathogenic changes in the host tissue. They concluded that as water soluble substances, these mycotoxins might diffuse into the host tissues from the surface before the pathogen invasion. Their work suggested that toxins alter the cell membrane permeability causing electrolyte losses from the host tissues. Occupation of the rachis and subsequent production of the toxins by the pathogen might block the water and nutrient stream thus causing the white head symptom above the point of infection (Kang & Buchenauer, 1999).

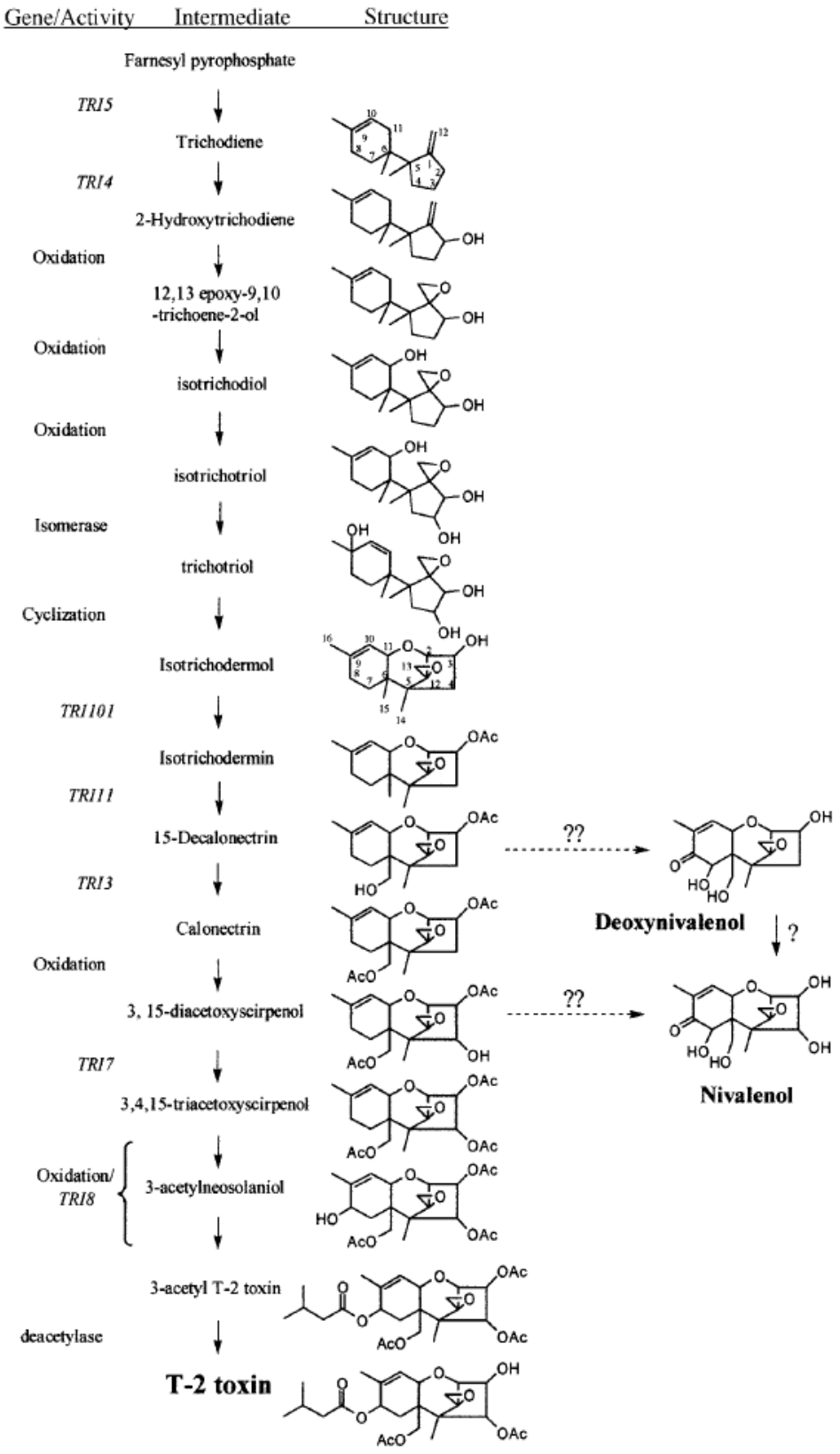

Figure 1.11 Pathway for trichothecene biosynthesis in *Fusarium* species (source: Brown *et al.,* 2001).

Testing *Tri5,* toxin non-producing mutants in the field, Desjardins *et al.* (1996) concluded that DON is a virulence factor contributing to FHB. Although DON is a phytotoxin and virulence factor it is not essential for the infection (McCormick, 2003). The complexity of trichothecene influence on *F. graminearum* virulence is shown in experiments of Maier *et al.* (2006) where non-toxin producing strains caused symptoms without spreading but the level of disease varied depending on pathogen strain (DON or NIV producing) and on the host plant. In the same study Maier *et al.* (2006) reported NIV as a virulence factor on maize. Bai *et al.* (2002) reported that DON is important for FHB spreading within a spike but it is not essential for the initial infection. It has been shown that DON has a role in the aggressiveness of its producer and colonization of the host (Eudes *et al.*, 2001; Mudge *et al.*, 2006) whereas no such information is available on the role of HT2+T2 toxins.

It is reported that DON does not inhibit germination of infected oat seeds but does affect seedling development (Tekle *et al.*, 2012b). It is not known what the effect of HT2+T2 on oat seedlings is. Further investigation of the role of HT2+T2 in aggressiveness and pathogenicity is needed.

Investigating the T2 influence on *Arabidopsis thaliana*, Nishiuchi *et al.* (2006) observed that T2 induced necrotic lesions on leaves. Cell death, accumulation of salicylic acid and expression of Arabidopsis defence genes were noted. On the contrary, DON did not induce elicitor like pathways. The influence on T2 was confirmed in a study by Masuda *et al.* (2007) where T2 toxin was infiltrated into the seedlings and acted as an elicitor, causing a defence response. Moreover, seedlings treated with T2 toxin showed signs of dwarfism by petiole shortening

and reduction in cell size. T2 might be connected with the development of lesions observed in detached leaf experiments by Imathiu *et al.* (2008). It would be useful to develop attached leaf assays as plant responses in detached assays may vary from the response when testing whole seedlings.

Within cereal grain samples, HT2 and T2 demonstrate mutual exclusion with DON and NIV (Edwards, 2007a, 2009; Opoku, 2012). The former two toxins are produced by different *Fusarium* species and exclusion might be a consequence of competition between different species of the same genus or due to different environmental niches required for fungal development.

1.1.3.2. HT2 and T2 mycotoxins

The T-2 toxin has historic implications as a possible cause of Alimentary Toxic Aleukia (ATA). Between 1941 and 1947 in Russia, there was a number of cases with high human mortality. It was suggested that fatal outcomes were due to eating wheat and barley left in the field during winter and harvested late in the spring. *Fusarium poae* and *F. sporotrichioides* were isolated from grain associated with ATA. Later the T2 toxin was characterized and it was found to produce symptoms similar to ATA in animal studies. It has been speculated that T2 contaminated grains caused the disease. As analysis of T2 were not done on original grain samples that caused ATA, the final proof is still missing (Desjardins, 2006). In 1981 T2 toxins were implicated in the controversy about 'the yellow rain' in northern Laos. Traces of toxin were found in blood and urine samples of some victims who fell ill after the 'yellow rain fell from sky'. USA accused Russia of biological warfare as species producing T2 toxin were not known to naturally occur in Southeast Asia. This was later weakened as trichothecenes were found in blood

samples of Thai people not exposed to yellow rain. But it should be taken into account that sampling after the yellow rain was difficult and performed only on smuggled samples by refugees (Desjardins, 2006).

In 2011, EFSA Panel on Contaminants in Food Chain issued a scientific opinion on the risk for animal and public health related to the presence of HT2 and T2 mycotoxins in food and feed. The highest concentration of HT2+T2 was detected in oats and oat products. The chronic dietary exposure across the population was the highest in toddlers but overall, based on data collected, there were no health concerns. They noted that the available information about the HT2+T2 toxicokinetics was sparse and HT2 was identified as a main metabolite of T2 toxin (Contam-EFSA, 2011). The European Commission is currently considering legislative limits for HT2+T2 in cereals and cereal products for human consumption and published an indicative limit of 1000 μg kg^{-1} HT2 and T2 combined (HT2+T2) in unprocessed oats (European Commission, 2013a). In previous studies across the UK, between 2002-2005, around 20% of harvested oat samples exceeded 1000 μg kg^{-1} HT2+T2 over the duration of this study and in 2005 around 30% of samples exceeded this level (Edwards, 2007a).

Mycotoxin legislation requires growers to use “Good Agricultural Practice” to minimise mycotoxins within farm produce and for food processors to use “Good Processing Practice” to minimise mycotoxins in finished products (Edwards et al., 2009; Scudamore et al., 2007). Previous studies have identified that there is little growers can do to minimise fusarium mycotoxins in cereals. For oats, varietal choice was one factor growers could use to reduce fusarium mycotoxins. Analysis of UK oat varieties at harvest from Agricultural and Horticultural Development Board (AHDB; previously Home-Grown Cereals Authority, HGCA) Recommended

List variety trials has identified differences in the susceptibility of oats to *Fusarium* infection. Spring oats have consistently low HT2+T2, with little differences between varieties while winter oats have a higher concentration of HT2+T2, and a greater concentration range between varieties (Edwards, 2015) . It is not known if this difference is due to genetics or differences in the agronomy of winter and spring sown varieties.

Conventional oat varieties are husked and the husk is removed during processing for human consumption. Naked oat varieties have a loose husk, which is removed during harvesting. The de-husked oat is called a groat and is further processed to produce a range of oat products e.g. oat flakes. Studies have shown that the majority of the mycotoxins are present in the husk (Scudamore *et al.*, 2007). Naked oat varieties tend to have a lower level of HT2+T2 compared to conventional husked varieties at harvest but that might be because their hull is removed during harvesting. De-hulling appears to reduce the level of HT2+T2 by around 90% (Edwards, 2007a; Scudamore *et al.*, 2007). It is not known how the mycotoxin level of naked varieties compares to conventional husked varieties before harvest.

Mycotoxin production of *F. langsethiae* can be expected in humid and cold conditions at around 15°C (Kokkonen *et al.*, 2010). Medina & Magan (2011) identified warmer optimal conditions for the production of HT2 and T2 at 20-30°C. The differences in these studies might be due to the different media used. Kokkonen *et al.* (2010) used a combination of oat, wheat and barley grains whereas Medina & Magan (2011) used only oat-based medium. The importance of different substrates in toxin production was documented earlier for *F. graminearum*

(Gardiner *et al.*, 2009). More work is required to clarify conditions conducive to mycotoxin production.

It is not known if the conditions for sporulation and mycotoxin production are the same but it can be speculated that humidity and temperature are equally important, as shown for other *Fusarium* species (Doohan *et al.*, 2003). Weather is of particular importance to spore production, dispersal and host infection with *Fusarium* species (Xu, 2003). Xu & Nicholson (2009) concluded that understanding FHB community structure is essential as relationships between different species involved in the complex is somewhat competitive, thus of importance when deciding on disease management.

1.1.4. Agronomic factors involved in mycotoxin risk

Much of the previous research on the impact of agronomy in *Fusarium*-cereal interactions was done on wheat. Those previous studies identified several factors that influence *Fusarium* contamination of wheat. Previous crop, cultivation practice and choice of varieties were identified as major agronomic factors contributing in *Fusarium*-wheat interactions. Early studies conducted in Germany identified five risk factors for DON contamination: a) growing wheat after maize, b) minimum tillage after maize, c) sowing of susceptible wheat variety, d) application of fungicides based on strobilurins, e) warm and wet period around flowering of wheat (Obst *et al.*, 2000).

Year, region, cultivation practice, previous crop and rotation were factors identified as important in reducing the risk of *F. langsethiae* (Edwards, 2007a). As oats are

closely related to wheat, it has some common diseases as wheat which makes crop rotation an important part of a good agricultural practice (Bjørnstad, 2012).

A model predicting occurrence of DON in durum wheat identified previous crop (such as maize or sorghum) and tillage practice (such as no-ploughing) as the most important factors, together with wet weather conditions during flowering. In this model, 40% of variance was unexplained. Some of the unexplained variance may be due to the weather conditions after flowering (Gourdain *et al.*, 2011).

In an observational study between 2002-2005 in the UK analysis showed that there was a significant interaction between year and region when modelling HT2+T2 mycotoxin concentration. This might be explained by weather differences except temperature differences, as there was not a trend from North to South (Edwards, 2007a). It was concluded that the highest concentration of HT2+T2 (30% of samples in 2005 above 1000 μg kg^{-1}) was during dry summers (Edwards, 2007a; van der Fels-Klerx & Stratakou, 2010) which is opposite to what is known about DON producing *Fusarium* species which are most commonly noted during wet conditions (Parry *et al.*, 1995; Matthäus *et al.*, 2004). In a later study by Edwards (2012b) the negative relationship between summer rainfall and the level of HT2+T2 mycotoxins was confirmed as significant ($p=0.008$, accounting for 74% of the observed variance).

There are contrasting reports on the effect of fungicides for control of trichothecene-producing *Fusarium* species. Reduction in FHB may not necessarily reduce the level of trichothecenes (Edwards, 2004). Conflicting results might be due to differences in infection time of different *Fusarium* species involved in FHB so what is assumed as a timely application of fungicides for one species, may not

necessarily mean that it is the correct timing for another species involved in the FHB complex. Another possibility is the variable efficacy of applied fungicides towards different species present within the disease complex.

Most of the studies comparing mycotoxin level are based on conventional agronomy. In one study comparing the level of HT2 and T2 in organic and conventional oat production, the levels detected in the former were five times lower, that being the largest quantified difference reported for any mycotoxin between organic and conventional production (Edwards, 2009). This is assumed to be due to differences in the agronomy employed in the two systems such as rotation or choice of varieties. However, there are few reports on HT2 and T2 levels in organic cereal production. Seed rate and nitrogen application was not found to be of significant importance (Edwards 2007, 2009; Edwards & Anderson, 2011).

For reduced risk of *Fusarium* infection, at least a one year break from another cereal crop is recommended (Bockus *et al.*, 2010). In observational studies in the UK, there was a lower level of HT2+T2 following a non-cereal suggesting that rotation is important. As seen for other *Fusarium* species, ploughing decreased the level of HT2+T2, highlighting the importance of crop debris (Edwards 2007, 2009). In a study by Edwards (2012b) the highest level of HT2+T2 was when oats were sown after other cereals (wheat, barley or oats) and after minimum tillage. There was an exception to this observation when oats were sown after oats with ploughing. Another exception was with oats after a non-cereal in the previous year and a cereal the year before with ploughing in which case as well the level of HT2+T2 was high . This might be due to ploughing that brought up the buried debris from two years ago (Edwards, 2012b). Cereal intensity as defined as a

number of cereal crops in the last four years and cereal sequence defined as a number of last four years in a continuous cereal production were significant ($p<0.001$) and accounted for 6.4% and 4.9% of variance respectively. The higher cereal intensity or sequence, it was resulting in the higher level of HT2+T2 mycotoxins (Edwards, 2012b).

The positive impact on soil conservation and economic cost has resulted in the promotion of no-till or direct drilling. Despite its other benefits, direct drilling can increase levels of certain mycotoxins in the following crop as many of the trichothecene producing *Fusarium* spp. survive saprophytically on crop debris (Parry *et al.*, 1995). Ploughing is advisable as part of management against FHB (Edwards, 2007a) however, this conflicts with other advice regarding soil and water conservation.

If growing wheat after maize, no-till can increase DON content by 1-3 ppm (Dill-Macky & Jones, 2000). In another study, contrasting but variable results showed that tillage might influence mycotoxin content but that warm and wet weather are probably more significant (Lori *et al.*, 2009). There are not many studies on the effect of cultivation practice with regards to the level of HT2+T2 on oats. Parikka *et al.* (2007) found that direct drilling increased *F. langsethiae* infection and consequently the level of HT2+T2.

Trials conducted in Norway suggested that fungicides currently used and active against *F. graminearum* are not effective against HT2+T2 producing *Fusarium* species such as *F. langsethiae* (Ingerd S. Hofgaard, NIBIO, Norway, personal communication).

Another group from Sweden reported that effects of fungicides such as Comet, Amistar, Tilt and Poline are low or not consistent (Pettersson *et al.*, 2008). However, our agricultural systems are becoming more and more dominated by a few plant species whose development is aided by different chemicals. Different microbial communities are an integral part of this system and they and the overall interaction of microorganisms including plant pathogens should be studied thoroughly in order to achieve the maximum plant potential (Trail, 2009).

1.1.5. Host resistance

Different physiological, morphological and developmental plant traits play a role in disease escape or prevention of fungal establishment (Walter *et al.*, 2010). Morphological also known as passive resistance can be the height of the plant, flowering time, spike density or presence of awns (Rudd *et al.*, 2001; Mesterházy, 2003; Gilsinger *et al.*, 2005). Active resistance involves physiological processes or recognition of the pathogen (Mesterházy, 1995; Hutcheson, 1998).

Mesterházy (1995) divided active (physiological) resistance into five types:
Type I – resistance to initial infection
Type II – resistance to pathogen spreading
Type III – resistance to kernel infection
Type IV – tolerance to FHB
Type V - resistance to toxins

They are no immune varieties of cereals to FHB but there is a range of varieties with different levels of resistance that has been shown to be quantitatively

inherited. A number of QTL have been reported for FHB resistance in wheat associated with resistance to fungal infection or associated with fungal spread but many of these have only a small impact on resistance on their own (Anderson *et al.*, 2001; Buerstmayr *et al.*, 2003; Steiner *et al.*, 2004; Jia *et al.*, 2005; Yang *et al.*, 2005). Most of the QTL identified were linked to Type I resistance. In conclusion, 15-60% of variation to FHB in different lines can be explained by *Fhb1* gene which was identified in Chinese Sumai-3 and made it a very popular breeding source (Lu *et al.*, 2012).

1.1.5.1. The impact of height and dwarfing genes

Dwarf varieties are short-strawed compared to conventional varieties. Introducing dwarf wheat varieties meant higher yields as lodging was reduced and plants put more energy into developing grains rather than producing tall straw (Evans, 1996). The plant hormone gibberellin (GA) has an important role during plant development in promoting stem and leaf growth among other functions. There are positive and negative regulators of GA signalling. DELLA proteins are a class of GA signal repressors. Examples of DELLA are *Rht* in wheat and *SLN1* in barley (Sun & Gubler, 2004). *Rht-B1b* and *Rht-D1b* are mutant alleles of Rht-1 loci (wild type alleles *Rht-B1a* and *Rht-D1a*), regulating height in wheat. These genes are suppressing growth but gibberellin is opposing that action (Peng *et al.*,1999).
In wild type alleles (*Rht-B1a* and *Rht-D1a*) in the presence of GA, DELLA interacts with GA receptor (GIBBERELLIN INSENSITIVE DWARF1, GID1) which leads to the degradation of DELLA proteins (Ueguchi-Tanaka *et al.*, 2007). Semi-dwarf wheat varieties are example where deletions within DELLA domain of an *Rht* gene (reduced height) resulted in GA-unresponsive, or gibberellin resistant thus dwarf phenotypes. These wheat lines are resistant to DELLA protein degradation as a

result of GA induced proteolysis (Sun & Gubler, 2004). A range of N-terminal mutations can convert *Rht-B1a* and *Rht-D1a* into mutants less affected by gibberellin thus increasing in plant gibberellin levels and causing dwarfism. Mutant variations *Rht-B1b* and *Rht-D1b* are known to produce active forms of these growth suppressors (Peng *et al.*, 1999).

Saville *et al.* (2011) investigated the role of DELLA proteins in wheat and barley through using lines with gain of function (GoF; semi-dwarf *Rht-B1b*, *Rht-D1b* and severe dwarf *Rht-B1c* and *Rht-D1c*) and loss of function (LoF) lines. GoF mutant alleles showed increased susceptibility to biotrophic pathogens while increasing resistance to necrotrophic pathogens. The authors proposed that DELLA has a part in regulating cell death. Similarly, Navarro *et al.* (2008) demonstrated working with Arabidopsis model species that DELLA promotes susceptibility to biotrophs and resistance to necrotrophs by altering salicylic acid (SA) and jasmonic acid (JA) balance. Accumulation of DELLA results in higher JA and lower SA signalling and cell death in reaction to introducing pathogens. Saville *et al.* (2011) suggested that the lines carrying semi-dwarf *Rht-B1b* and severe dwarf *Rht-B1c alleles* and thus accumulating DELLA have increased susceptibility to initial infection as a consequence of GoF. Due to the DELLA influence on plant height, there was higher resistance to fungal spread (Type II resistance) and cell death compared with the wild type (Saville *et al.*, 2011). This might be a reflection of two different phases of *F. graminearum* infection which starts with a biotrophic phase followed by necrotrophic phase (Brown *et al.*, 2010). Overall GoF DELLA lines might be more resistant as they limit cell death (Saville *et al.*, 2011) .

Graham & Browne (2009) observed that the plant height of glasshouse grown wheat plants were negatively correlated with FHB ($r = -0.22$, $p < 0.05$) but the

repeated experiment was not significant. Repeating the experiment in the field, significant correlation was reached ($p < 0.001$). Klahr *et al.* (2007) found a correlation between FHB and days to heading ($r = 0.23$; $p = 0.01$) and plant height ($r = -0.42$; $p = 0.01$) after conducting experiments at three consecutive years at one location and having an additional experiment in the following year at another location. Hilton *et al.* (1999) also observed a negative relationship between height and FHB ($p < 0.001$) where taller plants had lesser infection but at around 90-95 cm of height the FHB severity varied between 25-65% in different lines. Within one population tested there was a clear indication of taller plants to be less diseased but that was not apparent with the tall lines of the second population included in the study. The authors suggested two possible explanations; one being linkage and another explanation being pleiotropy (when one gene influences two or more seemingly unrelated phenotypic traits) and not plant height per se.

Draeger *et al.* (2007) found a QTL conferring FHB resistance on wheat chromosome 4D to co-localise with the *Rht-D1* height locus, accounting for 24% phenotypic variance for FHB. It appears that FHB susceptibility genes are linked to *Rht-D1b* semi-dwarfing allele (formerly called *Rht2*) and that higher infection rate of dwarf varieties is due to gene linkage rather than differences in height. Similarly Srinivasachary *et al.* (2009) found a major QTL (Qfhs.jic-4D) that is also in the region of the *Rht-D1* locus. This finding supports the view that there might be the pleiotropic effect on susceptibility to FHB. While Hilton *et al.* (1999) found that there was no difference in FHB infection observed between lines with either *Rht1* (*Rht-B1b*) or *Rht2* (*Rht-D1b*) this is the opposite to Srinivasachary *et al.* (2009) who observed that although FHB QTL was linked to the *Rht-D1* locus but not to the *Rht-B1b* allele that had no detrimental influence on the FHB resistance. The resistance to initial infection (Type I) in lines with either alleles was reduced under

high disease pressure but the resistance to the fungal spread (Type II) increased in lines with the *Rht-B1b* allele while *Rht-D1b* did not have an effect (Srinivasachary *et al.*, 2009). This would encourage the use of semi-dwarfing *Rht-B1b* allele for achieving desired wheat height without trading off the resistance to FHB.

In support of the view that the differences in FHB infection among wheat lines of different height are due to height is work by Yan *et al.* (2011). Initial infection with *F. graminearum* (Type I resistance) was significantly less severe in eight of ten tall lines ($p<0.01$) but there was no difference when dwarf lines were physically raised to the height of tall ones. The only exception that was still significant was the double dwarf line with *Rht8* and *Rht9*. The authors suggested that one possibility aiding resistance is microclimate of tall plants although they did not measure it. This was not the outcome of experiments done by Hilton *et al.* (1999) where microclimate (i.e. relative humidity) was ruled out based on the results from humidity sensors placed at the ear height at two ends of a plot. Yan *et al.* (2011) found support in a publication by Ellis *et al.* (2005) that showed *Rht* genes are found in different genomic regions and thus this can be an indication that there is no linkage but direct or indirect effects of plant height.

Miedaner & Voss (2008) tested wheat NIL (near isogenic lines) carrying different sets of *Rht* genes inoculated with *F. culmorum*. Although in dwarf lines carrying *Rht-B1b* or *Rht8c* an increase in FHB infection was seen (in comparison with *rht* wild type, $p<0.05$), a Tukey's test could not distinguish between lines carrying *Rht-B1b* or *Rht8c.* Authors pointed out that *Rht8c* from chromosome 2D is closely linked with the photoperiod insensitive *Ppd1* allele that affected heading by shortening of the cereal lifecycle and earlier flowering and might be affecting other

characteristics as well. It is worth further exploiting *Rht8c* if it can be separated from *Ppd1* which could allow less susceptible but high yielding wheat lines for the climate of Northern Europe to be selected.

In studies by Zhu *et al.* (1999) on barley, four QTL for FHB resistance corresponded with significant QTL for plant height and a few other phenotypic characters such as seeds per inflorescence and lateral floret size were identified. The authors concluded that FHB is associated with plant height and the coincidence QTL could be due to linkage or pleiotropy.

Successes with dwarfing genes in wheat and high yielding varieties encouraged research with dwarfism in oats. Contrary to wheat dwarfing genes, oat-dwarfing genes are GA-sensitive and dominant. Out of eight identified dwarf genes in oats only three are in use and out of these only one is in use at IBERS, the main public good breeding institution in the UK. Included in the AHDB Recommended List of varieties (2003), Buffalo was the first variety with the *Dw6* dwarfing gene. Besides its effect on height of the plant, oat dwarfing genes affect the length of the internode which can have a negative effect on panicles not fully emerging resulting in some sterile spikelets thus reducing the yield. These obstacles are still to be overcome and some promising results were seen in Balado, a dwarf variety on the AHDB Recommended List since 2010 (Marshall *et al.*, 2013).

The mycotoxin concentration of dwarf oat varieties tends to be higher than conventional varieties although the relationship between plant height and mycotoxin levels is not consistent (Edwards, 2015). Differences may be due directly to the morphological trait of crop height, or associated traits such as panicle length or maybe due to genetic linkage. There are no other studies

published on the influence on height to FHB in oats but a wealth of literature on wheat is divided whether the observed more severe FHB on dwarf varieties is due to height per se or due to genetic linkage (Hilton *et al.*, 1999; Draeger *et al.*, 2007; Srinivasachary *et al.*, 2009; Saville *et al.*, 2011; Yan *et al.*, 2011; Lu *et al.*, 2012).

Recently, He *et al.* (2013) published a list of QTL found in two spring oat populations. A major QTL for DON mycotoxin was identified in three consecutive years on a linkage group 17A/7C. Beside that QTL, a number of QTL with a smaller effect were detected and many of these co-localised with plant height and days to heading and maturity. This was a single study identifying QTLs for FHB on oats which was conducted in Norway; plots were spawn inoculated with *F. graminearum* as this is the main *Fusarium s*pecies in Norway.

1.1.5.2. Flowering and anther extrusion

One of the proposed traits aiding resistance is anther extrusion with higher anther extrusion resulting in lower infection with *Fusarium* species. Anther extrusion has been proposed as an avoidance mechanism. It has been suggested that wide flower opening and prolonged flowering might be a trap for *Fusarium* spores and in that way it can enhance infection (Skinnes *et al.*, 2010) In *Gramineae,* anther extrusion is explained as an elongation of anther filaments due to separation of lemma and palea pushed by swelling lodicule. Extension of anther filaments is a result of inflow of water as a response to increased osmotic pressure due to accumulation of potassium ions (Heslop-Harrison & Heslop-Harrison, 1996). It was proved that auxin and jasmonic acid play a role in anther dehiscence and flower opening in Arabidopsis lines. Anther dehiscence1 gene (*dad1*) encodes for the biosynthesis of jasmonic acid which promotes water transport leading to flower opening and anther extrusion (Ishiguro *et al.*, 2001). De Vries (1971) reviewed

literature on flowering of wheat and concluded that flowering duration is normally around one hour and weather dependent, with low temperatures and humidity promoting anther extrusion without specifying the range.

In 1971, Strange & Smith (1971) observed that within 48 h of inoculation with *F. graminearum*, extruded wheat anthers were heavily infected. They found that the supernatant of homogenized anthers showed strong stimulation of *Fusarium* growth. This subsequently led to discovery of choline and betaine as *Fusarium* growth stimulants (Strange *et al.*, 1974). Pearce *et al.* (1976) showed that glycine, betaine and choline are present in highest amounts in anthers. However, in another study, Engle *et al.* (2004) evaluated nine wheat genotypes and three *F. graminearum* isolates and found no significant relationship between *Fusarium* infection of wheat and betaine or choline concentration within anthers (Engle *et al.*, 2004). Describing the infection process of *F. culmorum* on wheat, Kang & Buchenauer (2000) found that anthers are not important for the infection but could promote the hyphal growth and consequently the disease severity. They noted that the penetration happens with the direct penetration of the epidermis by the infection hyphae and occasionally through the stomatal opening.

Anther extrusion has been shown to be highly heritable (H^2 = 0.91) in wheat with minimal genotype x environment interaction and negatively correlated with FHB (r = -0.53 to -0.69, p = 0.0001) and DON (r = -0.39 to -0.46, p = 0.0001) (Skinnes *et al.*, 2010). The relationship was described as ‘fan shaped’ where genotypes with higher anther extrusion were showing less of FHB infection and DON contamination whereas genotypes with lower anther extrusion gave a broad range of FHB and DON which would indicate involvement of several genes with small to moderate individual influences. On chromosome 1BL a QTL for both anther

extrusion and FHB was found at an overlapping location, which could indicate tight linkage or pleiotropy.

Graham & Browne (2009) distinguished between anther retention where anthers are held within the spikelets and partially extruded anthers (in their work referred to as trapped anthers between lemma and palea). They found a correlation between anther retention and FHB. In two experimental years they found the higher the anther retention (e.g. anthers held within the spikelets) the higher the FHB infection was. Kubo *et al.* (2012) reported that minor differences in wheat anther extrusion played a role in FHB escape where closed flowering wheat phenotypes and those with rapid ejection contributed to the enhanced avoidance. Partially extruded anthers were the most susceptible to FHB infection. Similar work was done on barley by Yoshida *et al.* (2007) studying open and closed flowering barley. Cleistogamus (closed flowering) cultivars showed greater resistance to FHB infection at anthesis but they were more susceptible 10 days after anthesis where chasmogamous (open flowering) cultivars were susceptible at both stages. Cleistogamous varieties also showed greater accumulation of DON and NIV when inoculated with *F. graminearum* after anthesis while DON and NIV concentration at chasmogamous cultivars was higher as a result of inoculation at anthesis. It was observed that in cleistogamous varieties that have closed flowering, anthers are not extruded during anthesis but after, when the developing grain pushes them between the tip of palea and lemma, between 7 and 10 days after anthesis. Previously Yoshida *et al.* (2005) reported that most two-rowed Japanese barley varieties are cleistogamous whereas most six-rowed varieties are chasmogamous . Chasmogamy contributed to FHB infection probably due to opening at anthesis and revealing anthers that would provide the pathogen with more accessible entering point for colonization. Another explanation might be that

six-rowed spikes possess three fertile spikelets at each rachis node while two-rowed spikes possess only one. Thus, six-row spikelets may retain more water which might induce more favourable conditions for the pathogen (Steffenson *et al.*, 2003). On wheat Kubo *et al.* (2010) experimentally confirmed that cleistogamous varieties have a lower risk of infection with FHB than chasmogamous varieties but did not find significant differences in DON accumulation.

Gilsinger *et al.* (2005) found that FHB incidence was correlated to mean flower opening width ($r = 0.61$, $p < 0.0001$). There was also a correlation between flower opening duration with flower opening width ($r = 0.863$, $p< 0.0001$). Results suggested this is a complex trait controlled by two or more genes as three markers associated with low FHB and narrow flower opening ($p<0.05$) were located at different regions of the genome.

As plant height is another mechanism of avoidance proposed, Lu *et al.* (2012) investigated the association of anther extrusion and plant height with FHB in bread wheat and determined that the resistance was Type I (resistance to initial infection). On the 4BS chromosome and in close proximity to the *Rht-B1* locus they found a major QTL to control anther extrusion and plant height. A correlation between anther extrusion and plant height was found ($r = 0.43$) but anther extrusion and plant height are under independent genetic control and combining the two is possible. FHB was negatively correlated with anther extrusion ($r = -0.45$ to -0.64) and plant height ($r = 0.37$ to -0.53). The authors pointed out that in some wheat lines considered to be resistant (such as SHA3/CBRD) most of the QTL for anther extrusion coincided with those for severity reduction and it is not clear whether reduction in FHB infection is due to avoidance or to active mechanisms of resistance.

It is unknown whether wide flower opening and duration of flowering results in a greater deposit of *Fusarium* spores (Gilsinger *et al.*, 2005) or certain nutrients increase the germination and growth of *Fusarium* species (Strange & Smith, 1971; Strange *et al.*, 1974) or the dead tissue itself is more easily colonized by *Fusarium* species (Skinnes, 2010). If anthers are trapped in florets, this can make a food substrate for germinating *Fusarium* spores. If anthers are not extruded, spores need first to germinate in order to reach the food source. In the case of anthers being extruded, they are further away from floral structures thus *Fusarium* spores might need longer to reach the floral cavity (Kubo *et al.*, 2013). Gilsinger *et al.* (2005) noted that wide open flowers tend to stay open for longer hence wheat lines with shorter flowering period will have a lower risk of *Fusarium* infection by simply escaping infection by decreasing the time when *Fusarium* spores can cause an infection. Thus a narrow flower opening might be a useful trait to select cereal lines for breeding.

Whilst there is not much work done on anther extrusion in oats, there is a wealth of literature on wheat. Rajala & Peltonen-Sainio (2011) indicated that individual oat spikelets can flower within 1-2 hours but the whole panicle does not flower at the same time, oat flowering can last for a few days whereas wheat flowering is more synchronized and faster so that whole wheat ear is pollinated in 4-5 days where it takes 10-11 days for the oat panicles.

1.1.5.3. Varietal susceptibility

In the UK, wheat is assessed for FHB resistance as part of the Agricultural and Horticultural Development Board (AHDB) trials. AHDB publishes the

Recommended List where varieties are scored from 1 to 9, with 9 being the most resistant. That is done for wheat but not for oats.

Varietal susceptibility to *Fusarium* infection has been identified as very important for wheat breeding (Kolb *et al.*, 2001; Snijders, 2004). Sowing resistant varieties, choosing appropriate cultivation practice, timely fungicide treatment together with weather based risk systems can minimize risk of *Fusarium* development (Osborne & Stein, 2007). Overall, all agronomic factors and interaction between different species must be taken in to consideration when providing guidelines on Good Agricultural Practice to reduce *Fusarium* mycotoxins (Edwards, 2004).

A specific relationship ('gene for gene') between the host and the pathogen where for each gene that causes reaction in the host there is a corresponding gene in the pathogen (Flor, 1971) is not found between cereals and *Fusarium* pathogens (Trail, 2009).

Edwards (2012b) analysed 48 winter and 30 spring oat trials for the HT2+T2 content, between 2006-2011. Significant differences were detected among winter varieties but with few differences between spring varieties. The dataset was divided into two parts. In the first dataset from 2006-2008, the range of HT2+T2 for winter varieties was between 150 to 400 μg kg^{-1}(with overall mean of 271 μg kg^{-1} HT2+T2) and for spring varieties 150-250 μg kg^{-1} (with overall mean of 222 μg kg^{-1} HT2+T2). In the second dataset for 2009-2011, winter varieties ranged between 250-1200 μg kg^{-1} (with overall mean of 708 μg kg^{-1} HT2+T2) and spring varieties were below 200 μg kg^{-1} HT2+T2 (with overall mean of 91 μg kg^{-1} HT2+T2). Winter varieties with the highest level of HT2+T2 were Gerald, Brochan and Balado and at the low level were Bastion, Dalguise and Grafton which is a naked variety.

Naked spring oats were among the varieties with the lowest HT2+T2. Spring variety Firth had the highest level of HT2+T2 among spring oats although level in general were lower when compared with winter oats. The AHDB Recommended List trials were further monitored for HT2+T2 between 2012-2015. This study (Edwards, 2015) confirmed findings of previous studies (Edwards, 2007a, 2012b) that there is a narrow range of HT2+T2 levels between spring oat varieties that tend to have lower levels of HT2+T2 mycotoxins in comparison with winter varieties. On the lowest end of the range from spring varieties were Gabby, Montrose, Ascot, Husky and Lennon which is a naked variety. On the higher end of the range were Firth, Aspen and Canyon. The range of HT2+T2 was narrow, between 68-169 μg kg^{-1} HT2+T2. On the other side winter varieties had a broader range between 218-848 μg kg^{-1} HT2+T2. Maestro, Dalguise and naked oat varieties Grafton and Beacon were on the lowest end whereas Gerald and Balado were with the highest concentration of HT2+T2 of 530 μg kg^{-1} and 848 μg kg^{-1} respectively.

1.2. Hypothesis, aims and objectives of the project

The aim of this project was to understand the variation in *F. langsethiae* infection and subsequent HT2+T2 contamination in UK oat varieties.

The project aimed to understand if observed differences in HT2+T2 concentration in harvested oats between spring and winter varieties, conventional husked and naked varieties, and conventional height and dwarf varieties are due to genetic or differences in agronomy and plant morphology. Currently, no studies have been conducted to understand the resistance of oats to HT2+T2-producting *Fusarium* species. This PhD project provided several opportunities to improve existing knowledge that will extend the direction of *Fusarium*-oat research.

This project was part of a large Defra/BBSRC LINK-funded project “Harnessing new technologies for sustainable oat production and utilisation” (QUOATS) in collaboration with academic partners and industry. The QUOATS project aimed to develop oats with improved agronomic qualities and quality traits. This would have little value if efforts were not taken to incorporate breeding for *F. langsethiae* resistance as the European Commission is considering legislative limits for HT2+T2 mycotoxins in oats intended for human consumption and animal feed (European Commission, 2013a).

The industry and society should see the benefit of developing new varieties and improvement of oats which has proven health benefits (EFSA NDA Panel (EFSA Panel on Dietetic Products Nutrition and Allergies), 2010). These benefits are in having safer crops with lower levels of mycotoxins and minimising the application

of fungicides in more resistant oat crops. Home-grown varieties within the EU mycotoxin limits will help the commercial sustainability of the UK oat industry.

The hypotheses tested were:

- There is no difference in susceptibility to *Fusarium langsethiae* infection between winter and spring oat varieties.
- There is no difference in susceptibility to *Fusarium langsethiae* infection between naked (huskless) oats and conventional (husked) oat varieties.
- There is no difference in susceptibility to *Fusarium langsethiae* infection between dwarf oats and conventional oat varieties.

Chapter 2: General materials and methods

The general methods used in experiments are described in this section. Where alterations were made they are mentioned in individual experimental chapters. All chemicals were sourced from Sigma-Aldrich (UK) unless specified otherwise.

2.1. Oat plant material

Oat seeds for experiments were sourced from Dr. Sandy Cowan from the University of Aberystwyth and all seed was treated with the single purpose seed dressing, Kinto (triticonazole and prochloraz, BASF Crop protection, UK).

2.1.1. Grain harvesting and milling

All plots were harvested when the crop was fully ripe at growth stage 92 (Zadoks *et al.*, 1974) and grain sub-samples of 1 kg were kept for milling. For the site at Harper Adams, due to a high volume of chaff within grain samples, all samples were cleaned with a grain sample cleaner with a 2 mm screen (Model M, a/s Rationel Cornservice, Denmark) before further processing. Milling of grains was done with a ZM200centrifugal laboratory mill (Retsch, Leeds, England) and panicles were milled with a laboratory mill (Christy Turner Ltd, Suffolk, England). Both mills were fitted with a 1 mm screen. Resulting samples were mixed thoroughly and ca. 200 g was taken for further analyses.

2.2. *Fusarium langsethiae* isolates

To isolate *Fusarium langsethiae* from grain material, the variety Gerald harvested in 2012 at three locations in the UK (Devon, Rosemaund, location in Scotland with subsamples Glenrothes and Balgonie) was used (Table 2.1). Grains were plated on PDA plates (39 g l^{-1}, Merck, UK) amended with 130 µg ml^{-1} streptomycin sulphate. Forty seeds were plated on PDA plates with 4 seeds per plate. Plates were incubated at room temperature (approximately 20°C) for five days to promote fungal growth. Fungal cultures that resembled *F. langsethiae* according to morphological characters (Torp & Langseth, 1999; Torp & Nirenberg, 2004) were sub-cultured on fresh PDA plates for seven days at the same conditions and after that sub-cultured again to allow growth before the mycelium could be harvested for DNA extraction and species identification by PCR. Those isolates identified as *F. langsethiae* were grown on PDA plates for purification to single spore cultures and further storage.

Table 2.1 Single spore isolates of *Fusarium langsethiae* used to produce spore suspensions used in experiments

Isolate code	Source location	Oat source variety	Year of harvesting
D5	Devon	Gerald	2012
R2	Rosemaund	Gerald	2012
B1	Balgonie	Gerald	2012
G1	Glenrothes	Gerald	2012

For inoculation experiments, purified single spore cultures were used. Fourteen-day-old PDA growing *F. langsethiae* mycelium was flooded with 5 ml SDW and a small portion of mycelium was scraped with a glass rod taking care not to pull any agar beneath. The suspension was filtrated through two layers of sterile muslin cloth to remove mycelium and diluted with SDW. Diluted spore suspension was vortexed and then streaked onto Water Agar (20g agar l^{-1} deionized H_20; Merck, UK) making a 'zig-zag' motion with a sterile loop. Plates were rinsed with SDW to additionally reduce the number of spores and kept for 24-48 h at room temperature. Only the germinating, single growing spores were selected, under dissection microscope, and plated on freshly prepared PDA plates.

To store isolates that were purified as single-spore isolates and confirmed as *F. langsethiae* by species-specific PCR (as detailed below), growing mycelium on PDA plates was sub-cultured onto PDA slopes into 20 ml sterile tubes. Slopes were incubated at room temperature for five days after which they were stored at 4°C.

2.3. DNA extraction and PCR

2.3.1. DNA extraction from PDA plates

For the purpose of identifying fungal cultures, a crude DNA extraction with Chelex Carbon Buffer was used. Seven-day old mycelium growing on PDA plates was removed from the surface, avoiding agar underneath, and transferred into 2 ml SnapLock Eppendorf tubes with addition of 250 µl Chelex Carbon Buffer (2.5 g activated charcoal, 5 g Chelex made up to 50 ml with SDW). Mycelium was

crushed with a sterile micropestle and incubated on a heat block at 56°C for 20 min. Eppendorf tubes were vortexed and spun at 12 000 x g for 15 min. New tubes were prepared with 50 µl TE buffer (10 mM Tris-Cl, pH 7.5. 1 mM EDTA) and 50 µl of supernatant was transferred. Contents of the new tubes were vortexed and stored at 4°C until used for PCR.

2.3.2. DNA extraction from plant material

F. langsethiae biomass was determined by quantitative PCR (qPCR). DNA was extracted from the milled samples. A subsample of 5 g was weighed into a 50 ml tube and mixed with 30 ml CTAB buffer (46 g sorbitol, 20 g N-lauryl sarcosine, 16 g hexadecyl trimethylammonium bromide, 16 g ethylenediamine tetraacetic acid (EDTA), 20 g polyvinylpolypyrolidone (PVPP) and 175.4 g sodium chloride (NaCl) made up to 2 L with deionised water). The solution was mixed with a magnetic stirrer during preparation and dispensing.

Extraction was performed as described by Opoku (2012). Samples were shaken vigorously by hand and incubated for 1 h in a water bath at 65°C. Tubes were shaken once again and centrifuged at 3000 x g for 15 min. From centrifuged samples 100 µl of supernatant was removed and transferred into a sterile 2 ml Eppendorf tube and mixed with 300 µl potassium acetate (5M). Samples were incubated at -20 °C for 1 hour then thawed before 600 µl chloroform was added. Tubes with samples were inverted 20 times and spun at 12000 x g for 15 min. From each sample 900 µl of aqueous layer was removed and added to a new 2 ml Eppendorf tube, mixed with 800 µl isopropanol (100%) and incubated at room temperature for 30 min. After the incubation samples were spun at 12000 x g for

15 min. The formed pellet was washed twice in 44% isopropanol and spun at 12000 x g for 15 min between washings. The pellet was air dried overnight. When all isopropanol had evaporated, the pellet was re-suspended in 200 µl TE buffer at 65°C for 2 hours. Samples were vortexed after each hour and left overnight at room temperature before the total DNA concentration was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Extracted total DNA was diluted to the working concentration of 20 ng μl^{-1} prior to quantifying with qPCR. If used within a few days, extracted DNA was stored at 4°C or at -20°C for a longer storage time.

2.3.4. Conventional PCR

Prior to quantification of *F. langsethiae* biomass with qPCR, a control reaction with conventional PCR was performed. This was performed with ITS 4 (5'-TCCTCCGCTTATTGATATGC) and ITS 5 primers (5'-GGAAGTAAAAGTCGTAACAAGG) from Eurofins MWG Operon (Germany). These are universal fungal primers (White *et al.*, 1990) that will amplify fungal and plant DNA with a 50°C anneal temperature. The PCR buffer used was prepared as described earlier by Edwards *et al.* (2012). It is a ready load PCR mix containing Cresol Red and glycerol. In a total volume of 25 µl, a sample of 5 µl was added. SDW was used as negative control.

The PCR reaction was performed on a Bio-Rad T100™ Thermal Cycler (UK) and it was as follows; denaturation started at 94 °C for 1:15 min, followed by 34 cycles of 15 s at 94°C (denaturation), 15 s at 50°C (annealing), 45 s at 72°C (extension) and ending with 4:15 min at 72°C before entering storing temperature at 4°C. Gel

electrophoresis was performed on a 2% agarose gel (Electran ®, BDH, UK) stained with GelRed™ (Gel Stain 10 000 x in water, Biotium, USA) on Kodak BioMax HR 2025, PCR products (around 600-700 bp in size) were viewed with GelLogic 212 PRO (Carestream, UK).

2.4.4. Quantitative PCR (qPCR)

A CFX96™ real-time PCR thermal cycler (Bio-Rad, UK) was used for amplification and quantification of *F. langsethiae* DNA from milled oat grain samples. Real-time PCR was performed as described by Edwards *et al.* (2012). *F. langsethiae* DNA standards (10^0 – 10^{-7} ng μl^{-1}) were made in TE buffer. PCR-grade water was used as a negative control. Total volume was 25 µl where 5 µl of DNA sample was used for each reaction. Primers (conc. 500 pmol μl^{-1}) used in the reaction mix, FlangF3 (5′-CAAAGTTCAGGGCGAAAACT) and LanspoR1 (5′-TACAAGAAGACGTGGCGATAT) were reported by Wilson *et al.* (2004) to be specific for *F. langsethiae*. Primers were ordered from Eurofins MWG Operon (Germany). Besides sample template and primers, HOT FIREPol ® EvaGreen ® qPCR Mix Plus (Solis Biodyne, Estonia) master mix was used. The initial denaturation was for 2 min at 95 °C, followed by 40 cycles with 15 s at 95°C (denaturation), 10 s at 62°C (annealing), 30 s at 72°C (extension) and 10 s at 82°C (for fluorescence measurement). Melting curves were obtained by heating at 95°C for 1 min, cooling to 55°C for further minute and rising to 95°C. Ramp temp rate was 0.05°C s^{-1} with continuous measurement of fluorescence.

2.4. Mycotoxin analyses

HT2 and T2 quantification was performed using Ridascreen® T-2 ELISA assay (R-Biopharm, AG, Germany). Manufacturer's instructions were followed with minor changes. Sample size for T2 extraction was increased and the volume of methanol/deionised water corrected accordingly. Modification was as follows: 8 g of milled samples was mixed with 40 ml of 70% Methanol (Fisher Scientific, USA). Mixture was shaken for 15 minutes at full speed (300 motions min^{-1}), on a digital shaker (HS 501, JK IKA Labortechnik, Germany). ELISA plates were read immediately after the reaction was finished with a BioTek Plate reader (ELx800, BioTek Instruments Limited, USA). The sum of HT2+T2 concentration was calculated based on the known cross-reactivity (11%) of T-2 antibody with HT2 and the known ratio (1:3.125) of these two mycotoxins in UK oats as determined from a previous study in commercial grain samples (Edwards *et al.*, 2012).

2.5. Statistical analyses

Results were analysed with GenStat (Version 13, VSN International Ltd). *Fusarium langsethiae* DNA concentration and the combined HT-2 and T-2 concentration was analysed by ANOVA (Analysis of Variance) to see whether there was a significant difference between treatments. If needed, data were log_{10} transformed to normalize the distribution of residuals prior to analysis. Where significant differences were detected Tukey's test (p=0.05) was used to distinguish

differences between individual treatments. All experiments were analysed separately blocked by repetition (blocks in experiments) and where possible experiments were combined, analysed together and blocked by repetition within experiment.

Chapter 3: Evaluating resistance of UK oat varieties

3.1 Introduction

Fusarium langsethiae is the predominant *Fusarium* species found on UK oats (Edwards *et al.*, 2012). It is isolated and reported from wheat, barley and oats (Opoku, 2012) and detected in 99% of UK oat samples that were tested in a survey between 2002-2004 (Edwards *et al.*, 2012). Several toxins are produced by *F. langsethiae* but HT2 and T2 are produced in the highest amounts (Thrane *et al.*, 2004). There was a significant relationship between the level of HT2 and T2 mycotoxins and *F. langsethiae* DNA from UK cereal samples, indicating that this species is the predominant producer of these toxins in the UK (Opoku *et al.*, 2013).

EFSA Panel on Contaminants in the Food Chain (2011) concluded as part of a risk assessment that these two mycotoxins are considered to be very potent but called for more research about their toxicity and the agronomy affecting their accumulation in a susceptible plant host. The European Commission issued a recommendation for the indicative levels for the combined concentration of HT2 and T2 (HT2+T2) mycotoxins in cereals and cereal products intended for human and animal consumption (European Commission, 2013b). In the recommendation the European Commission called Member States to monitor levels of HT2+T2 mycotoxins and investigate where indicative levels are exceeded. Investigations should identify why levels of HT2+T2 are exceeded and what mitigation can be used to prevent in future high levels. If legislative limits are set this might have serious consequences for the UK oat producers and processors as results from

observational studies conducted from 2002 to 2008, showed that around 16% of UK oats exceeded the indicative level of 1000 µg kg $^{-1}$ HT2+T2 in unprocessed cereals (Edwards, 2012b).

Year, region, cultivation practice, previous crop and rotation were all factors identified as having a significant impact on HT2+T2 concentration (Edwards, 2007a). The interaction between year and region might be due to different weather conditions within years and regions. Not many studies analysed the effect of cultivation practice and its influence on the level of HT2+T2 on oats. In Finland, Parikka *et al.* (2007) found that direct drilling increased *F. langsethiae* infection and subsequently the level of HT2+T2. In the UK, a study by Edwards (2012b) identified cereal intensity as the most significant. The cereal intensity was defined as number of cereals grown in rotation, where fewer cereals in the rotation resulted in lower concentrations of HT-2 and T-2.

Lower levels of HT2+T2 were observed when oats were grown after non-cereal, which would indicate the importance of rotation. The same was observed when ploughing was employed and the level of HT2+T2 was lower than on min-till soil. This demonstrated the importance of crop debris. Factors not found to be of significance were seed rate and nitrogen application (Edwards, 2007a, 2009). It appears that fungicides (such as azoxystrobin, fluoxastrobin, prothioconazole, tebuconazole, epoxiconazole) in use for other *Fusarium* species are not effective against *F. langsethiae* (Pettersson *et al.*, 2008; Edwards & Anderson, 2011). It is also reported that the reduction in FHB after fungicide application (such as with azoxystrobin) does not necessarily result in a reduced level of trichothecenes (Edwards, 2004).

The above mentioned are all studies based on conventional agronomy. Edwards (2007) compared conventional and organic production systems and showed that five fold lower levels were detected in the latter. This is the largest difference reported for mycotoxin content on cereals between these two production systems. The difference is assumed to be due to different crop rotation employed at organic farms and due to the choice of varieties grown within the two systems.

The choice of varieties was identified as very important in reducing fusarium head blight (FHB) on wheat (Kolb *et al.*, 2001; Snijders, 2004). A research group from France working on barley suggested that varietal susceptibility is less important for the accumulation of HT2+T2 mycotoxins (Orlando *et al.*, 2010). In their 2 year study, 451 barley samples were analysed. Spring barley was four times more contaminated than barley sown in autumn and winter ($p < 0.05$). The sowing date explained around 12% of variation in HT2+T2 mycotoxin content. The conclusion of the study was that the later sowing resulted in higher HT2+T2 contamination (Orlando *et al.*, 2010). The authors reported that there was no difference between 22 varieties tested but they did not report any statistics for this conclusion. It should be noted that out of 435 samples, there were only 20 samples of winter barley included, so the dataset was highly unbalanced.

In the UK, the Agricultural and Horticultural Development Board (AHDB) publishes Recommended List of cereal varieties. With regards to disease resistance, oats are assessed for mildew and crown rust but not to FHB. Certain trends of *F. langsethiae* infection and HT2+T2 mycotoxin accumulation were observed in a few observational studies (Edwards, 2007a, 2012b, 2015) conducted so far but it is not clear what the cause of the differences observed in contamination is and as the

samples are dominated by the most popular varieties the dataset is highly unbalanced.

The aim of this study was to evaluate the susceptibility of UK oat varieties to *F. langsethiae* and determine the difference observed between winter and spring oat varieties is due to the difference in sowing date or genetics of different varieties. Twelve (six winter and six spring) oat varieties were sown in winter and in spring of two consecutive growing seasons at three locations in the UK. Harvested grain was tested for *F. langsethiae* biomass and HT2+T2 mycotoxins.

3.2 Materials and methods

The experiment was conducted in 2011/2012 and repeated in the 2012/2013 growing season with spring sowing in March and autumn sowing in October at three sites (Scottish Agronomy, GPS position 56.189816, -3.118383, Glenrothes, Scotland; Saaten Union, GPS position 52.161677, 0.487297, Cowlinge, England; Harper Adams University, GPS position 52.784974, -2.436103, Edgmond, England). Due to a lack of methods for artificial field inoculation, field trials were not inoculated and detected levels of mycotoxins and fungal biomass were due to natural infection. When possible, sites were selected based on a high or detectable concentration of HT2+T2 mycotoxins in previous harvests. All experiments included followed the standard farm practice for milling oats applied, as recommended by agronomists at each site. Seed rate was calculated based on thousand grain weight (TGW) corrected with germination rate of selected varieties,

with a target of 300 plants m^{-2} for winter sowing, and 325 plants m^{-2} for spring sowing.

In the 2011/2012 growing season each trial consisted of four randomized blocks of 12 plots (ca. 20 m^2). Due to difficulties in harvesting at one site, a split-plot design was applied to all trials in 2012/2013 growing season with the trials randomised by type (winter or spring) within plots and by variety within subplots. Varieties chosen for the experiment were selected from the AHDB Recommended List of oat varieties. Winter varieties were: Gerald, Dalguise, Mascani, Balado, Grafton and Fusion. Chosen spring varieties were Canyon, Ascot, Husky, Firth, Lennon and Zuton. Varieties were selected to represent the range of HT2+T2 observed from previous studies of Recommended List varieties (Edwards, 2007a, 2012b, 2015) and to include four conventional and two naked varieties.

Standard methods as described in Chapter 2 were applied for the harvesting, milling, HT2+T2 quantification, DNA extraction and PCR quantification of *F. langsethiae*. Data was analysed as described in Chapter 2. For combined analysis of two years and all locations, the block structure was block within trial.

3.3. Results

All experiments were analysed by sowing date and year and results are presented in the Appendix. Because of their equivalent variance it was possible to combine them and analyse together and results are presented here.

3.3.1 Field trials 2011/2012

The winter sown trial at the Scottish Agronomy site had a consistently low level of HT2+T2 (general mean = 96 µg kg^{-1}) and *F. langsethiae* DNA (general mean = 0.00245 pg ng^{-1}) with no significant difference between varieties ($p = 0.403$ and $p = 0.882$ respectively). Consequently this trial was excluded from further analysis of trials in that growing season. Two remaining winter trials (Saaten Union and Harper Adams) showed a significant difference between varieties and had an equivalent variance so were analysed together for the concentration of HT2+T2 (Appendix 1, Table A1) and for the *F. langsethiae* DNA (Appendix 1, Table A2). Due to severe lodging, the spring trial at Harper Adams in 2012 could not be harvested. The general mean for the concentration of HT2+T2 in winter sown trials was 129 µg kg^{-1} and 176 µg kg^{-1} in spring sown trials. The general mean for the concentration of *F. langsethiae* DNA was 0.0137 pg ng^{-1}) in winter sown trials and 0.0119 pg ng^{-1} in spring sown trials. Across all trials the influence of variety was significant ($p < 0.001$).

3.3.2 Field trials 2012/2013

All three winter trials had an equivalent variance and could therefore be analysed together for the concentration of HT2+T2 (Appendix 1, Table A1) and for *F. langsethiae* DNA (Appendix 2, Table A2), blocked by blocks within the trials. Combined analysis was possible as individual trials had equivalent variance.
In this year there was no problem with lodging and all spring sown experiments were harvested. An equivalent variance made it possible to analyse trials together

for the concentration of HT2+T2 and for the *F. langsethiae* DNA, blocked in the same way as previously analysed trials by location and blocks within the trials.

The general mean for the concentration of HT2+T2 in winter sown trials was 387 μg kg^{-1} and 268 μg kg^{-1} for spring sown trials. The general mean for the concentration of *F. langsethiae* DNA was 0.1570 pg ng^{-1} in winter sown trials and 0.0492 pg ng^{-1}) in spring sown trials. Across all trials the influence of variety was significant ($p < 0.001$).

3.3.3 Combined analysis of both years

Experiments were conducted in 2011/2012 and 2012/2013 growing season. The locations were the same in both years. The experiment at Harper Adams in 2012 was completely lodged and none of the plots were harvested. The winter sown experiment at Scottish Agronomy in 2012 had very low levels of HT2+T2 and *F. langsethiae* DNA. These two trials were excluded from analysis and the remaining experiments were harvested and analysed. Spring sown experiments from both years were analysed together and winter sown experiments from both years were analysed together. This was possible due to experiments having equivalent variance. Samples were analysed for HT2+T2 concentration and *F. langsethiae* DNA. Tukey's test was used to distinguish the difference between varieties when quantifying HT2+T2 within winter sown trials (Figure 3.1) and spring sown trials (Figure 3.2) and *F. langsethiae* biomass within trials sown in winter (Figure 3.3) and trials sown in spring (Figure 3.4).

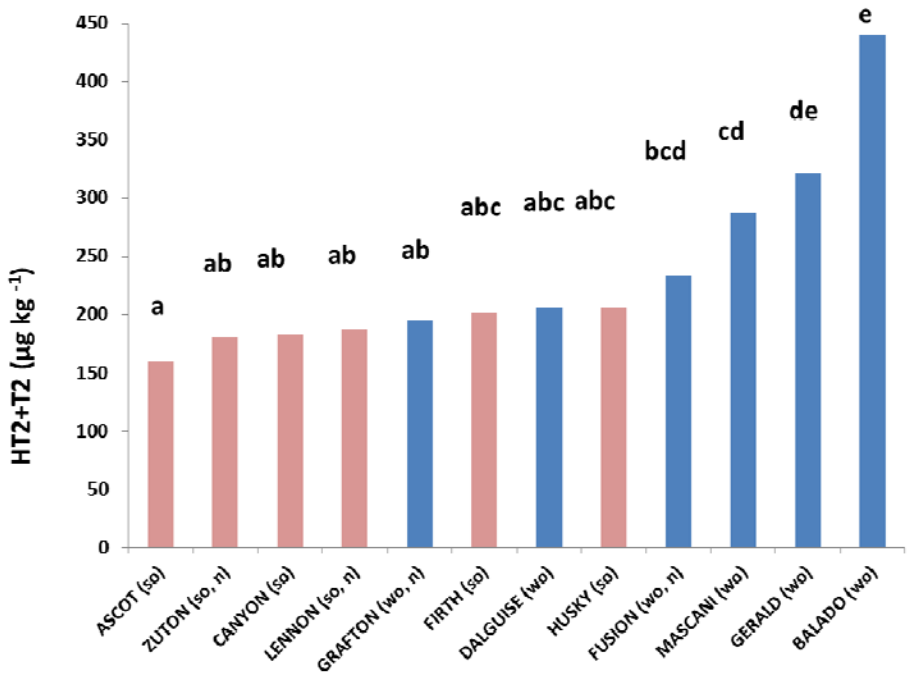

Figure 3.1 Concentration of HT2 and T2 mycotoxins (µg kg $^{-1}$) in winter sown winter and spring oat varieties. Two winter trials (Saaten Union and Harper Adams) from 2011/2012 and three winter trials (Scottish Agronomy, Saaten Union and Harper Adams) from 2012/2013 were analysed together blocked by block within trial. Varieties with the same letter were not significantly different according to Tukey's test (p=0.05). After variety names; (so) spring oat variety (red), (wo) winter oat variety (blue), (n) naked oat variety.

Significant differences between varieties were detected in both years ($p < 0.001$). Regardless of the sowing date, the same winter varieties always had higher concentration of HT2+T2, specifically Balado and Gerald. When analysing both years together, the mean concentration of HT2+T2 in spring sown experiments (Figure 3.2) was 222 µg kg $^{-1}$ and 258 µg kg $^{-1}$ in winter sown experiments (Figure 3.1). In winter sown experiments the mean concentration of HT2+T2 for Balado was 441 µg kg $^{-1}$ and for Gerald 321 µg kg $^{-1}$. In spring sown experiments Balado

and Gerald again had the highest concentration of HT2+T2 of 794 µg kg $^{-1}$ and 316 µg kg $^{-1}$ respectively.

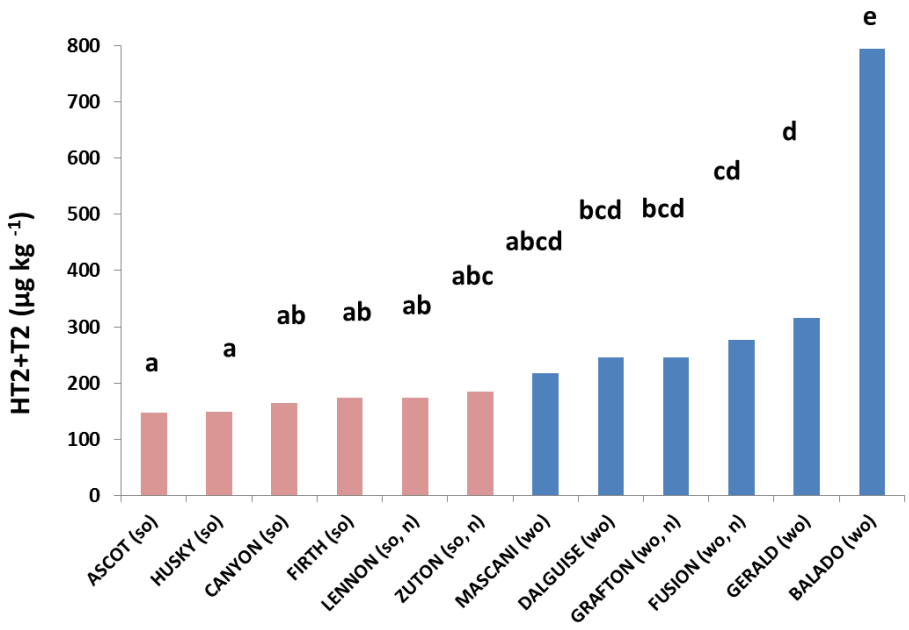

Figure 3.2 Concentration of HT2 and T2 mycotoxins (µg kg $^{-1}$) in spring sown winter and spring oat varieties. Two spring trials (Scottish Agronomy and Saaten Union) from 2011/2012 and three spring trials (Scottish Agronomy, Saaten Union and Harper Adams) from 2012/2013 were analysed together blocked by block within trial. Varieties with the same letter were not significantly different according to Tukey's test (p=0.05). After variety names; (so) spring oat variety (red), (wo) winter oat variety (blue), (n) naked oat variety.

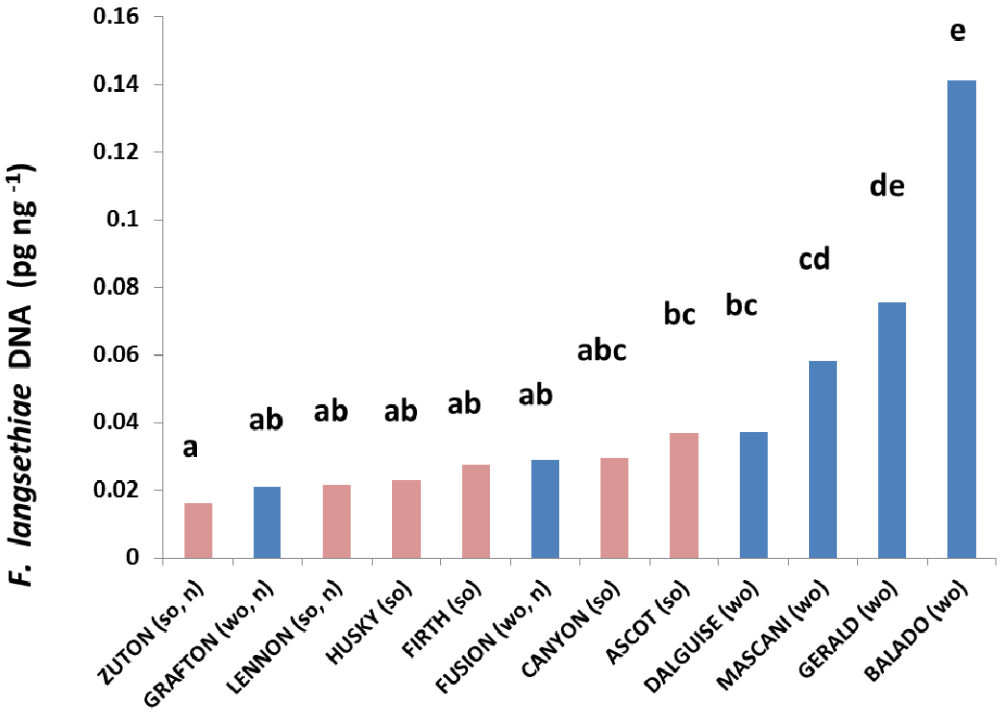

Figure 3.3 Fusarium langsethiae DNA (pg ng $^{-1}$) in winter sown winter and spring oat varieties. Two winter trials (Saaten Union and Harper Adams) from 2011/2012 and three winter sown trials (Scottish Agronomy, Saaten Union and Harper Adams) from 2012/2013 were analysed together blocked by block within trial. Varieties with the same letter were not significantly different according to Tukey's test (p=0.05). After variety names; (so) spring oat variety (red), (wo) winter oat variety (blue), (n) naked oat variety.

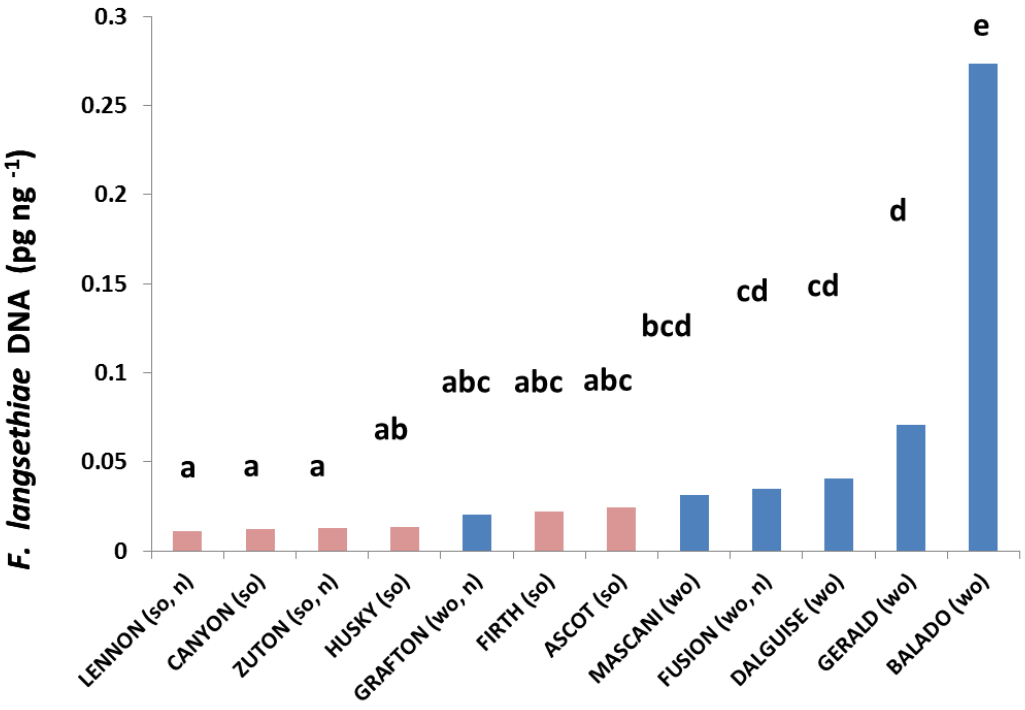

Figure 3.4 *Fusarium langsethiae* DNA (pg ng $^{-1}$) in spring sown winter and spring oat varieties. Two winter trials (Saaten Union and Harper Adams) from 2011/2012 and three winter trials (Scottish Agronomy, Saaten Union and Harper Adams) from 2012/2013 were analysed together blocked by block within trial. Varieties with the same letter were not significantly different according to Tukey's test (p=0.05). After variety names; (so) spring oat variety (red), (wo) winter oat variety (blue), (n) naked oat variety.

As it was expected, varieties performed differently and there was a fluctuation of the overall mean of the individual varieties from different environments. Despite this, certain trends were constant. Apart from one trial (winter sown at Harper Adams in 2012), Balado always had the highest level of HT2+T2 mycotoxins and that was true regardless of the sowing date (Figure 3.5 and Figure 3.6)

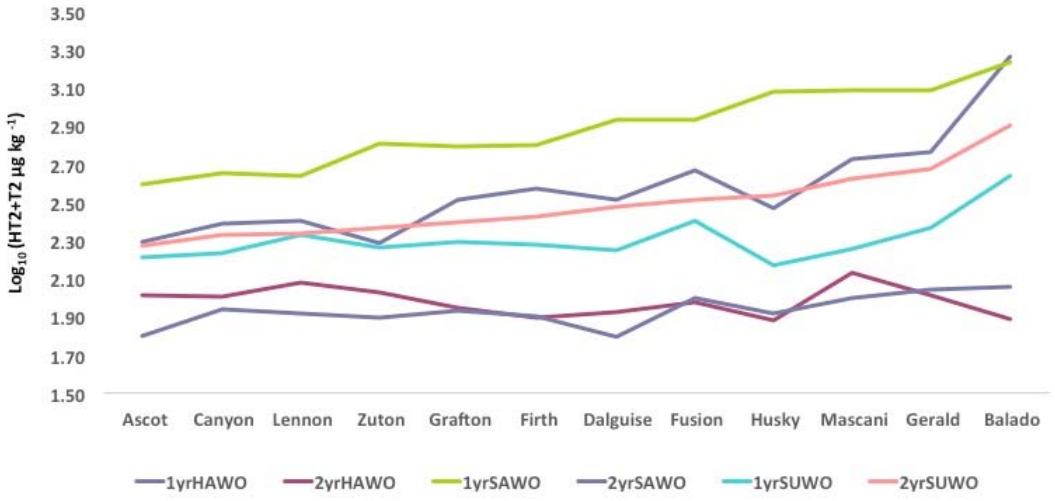

Figure 3.5 Concentration of HT2 and T2 mycotoxins (µg kg $^{-1}$) presented as Log_{10} (HT2+T2 µg kg^{-1}) in winter sown winter and spring oat varieties. In the legend below the graph; (1yrHAWO) winter sown trial at Harper Adams in 2011/2012, (2yrHAWO) winter sown trial at Harper Adams in 2012/2013, (1yrSAWO) winter sown trial at Scottish Agronomy in 2011/2012, (2yrSAWO) winter sown trial at Scottish Agronomy in 2012/2013, (1yrSUWO) winter sown trial at Saaten Union in 2011/2012, (2yrSUWO) winter sown trial at Saaten Union in 2012/2013

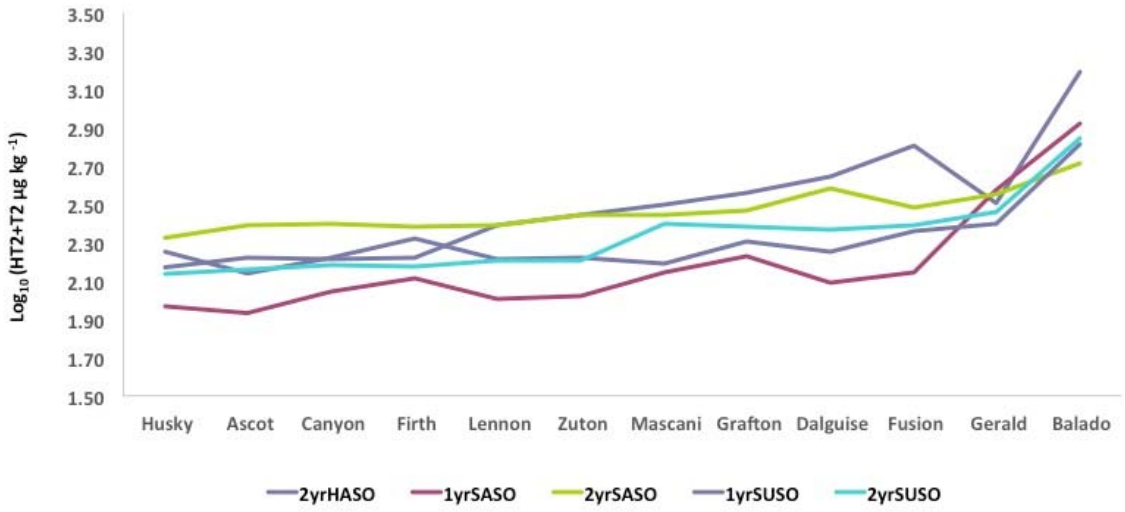

Figure 3.6 Concentration of HT2 and T2 mycotoxins (µg kg $^{-1}$) presented as Log_{10} (HT2+T2 µg kg^{-1}) in spring sown winter and spring oat varieties. In the legend below the graph; (2yrHASO) spring sown trial at Harper Adams in 2012/2013, (1yrSASO) spring sown trial at Scottish Agronomy in 2011/2012, (2yrSASO) spring sown trial at Scottish Agronomy in 2012/2013, (1yrSUSO) spring sown trial at Saaten Union in 2011/2012, (2yrSUSO) spring sown trial at Saaten Union in 2012/2013

The ability of varieties to perform the same across different environments was tested using the Finlay-Wilkinson regression (Finlay & Wilkinson, 1963). The Finlay-Wilkinson regression coefficient is a measure of variety adaptation or consistency. Varieties closer to the middle of y axis (y=1) were considered to have an average consistency or ranking of mycotoxin contamination across different environments. Varieties closer to x axis can be expected not to have constant ranking in different environments and further from y axis are varieties with a constant mycotoxin levels across a wide range of environments. Below the line (axis y=1) are varieties with more consistent HT2+T2 levels in environments with

no optimal conditions and above the axis are varieties with more consistent mycotoxin levels in favourable environments. Varieties are ranked according to sensitivity or consistency of ranking. When sown in winter, Lennon, Ascot and Canyon tend to be the most stable across different environments (Appendix Table A3, Figure 3.7). But when sown in spring, Gerald, Balado and Firth are the most consistent in their HT2+T2 levels, performing similarly when sown in different environments (Appendix Table A4; Figure 3.8). It could be postulated that highly contaminated varieties such as Balado and Gerald are more variable in HT2+T2 concentration across a range of environments.

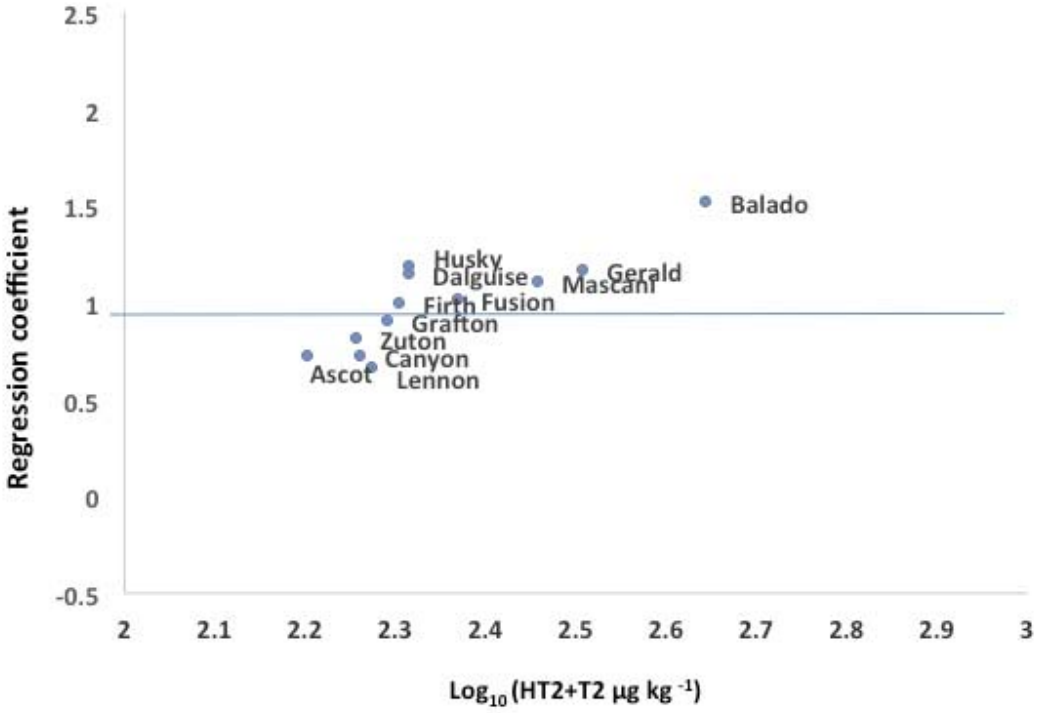

Figure 3.7 Finlay-Wilkinson regression for experiments sown in winter. All trials were analysed together. Presented is mean HT2+T2 µg kg^{-1} value for individual varieties

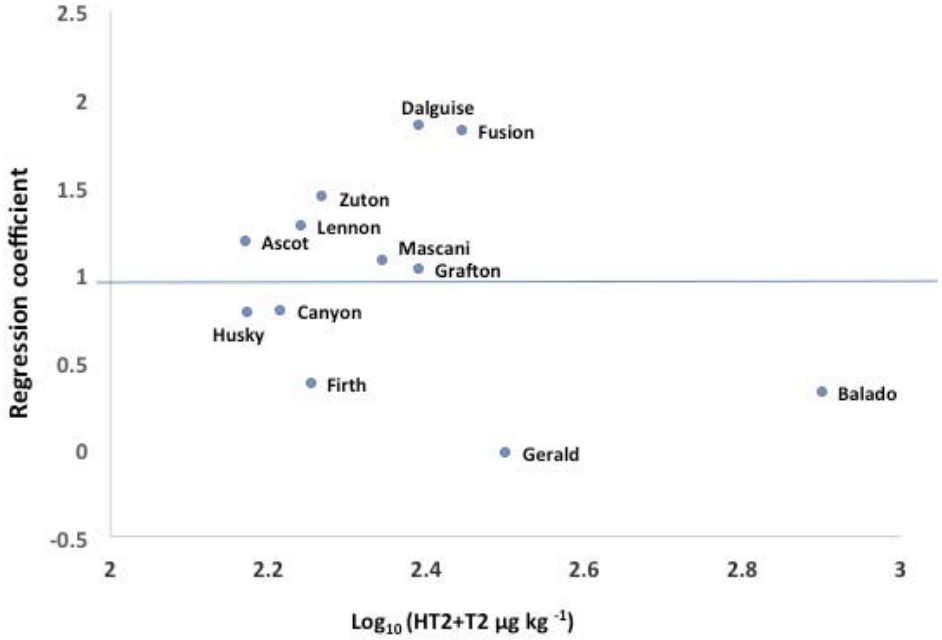

Figure 3.8 Finlay-Wilkinson regression for experiments sown in spring. All trials were analysed together. Presented is mean HT2+T2 µg kg^{-1} value for individual varieties

Grouping by year and sowing time (i.e. winter or spring), regression analysis showed the relationship between the concentration of HT2+T2 mycotoxins and the level of *F. langsethiae* DNA ($p<0.001$). The single lines could be fitted for all trials together which accounted for 50% of the variance (Figure 3.9). From Figure 3.9 it is noticeable that the spring trial in the second year had a very weak relationship between the level of *F. langsethiae* DNA and the level of HT2+T2 mycotoxins ($R^2 = 0.15$). If separate lines were fitted, 52% of variance could be explained with a significant regression. Lines fitted would be as follows: winter trials in the first year ($p < 0.001$, $R^2 = 0.56$), spring trials in the first year ($p < 0.001$, $R^2 = 0.40$), winter trials in the second year ($p < 0.001$, $R^2 = 0.53$) and spring trials in the second year ($p = 0.31$, $R^2 = 0.15$).

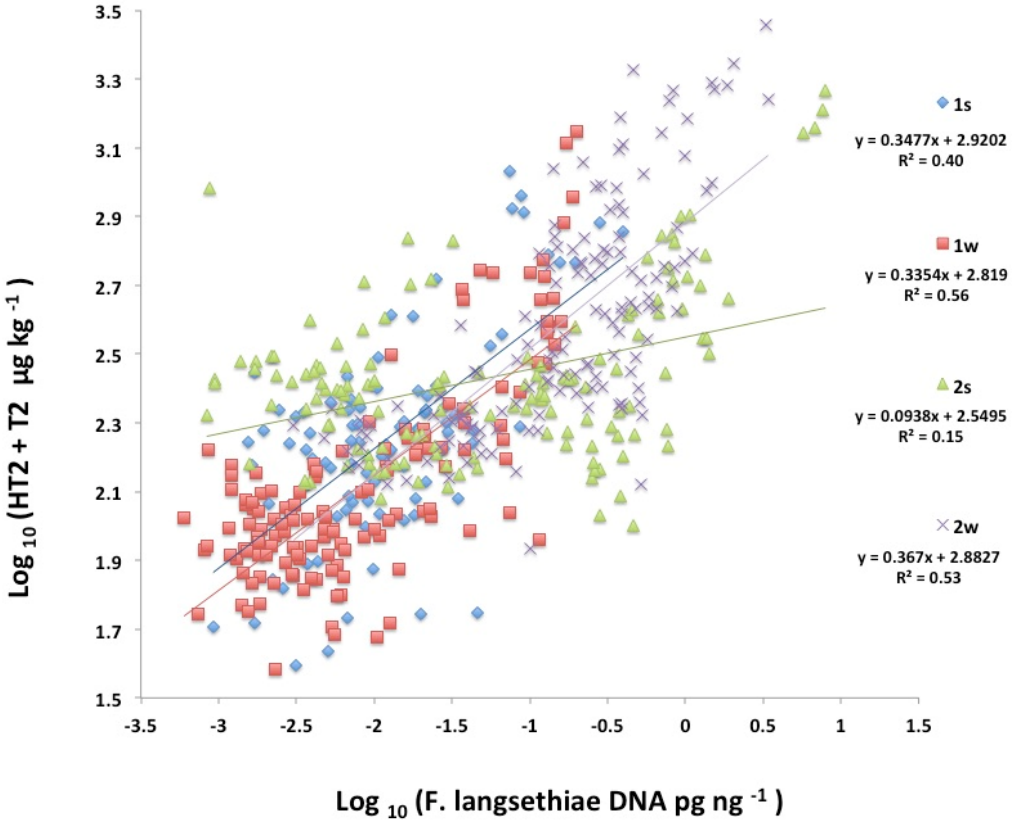

Figure 3.9 Relationship between Log_{10} transformed Fusarium langsethiae DNA and HT2+T2 concentration ($p<0.001$) in winter and spring sown trials from three different locations in 2011/2012 and 2012/2013. In each year there were three locations and both winter and spring trials at each location with exception of 2012 where one spring sown trial was completely lodged. On the figure; (1w) winter trials in the first year, (1s) spring trials in the first year, (2w) winter trials in the second year, (2s) spring trials in the second year.

3.4 Discussion

This Chapter evaluated the resistance of UK oat varieties to *F. langsethiae* and HT2+T2 mycotoxin contamination. In order to do so experiments at three locations across the UK and in two consecutive seasons were performed. In each year and at each location experiments were sown in the spring and in the winter to determine if sowing date is a predominant factor in infection and mycotoxin contamination and as such explains the disparities seen between winter and spring oats in previous observational studies.

Results from these experiments have indicated that the sowing date might not be of major importance for the accumulation of HT2+T2 mycotoxins and *F. langsethiae* infection. Winter varieties, Gerald and Balado sown in spring still had significantly higher levels of HT2+T2 when compared to spring varieties from the same trials. The same experiments were repeated across three sites and over two years indicating the observed differences are stable phenotypes.

For winter sown trials in the first year, low levels were recorded at the sites of Scottish Agronomy and Saaten Union. High levels were recorded at the site of Harper Adams where the highest level of HT2+T2 was detected in samples of Balado (1399µg kg^{-1}) and Gerald (1295 µg kg^{-1}) which was the highest level of HT2+T2 in that year. Only those two varieties sown at the Harper Adams site had levels exceeding the currently recommended 1000 µg kg^{-1}. In the second experimental year, the site of Saaten Union had a low level of HT2+T2 while a high level was recorded at the Scottish Agronomy site. Harper Adams had a

mixture of HT2+T2 levels with some at the lowest end that year and some of the highest such as Balado with the highest overall mean that year across sites of 917 μgkg $^{-1}$. Spring trials sown in the first experimental year at Saaten Union and Scottish Agronomy had some low levels of HT2+T2 recorded. They also had some of the highest recorded levels in samples and an example is Balado sown at the site of Scottish Agronomy having the highest mean HT2+T2 (1072 μg kg $^{-1}$) followed by Gerald (816 μg kg $^{-1}$). Spring trials sown in the second experimental year had the lowest levels of HT2+T2 at the site of Saaten Union and a range of levels at the site of Harper Adams with the highest level from all varieties across sites recorded for Balado (591 μg kg $^{-1}$) from samples harvested at Harper Adams.

Due to the uncertainty of weather conditions, and a dependence solely on natural infection, not all trials showed quantifiable differences between varieties. Some of the trials did not show significant difference among varieties. In those cases the overall mean of HT2+T2 concentration was low. It is likely that due to a wet summer in 2012 when average rainfall for summer months was 180% higher than the hundred-year average (www.metoffice.gov.uk), levels of HT2+T2 were not high and when the low levels occur it is unlikely that differences between varieties can be detected (Edwards, 2012a).

However, even winter trials that did not show significant differences had a trend of winter varieties Balado or Gerald, having the highest concentration of HT2+T2. Differences that were observed, with higher levels detected in Gerald and Balado match those observed from AHDB Recommended List trials (Edwards, 2007a, 2012b, 2015). This is contrasting with results from France, although on barley, where sowing date was found to be the most important factor for the HT2+T2

contamination (Orlando *et al.*, 2010) with higher concentrations detected in spring compared with winter sown barley.

All except the spring trials in the second year had a significant relationship between *F. langsethiae* DNA and the concentration of HT2+T2 mycotoxins. The disparities observed in the spring trial of the second year should encourage further investigation into details of why such differences occur. The recommendation for future study would be to monitor oat growth stage and the weather conditions especially around flowering time. This is especially important given that anther extrusion is weather dependent (De Vries, 1971b).Additionally, flower opening has been suggested as a mechanism of disease avoidance (Skinnes *et al.*, 2010; Lu *et al.*, 2012; Kubo *et al.*, 2013) and there is a current lack of understanding of how and when infection with *F. langsethiae* happens in the field.

These results have identified that the sowing date might not be the predominant factor for *F. langsethiae* infection and grain contamination with HT2+T2 mycotoxins in oats. As winter varieties still have greater level of HT2+T2, despite different sowing dates, genetic resistance should be investigated in greater detail. To do so a mapping population constructed from parent varieties that differ in their level of susceptibility to *F. langsethiae* and HT2+T2 mycotoxins could be used for phenotyping and accordingly QTL analysis. The QTL analyses are used for complex quantitative traits such as disease resistance. They rely on the association between genotypic with phenotypic data, which underlines the need for developing a method of artificial inoculation.

The European Commission is currently considering legislative limits for the joint concentration of HT2+T2 mycotoxins as they occur together. An indicative limit of

1000 μg kg^{-1} HT2 and T2 combined (HT2+T2) in unprocessed oats is published in the recommendation by the European Commission (2013). Results from the experiment presented in this Chapter did not show many samples to exceed this indicative limit but previous studies by Edwards (2007, 2012a,b, 2015) have identified up to 30% of samples can exceed 1000 μg kg^{-1} HT2+T2 in some years.

A major issue in studying *F. langsethiae* is the lack of disease symptoms in the field or on harvested grain (Opoku *et al.*, 2013). *Fusarium langsethiae* is reported in several countries and in all cases, reports are based on either the levels of HT2+T2 toxins produced or on the levels of DNA. There are no reports of successful artificial inoculation in the field and due to this, it was necessary to rely on natural infection of *F. langsethiae*.

Sowing resistant varieties, choosing appropriate cultivation practices, timely fungicide treatment together with weather based risk systems can minimize risk of *Fusarium* development (Osborne & Stein, 2007). Overall, all agronomic factors and the interaction between different species must be taken in to consideration when providing guidelines on Good Agricultural Practice to reduce *Fusarium* mycotoxins (Edwards, 2004). Publishing legislation with the indicative limits from the European Commission would have economic implications for UK growers and oat processors. Due to the cost implicated with some of the changes in agronomy, selecting the less susceptible variety is the measure of reducing HT2+T2 mycotoxin levels that can be expected from farmers (Edwards, 2012a). Results from this chapter present information on more resistant varieties across environments with lower level of HT2+T2 mycotoxins. Growing more spring oats would be one of the recommendations based on results of the experiments presented in this Chapter.

Chapter 4: Susceptibility of naked oats compared with conventional oats

4.1 Introduction

Naked oats is a term used to describe such varieties with a loose hull that is easily removed during harvesting. Naked oats are used as an animal feed. Conventionally or husked oats intended for human consumption need to be de-hulled prior to consumption. De-hulling is a process of removing oat husk so only the groat is left (Valentine, 1995).

Oat husk of conventional varieties, that can contribute to around 30% of the oat grain weight, can be included as a small percentage of ruminant diets (Edwards, 2007a) and therefore it is important to know the mycotoxin level of the husk. In the studies of the AHDB Recommended List oat variety trials in the UK, it was observed that naked varieties had a notably lower level of HT2+T2 mycotoxins at harvest. Where naked oats were dwarf or short-strawed varieties, the level of HT2+T2 mycotoxins was intermediate (Edwards, 2007a, 2012b, 2015). Similar results were found in Austria and Czech Republic, where naked oats from different locations had a lower lever of mycotoxins at harvest compared with conventional (husked) oats (Adler *et al.*, 2003). Evaluating a Russian collection of oat germplasm, Gagkaeva *et al.* (2011) concluded that all naked oats are less susceptible to FHB. Lower levels of mycotoxins in naked oats might be explained by the absence of the husk after harvesting as de-hulling is known to reduce the

level of HT2 and T2 by around 90% (Scudamore *et al.*, 2007; Pettersson *et al.*, 2008).

In experiments to determine the impact of de-hulling on HT2+T2 concentration, removing the husk resulted in a lower concentration of mycotoxins on groat and comparably high concentration in the husk. The reduction observed as a result of the de-hulling of conventional husked oats also resulted in a lower level of HT2+T2 that could be compared with naked oats during harvest. When de-hulled, husked oats had a much lower level of HT2+T2. It was suggested that the HT2+T2 level would be higher on naked oats if compared with conventional oats after de-hulling (Edwards, 2007a).

All oats for human consumption are de-hulled and the low levels of HT2+T2 of shop marketed oat products is in line with the low levels reached by de-hulling (Edwards, 2007a; Scudamore *et al.*, 2007).

It is not known how the HT2+T2 level of naked varieties compares to conventional husked varieties before harvest and how panicles of naked oats collected before harvest compare to the harvested grain. There are no studies comparing the level of HT2+T2 mycotoxins on the ripe plants from the field with the harvested grain. Analysing Russian oat germplasm collection, Gagkaeva *et al.* (2011) concluded that all huskless oat varieties are more resistant to FHB. The authors also noted that after manual de-hulling of husked varieties, the level of mycotoxins was reduced. However this study analysed huskless varieties after the harvest during which they loose their husk and therefore the levels can be expected to be lower. To date there are no studies comparing the level of mycotoxins of huskless varieties before their husk is naturally removed during the harvest. Furthermore,

there are no reports to date comparing the level of mycotoxins between conventional oat varieties and huskless varieties before they are harvested. In this Chapter the concentration of mycotoxins on panicles from the varietal experiment detailed in Chapter 3 were analysed. The Null hypothesis was that there is no difference in HT2+T2 concentration between panicles of different varieties.

4.2 Materials and methods

The material analysed for this study was collected from the experiments described in Chapter 3 that was conducted in 2011/2012 and repeated in the 2012/2013 growing season, with spring sowing in March and autumn sowing in October at three sites (site of Scottish Agronomy, Glenrothes, Scotland; site of Saaten Union, Cowlinge, England; site of Harper Adams University, Edgmond, England).

As described earlier in Chapter 3, the field was not inoculated artificially and HT2+T2 levels resulted from naturally occurring infection in the field. Varieties included as winter were: Gerald, Dalguise, Mascani, Balado and naked Grafton and Fusion. Chosen spring varieties were Canyon, Ascot, Husky, Firth and naked Lennon and Zuton. In addition to collecting grain at harvest for analysis presented in Chapter 3, samples of panicles were collected just before harvest at the growth stage (GS92; Zadoks *et al.*, 1974) and analysed for HT2+T2.

Each trial comprised of four randomised blocks of 12 plots of the same size. Each plot was sown with one randomly selected variety. From each plot 30 panicles were collected when the crop was fully ripe at growth stage 92 (Zadoks *et al.*,

1974). Panicles were randomly selected from each plot, avoiding the plot edges and evenly selecting across the remaining area of the plot. To standardise selection, all panicles selected were cut above the flag leaf. Panicles were milled with a laboratory mill (Christy Turner Ltd, Suffolk, England) fitted with 1 mm screen. HT2+T2 quantification and data analysis was conducted as described in Chapter 2.

4.3 Results

All experiments were analysed by sowing date and year and results are presented in the Appendix. Because of their equivalent variance it was possible to combine them and analyse together and results are presented here.

4.3.1 Field trials 2011/2012

For both the winter and spring sown experiments in each year ANOVA of each experiment showed that they had near equivalent variance and were analysed together by blocks within trial. Mean concentration of HT2+T2 was 405 µg kg $^{-1}$ in winter sown experiments and 711 µg kg $^{-1}$ in spring sown experiments (Appendix Table A5). Sown in winter, Ascot had the lowest level of HT2+T2 (285 µg kg $^{-1}$) and the maximum level was recorded for Balado samples (578 µg kg $^{-1}$). At the higher end of HT2+T2 levels were naked varieties Grafton (434 µg kg $^{-1}$) and Fusion (487 µg kg $^{-1}$). Spring sown trials had overall higher level of HT2+T2 with the highest concentration for Balado samples (2582 µg kg $^{-1}$) followed by Fusion (1778 µg kg $^{-1}$) and the lowest concentration was for Firth samples (285 µg kg $^{-1}$).

4.3.2. Field trials 2012/2013

Panicles from the winter trial at Saaten Union in the second year were lost in transport. In the second experimental year, all trials had a similar variance thus it was possible to analyse together panicles from winter and then together panicles from spring sown experiments. Panicles were analysed for the concentration of HT2+T2. In statistical analysis experiments were blocked by blocks within trial. Mean concentration of HT2+T2 was 1263 µg kg $^{-1}$ in winter sown experiments and 466 µg kg $^{-1}$ in spring sown experiments (Appendix Table A5). The highest overall concentration of HT2+T2 was for Fusion samples from winter sowing (4875 µg kg $^{-1}$) with the lowest concentration in winter sown experiments for Ascot samples (329 µg kg $^{-1}$). Sown in spring, Balado samples had the highest overall mean of HT2+T2 (1726 µg kg $^{-1}$) while the lowest concentration was obtained from Husky samples (171 µg kg $^{-1}$).

4.3.3 Combined analysis of both years

Experiments were conducted in two growing seasons at three different locations that were the same in both years. Spring sown experiments were analysed together and winter sown experiments were analysed together. All spring sown and winter sown experiments had equivalent variance. In analysing all experiments, Tukey's test was used to differentiate between the varieties HT2+T2 concentration sown in winter (Figure 4.1) and sown in spring (Figure 4.2). There were significant differences between varieties in both the winter sown and spring sown varieties ($p<0.001$).

In winter sown experiments, Ascot, Canyon and Dalguise were varieties which showed lower concentrations of HT2+T2. Lennon and Zuton had a moderate concentration and amongst varieties with the highest concentration of HT2+T2 mycotoxins were Balado, Grafton and Fusion (Figure 4.1).

In spring sown trials, Firth, Ascot and Husky had the lowest HT2+T2 concentration. Intermediate was the concentration of Canyon and Lennon and, similarly to winter trials, the highest concentration of HT2+T2 was found on panicle samples from Grafton, Fusion and Balado (Figure 4.2).

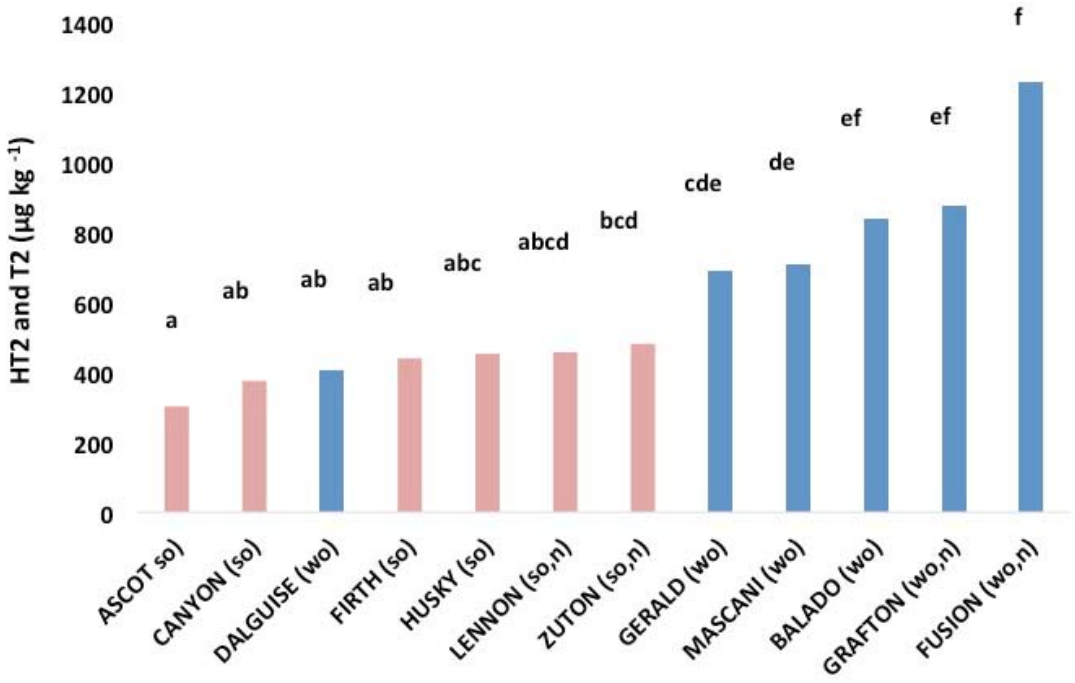

Figure 4.1. Concentration of HT2 and T2 mycotoxins (µg kg $^{-1}$) of panicles in winter sown oat varieties. Varieties with the same letter were not significantly different, according to Tukey's test (p=0.05). After variety names; (so) spring oat variety (red), (wo) winter oat variety (blue), (n) naked oat variety.

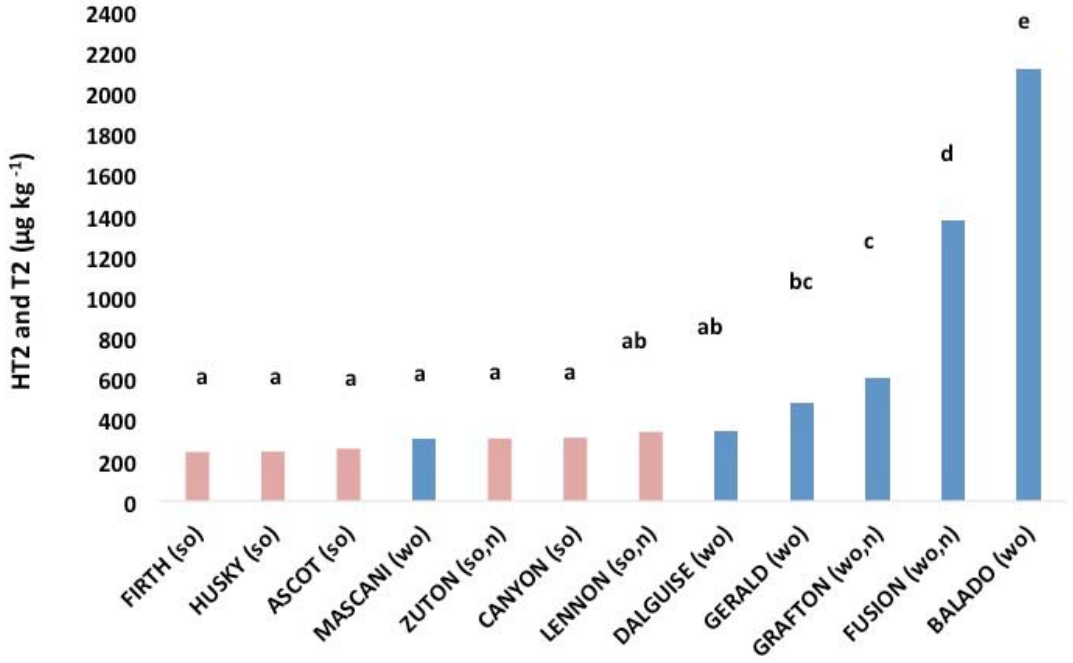

Figure 4.2. Concentration of HT2 and T2 mycotoxins (µg kg $^{-1}$) of panicles in spring sown oat varieties. Varieties with the same letter were not significantly different according to Tukey's test (p=0.05). After variety names; (so) spring oat variety (red), (wo) winter oat variety (blue), (n) naked oat variety.

Although, as expected, different varieties performed differently in different environments there was a trend that was similar across environments for all varieties (Figure 4.3 and Figure 4.4). Regardless of the sowing date, varieties Fusion, Grafton and Balado had the highest level of HT2+T2 across all environments. The consistent ranking of varieties with regards to the level of contamination of mycotoxins at different environments was examined using the Finlay-Wilkinson regression (Finlay & Wilkinson, 1963). The measure of consistency of variety was determined by Finlay-Wilkinson regression coefficient. Closer to the middle of the axis for where y=1, were varieties considered to have

an average consistency of mycotoxin contamination across different environments. Below that line (axis y=1) were varieties whose contamination with HT2+T2 was more constant at environments without optimal conditions and above the axis are varieties with more constant mycotoxin levels and ranking across favourable environments. Close to the y axis, were varieties whose ranking will be diverse in different environments and further from that point are varieties who tend to have similar level of mycotoxins across a wide range of environments.

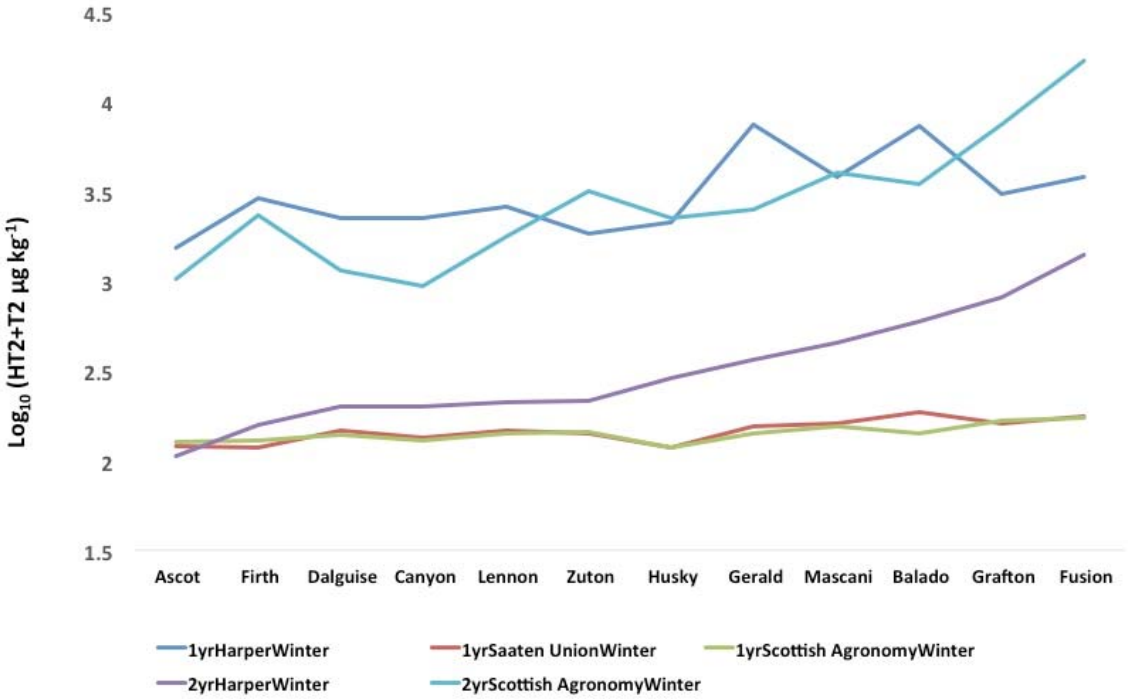

Figure 4.3 Concentration of HT2 and T2 mycotoxins (µg kg $^{-1}$) of harvested panicles presented as Log_{10} (HT2+T2 µg kg^{-1}) in winter sown oat varieties. In the legend below the graph; (1yrHarperWinter) winter sown trial at Harper Adams in 2011/2012, (2yrHarperWinter) winter sown trial at Harper Adams in 2012/2013, (1yrSaaten UnionWinter) winter sown trial at Saaten Union in 2011/2012, (1yrScottish AgronomyWinter) winter sown trial at Scottish Agronomy in 2012/2013, (2yrScottish AgronomyWinter) winter sown trial at Scottish Agronomy in 2012/2013

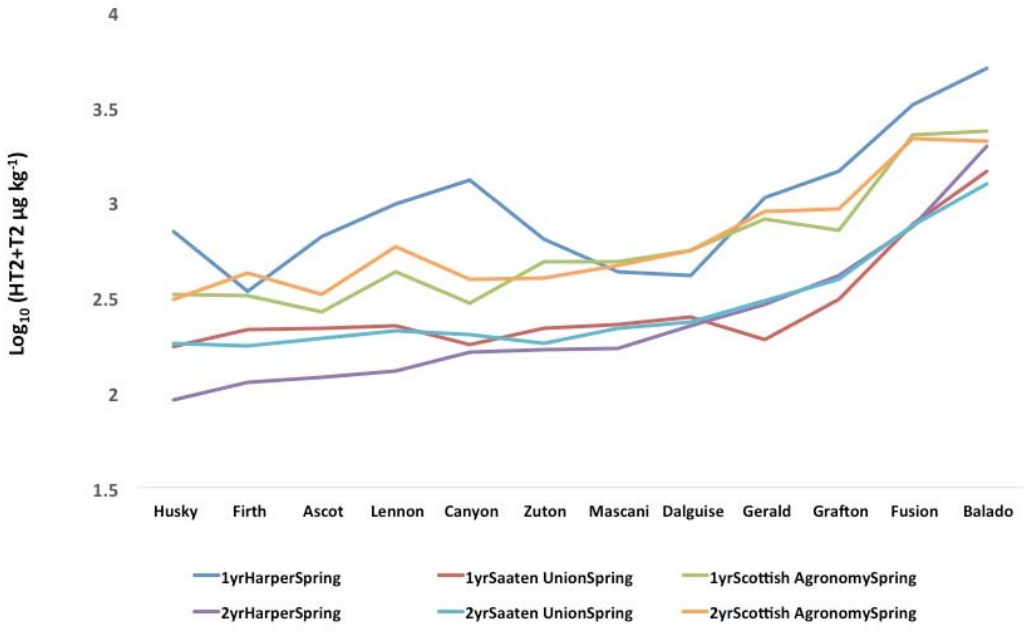

Figure 4.4 Concentration of HT2 and T2 mycotoxins (µg kg $^{-1}$) presented as Log_{10} (HT2+T2 µg kg^{-1}) of harvested panicles from spring sown oat varieties. In the legend below the graph; (1yrHarper Spring) spring sown trial at Harper Adams in 2011/2012, (1yrScottish Ag Spring) spring sown trial at Scottish Agronomy in 2011/2012, (2yrSaaten Union Spring) spring sown trial at Saaten Union in 2012/2013, (1yrSaaten UnionSpring) spring sown trial at Saaten Union in 2011/2012, (2yr HarperSpring) spring sown trial at Harper Adams University in 2012/2013, (2yrScottish AgronomySpring) spring sown trial at Scottish Agronomy in 2012/2013

Varieties are ranked according to the consistency of their ranking across different environments. When sown in winter, Canyon, Ascot and Dalguise tended to have a similar level of mycotoxins across different environments (Table 4.2, Figure 4.5).

Dalguise, Balado and Mascani were the most consistent in ranking from varieties sown in spring (Figure 4.6). These results show that although the level of mycotoxins are not the same across different environments, there is a certain trend. The consistent ranking of varieties can be seen as following that trend. Winter sown experiments (Figure 4.5) show a consistent trend that more highly contaminated varieties were more variable in HT2+T2 concentration across different environments. The least consistent varieties were the ones with the highest concentration of HT2+T2 such as Fusion, Gerald and Balado. This would suggest that they can be more variable across different environments. When sown in spring (Figure 4.6) they were more constant. This is in accordance to the previous finding that HT2+T2 levels from spring trials are in general lower than from the winter trials. However, as demonstrated in Chapter 3, even with lower overall levels of HT2+T2 in spring, certain winter varieties are still more susceptible regardless of the sowing date.

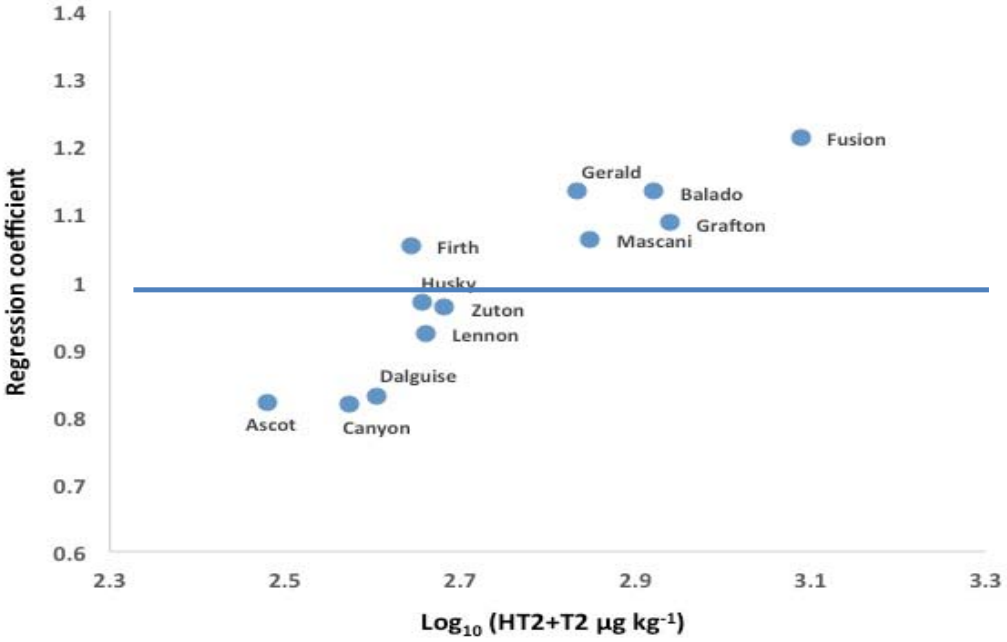

Figure 4.5 Finlay-Wilkinson regression for HT2+T2 concentration of harvested panicles from winter sown experiments.

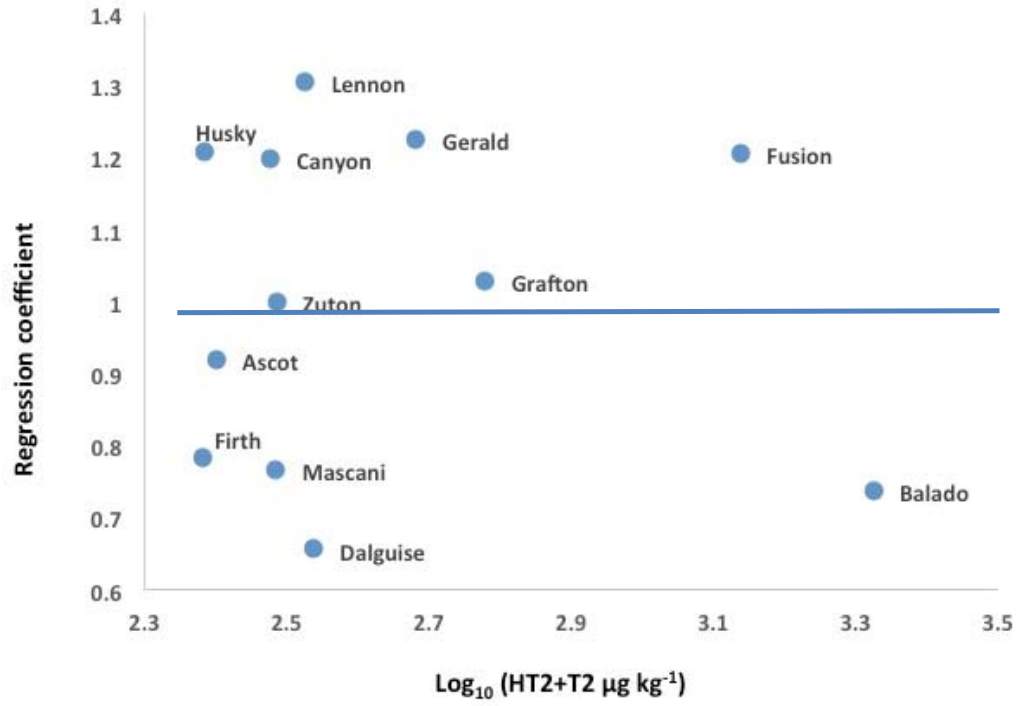

Figure 4.6 Finlay-Wilkinson regression for the HT2+T2 concentration of harvested panicles from spring sown experiments.

4.3.4 Comparing mycotoxin levels of harvested panicles with grain samples

Results from panicles collected in this study were compared with results from Chapter 3 when grain samples were analysed. Panicles originated from the same plots and were typically harvested two days before the grain samples were harvested.

For the winter sown experiments most panicles are about 2-fold greater in HT2+T2 concentration compared to the equivalent grain concentration except for the naked spring sown varieties that are about 2.5-fold higher in concentration and winter oat varieties which have a 4 to 5-fold higher concentration of HT2+T2 in panicles before harvest compared to the grains (Figure 4.7). For the spring sown experiments the concentration in panicles is generally lower at about 1.5 to 2-fold higher compared to the HT2+T2 concentration in grain except for Balado and Grafton at 2.5-times higher and Fusion at 5-times higher (Figure 4.8).

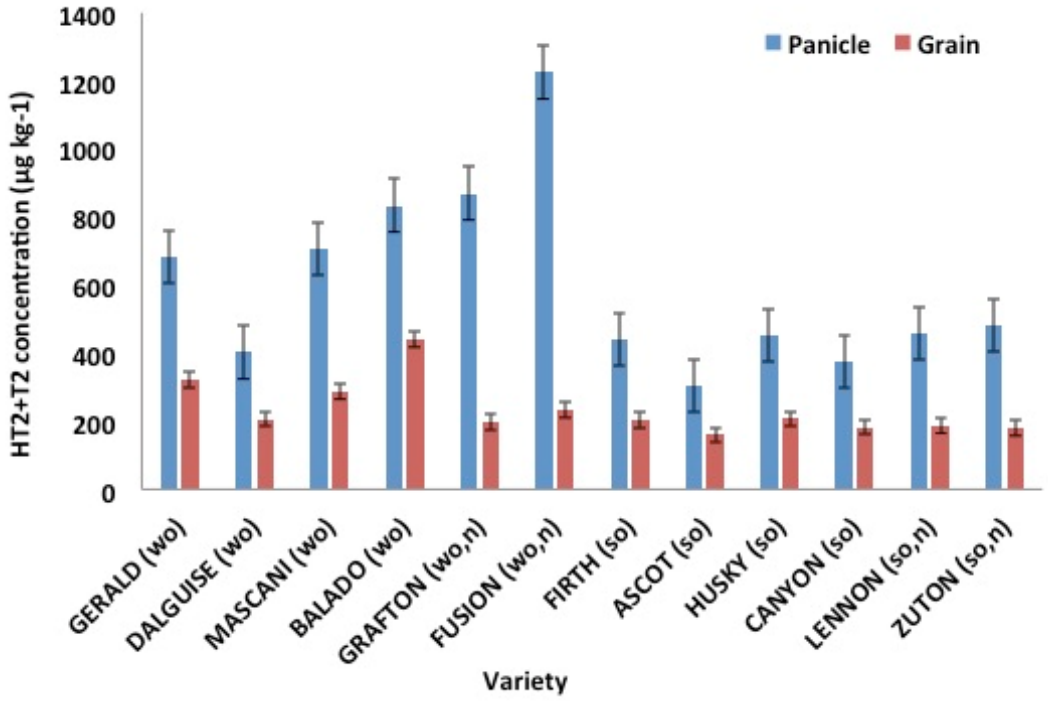

Figure 4.7 Concentration of HT2 and T2 mycotoxins (µg kg $^{-1}$) in panicle and grain samples from winter sown oat varieties. Error bars represent standard errors. After variety names; (so) spring oat variety, (wo) winter oat variety, (n) naked oat variety

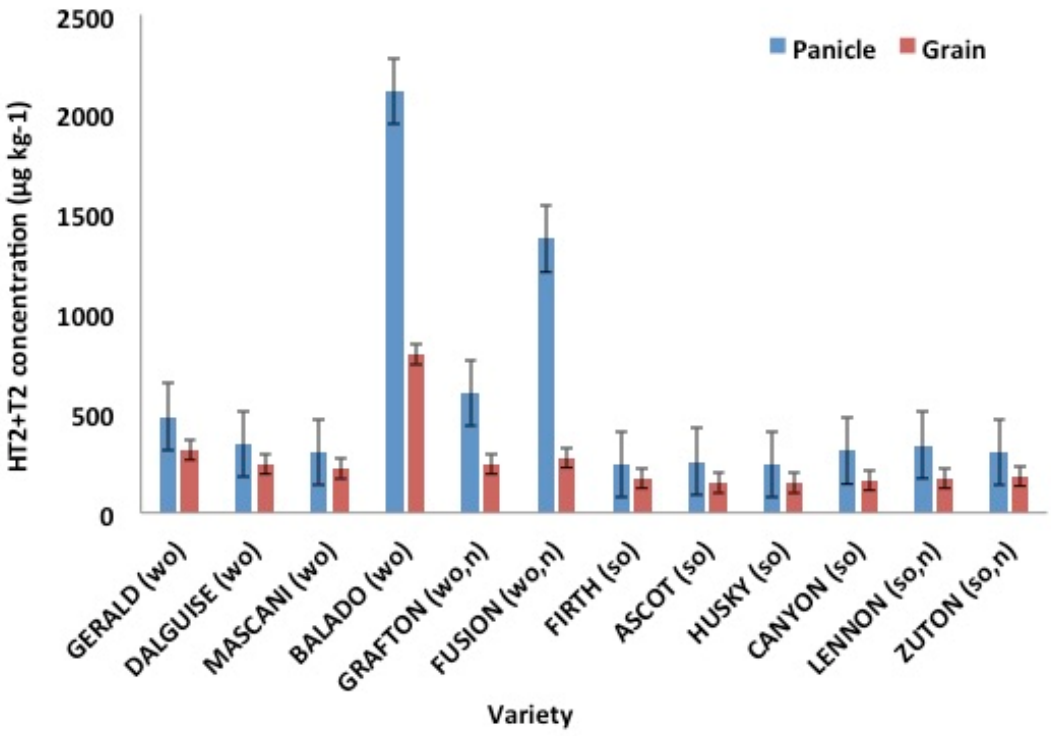

Figure 4.8 Concentration of HT2 and T2 mycotoxins (µg kg $^{-1}$) in panicles and grain samples from spring sown oat varieties. Error bars represent standard errors. After variety names; (so) spring oat variety, (wo) winter oat variety, (n) naked oat variety.

4.4. Discussion

The concentration of HT2+T2 in naked oats is considered to be lower than in the conventional husked oats. Assumptions are made on studies comparing the grains samples after the harvest (Adler *et al.*, 2003; Edwards, 2007a; Gagkaeva *et al.*, 2011). Nonetheless, huskless and conventional oats differ at the point of harvest as the husk is easily removed from huskless oats while husked varieties still have

their husk after the harvest (Valentine, 1995). Studies agree that the majority of mycotoxins are found in the husk (Edwards, 2007a; Scudamore *et al.*, 2007; Pettersson *et al.*, 2008). The question when comparing husked and huskless varieties is whether the equal material is compared after the harvest given the loose husk of huskless varieties. It is not clear how levels of mycotoxins correspond before the harvest.

Experiments conducted in this Chapter tried to evaluate the concentration of HT2+T2 mycotoxins in UK oat varieties by collecting and analysing panicles before the harvest. Differences were identified between varieties with naked varieties resulting in the highest level of HT2+T2 mycotoxins. Especially high were winter naked varieties Grafton and Fusion that when previously analysed as grain samples (in Chapter 3), showed a lower level of contamination. The greatest difference was for panicles of Fusion which had five times more HT2+T2 than grain samples of the same variety in the spring sown experiments.

The consistently lower levels of mycotoxins were previously detected on naked oats in the UK (Edwards, 2007a), in Austria, Czech Republic (Adler *et al.*, 2003) and in Russia (Gagkaeva *et al.*, 2011). Nevertheless all studies compared only the grain after the harvest where lower levels are to be expected as a result of de-hulling (Scudamore *et al.*, 2007). Comparing naked oats with conventional oats, (Edwards, 2007a) showed that HT2+T2 levels can be higher on naked oats at harvest compared to the groat of de-hulled conventional oats.

Results from these experiments have indicated that naked varieties before harvest do not have the lowest level of HT2+T2 mycotoxins but rather an intermediate to high level. This would suggest that naked varieties are not genetically more

resistant to HT2+T2 contamination but that it is rather the result of their loose hull, removed during harvest, which contributes to the reduction of HT2+T2 mycotoxins. This is in agreement with Edwards (2012a) where naked varieties Grafton and Fusion had a lower level and an intermediate level of HT2+T2 compared with other varieties. As Fusion is a short-strawed variety, its intermediate rather than low level of HT2+T2 might be a consequence of height. Nevertheless, panicles of Fusion had distinctly higher level of HT2+T2 than grain samples collected after harvest.

Despite lower levels in some trials, there was a trend of winter varieties Balado or Gerald to show the highest concentration of HT2+T2 when grain samples were analysed. The differences observed, with the highest levels detected in Gerald and Balado are in accordance with observations from AHDB Recommended List trials (Edwards, 2007a, 2012b, 2015). Results presented in this chapter suggest that when panicles were collected and analysed some naked varieties such as Grafton and Fusion had the highest level of HT2+T2, indicating these varieties actually have a high susceptibility to *F. langsethiae*.

The differences observed can be explained as the husk of naked oats is loosely attached and easily removed during the harvest and by knowing that de-hulling significantly reduces the amount of HT2+T2 mycotoxins (Scudamore *et al.*, 2007). It was previously identified that de-hulling can significantly reduce the level of mycotoxins. The reduction is reported to be 90% although there are reports of a wider range of reduction and this inconsistency might be due to the inadequate sample size in some studies (Edwards, 2007).

Another contributing factor suggested is that the spikelet of naked oats differ from conventional husked oats due to a thinner husk and that this might be creating a different microclimate that is less favourable for the growth of microorganism as pointed out by Adler *et al.* (2003). This is unlikely as in the results presented here it is not the case of less HT2+T2 found on panicles but rather on grain samples once husks are removed in the process of harvesting.

As the differences and trends were relatively stable across environments, it suggest that these differences to the level of contamination to HT2+T2 are under strong genetic control. It is important in studies comparing the level of resistance between different varieties to *Fusarium* based on DNA or mycotoxin concentration that equivalent material is analysed. Results show that there are large differences in HT2+T2 concentration in groats compared to oats, which does not reflect the differences in the whole plant resistance to *F. langsethiae*.

Chapter 5: Height as a susceptibility trait of oats to *Fusarium* infection

5.1 Introduction

In comparison to conventional varieties, dwarf varieties of cereals are short-strawed. In observational studies across the UK, it was identified that dwarf oat varieties had higher concentrations of HT2+T2 mycotoxins compared with varieties of conventional height (Edwards, 2007a, 2012b, 2015). Nevertheless, the correlation between plant height and mycotoxin levels is not clear and the relationship may be due directly to the morphological trait of the crop height or maybe due to genetic linkage.

Plant development and regulation of stem and leaf growth is supported by gibberellin (GA), a plant hormone. Both positive and negative regulators of GA signalling exist within the plant. One example is DELLA proteins, which are GA signalling repressors and which are coded for by *Rht* genes present in wheat. Wheat varieties with reduced height (*Rht*) genes are GA-unresponsive or resistant and resistant to DELLA protein degradation, which results in dwarfism (Sun & Gubler, 2004).

Contrary to the majority of wheat dwarfing genes used in breeding, dwarf oat varieties are GA-sensitive and involved in GA metabolism and biosynthesis. Oat lines carrying dwarfing genes are responsive to the exogenous application of GA resulting in increased height (Milach & Federizzi, 2001) . There are eight dwarfing

genes identified in oats of which three have been used in breeding programs. Out of these, *Dw6* is the only gene used in UK oat breeding programs. Oat dwarfing genes affect the height of the plant and the length of the internode (Marshall *et al.*, 2013).

The relationship between the plant height and FHB is still not clear. In wheat there are reports of co-localisation of genes for height reduction and FHB QTLs for resistance (Hilton *et al.*, 1999; Draeger *et al.*, 2007; Srinivasachary *et al.*, 2009) and contribution of height per se to FHB susceptibility (Yan *et al.*, 2011). There are no previous studies on plant height and HT2+T2 susceptibility in oats.

A study by Draeger *et al.* (2007) found FHB resistance QTL on wheat chromosome 4D to co-localise with the *Rht-D1* height locus, accounting for 24% phenotypic variance for FHB. Similarly, another study by Srinivasachary *et al.* (2009), where wheat plants were spray inoculated with conidial suspension of *F. culmorum*, found a major QTL (*Qfhs.jic-4D*) located in the region of *Rht-D1* locus as well. These findings would indicate a linkage rather than difference in height as such to contribute in a higher infection rate of dwarf varieties. In the same study the resistance to the fungal spread improved in lines with *Rht-B1b* allele (*Rht-D1b* had the greatest AUDPC, $p< 0.001$). Interestingly, after the point inoculation, *Rht-B1b* lines were less diseased then either tall or *Rht-D1b* lines ($p<0.001$) (Srinivasachary *et al.*, 2009).

Another study using field experiments (Graham & Browne, 2009) showed a significant negative correlation between plant height and FHB in two experimental years ($p < 0.001$; $r = -0.46$ and $r = -0.62$ respectively).

from field experiments conducted over three consecutive years at one location and with an additional experiment in the following year at another location in Germany, Klahr *et al.* (2007) found a relationship between FHB and plant height ($r = -0.42$, $p = 0.01$). Seven QTL were found to be associated with FHB resistance explaining 57% of the phenotypic variance in FHB infection.

Hilton *et al.* (1999) established that a higher infection of dwarf lines ($p < 0.001$) resulted from a contribution of different factors and not only because of height. Wheat lines having *Rht-B1b* allele had a higher level of infection when compared with tall lines and there was a negative relationship between height and disease severity ($p < 0.001$).

A study by Yan *et al.* (2011) provided a contrasting view, supporting that the differences in FHB infection in wheat lines of different height were due to height. The authors aimed to assess the impact of *Rht* genes and to distinguish the impact that height had on both types of resistance. Less severe infection was observed in tall lines ($p<0.01$) but there was no difference when dwarf lines were physically raised to the level of tall ones and point or spray inoculated. Only lines that had a combination of two dwarfing genes (*Rht8* and *Rht9*) showed higher susceptibility. The authors pointed out from a study by Ellis *et al.* (2005) that Rht genes are located at different genomic regions so the differences observed are an effect of plant height per se. What this study omitted to do is to test ground spawn inoculum, given that the ground debris is an important source of inoculum at natural conditions. Authors suggested that morphological and structural differences were implied and controlled by *Rht* genes.

Plant growth regulators (PGR) are mainly used to reduce lodging by shortening the stem of a plant. They alter plant metabolism by regulating plant hormones. Commonly used PGR for oats are those with active ingredients such as trinexapac-ethyl and chlormequat that act as gibberellin biosynthesis inhibitors at various stages of its pathway. While various studies agree that the effect of PGR application is stem shortening, there are inconsistent reports of other traits that might be affected (Rajala, 2004). In previous studies by Edwards (2007) and Edwards & Anderson (2011) there was no significant effect of the use of PGR on HT2+T2 content on oats.

To test whether a high concentration of HT2+T2 mycotoxins observed in dwarf varieties is due to morphological trait of height or genetics, the height of two winter oat varieties (Gerald and Balado) were additionally manipulated with plant growth regulators (PGR) Moddus (trinexapac-ethyl, Syngenta) and 3C (chlormequat, BASF). They were selected as they are the most common PGR used for oats.

The null hypothesis for this study were:

- There is no difference in HT2+T2 concentration between varieties Gerald and Balado
- There is no relationship between height and HT2+T2 mycotoxin concentration
- There is no interaction between variety and height on HT2+T2 mycotoxin concentration

5.2 Materials and methods

The experiment was conducted in 2012/2013 and repeated in 2013/2014 growing season. It was sown in October at the site of Harper Adams University (Edgmond, Shropshire, England). As in earlier experiments and due to the lack of a reliable method of field inoculation, the detected level of mycotoxins and fungal biomass was a result of natural infection in the field. The site chosen for the experiment had high concentrations of *F. langsethiae* in previous seasons. Different concentrations of PGR were applied as detailed in Table 5.1. The PGR treatments were selected to maximise the range of heights achieved across the treatments without causing any phytotoxicity and were based on advice from the PGR manufacturer's and industry experts (Dr S. Cowan, IBERS and Dr A. Wade, ADAS) . In the first year, unfortunately a PGR treatment was applied in error as a blanket spray to the trial as part of the standard agronomy. Consequently the PGR application treatments were modified so that this single application became a base rate instead of the zero PGR treatment. The second year was treated as originally planned.

Other than the PGR application, both experiments had standard farm agronomy inputs. An average seed rate of 300 plants m^2 was used, calculated based on thousand grain weight (TGW) and germination rate. Each experiment included four randomized blocks of twelve plots (6 PGR x 2 variety). Varieties used in experiments were Gerald and Balado. They are both very common among oat growers in the UK. Balado is a dwarf variety on the AHDB Recommended List since 2010 (Marshall *et al.*, 2013). The average height of Gerald in AHDB Recommended List trials is 120 cm and the average height of Balado is 94 cm (AHDB, 2009).

Table 5.1 Treatments with plant growth regulators (PGR) applied as two doses in 2012/2013 at GS30 and as a single dose in 2013/2014 between GS30 and GS31

PGR treatments	Dose 1 in 2012/2013 (applied at GS30)	Dose 2 in 2012/2013 (applied at GS31)	Dose in 2013/2014 (applied at GS30-GS31)
1.	3C 1.125 + Moddus 0.1 L/ha	Untreated	Untreated
2.	3C 1.125 + Moddus 0.1 L/ha	3C 1.125 + Moddus 0.1 L/ha	3C 1 L/ha
3.	3C 1.125 + Moddus 0.1 L/ha	3C 2.250 + Moddus 0.1 L/ha	Moddus 0.2 L/ha
4.	3C 1.125 + Moddus 0.1 L/ha	3C 1.125 + Moddus 0.2 L/ha	3C 1 + Moddus 0.2 L/ha
5.	3C 1.125 + Moddus 0.1 L/ha	3C 2.250 + Moddus 0.2 L/ha	3C 2 + Moddus 0.4 L/ha
6.	3C 1.125 + Moddus 0.1 L/ha	3C 2.250 + Moddus 0.4 L/ha	3C 4 +Moddus 0.4 L/ha

Prior to harvesting, height measurements of plants were taken in each plot from ground to panicle tip. Harvesting and milling was done as described in Chapter 2. Harvested grain was analysed for the combined concentration of HT2+T2 mycotoxins using the ELISA assay as described in Chapter 2. To statistically analyse data, ANOVA and linear regression with groups was used. Grouping was done by variety. When analysing for variance, height was set as a covariate.

5.3 Results

The experiment was conducted in two growing seasons (2012/2013 and 2013/2014) and harvested grain samples were analysed for the concentration of HT2+T2 mycotoxins. HT2+T2 mycotoxin concentrations were plotted against

height grouped by variety, both for the experiment in the first year (Figure 5.1) and for the second year (Figure 5.2).

On both graphs it can be observed that there are two distinct groups. One formed of Balado samples and another of Gerald samples. In the first year the height of Balado was from around 66 to 95 cm and the height of Gerald was from 96 to 115cm (Figure 5.1). In the second year the height of Balado was from around 89 cm to 119 cm and the height of Gerald was from 120 to 155 cm (Figure 5.2). In the first experiment it was noticeable that a taller height meant a lower level of HT2+T2 and at a shorter height there was a range of concentrations (Figure 5.1). In the second year, results at shorter heights were similar but results from taller samples were more inconclusive where there was also a range of heights (Figure 5.2). There was not a strong relationship between plant height of either variety and HT2+T2 mycotoxins in either of the years (Figure 5.1 and Figure 5.2). In the first experimental year, there were distinct differences between varieties but no indication of an effect of height (Figure 5.1).

The relationship between plant height and the concentration of HT2+T2 mycotoxins was tested with regression analysis. To do this, HT2+T2 values were $\log_{10}$ transformed and varieties were grouped into two groups. In the first experimental year the data could be best fitted with two non-parallel lines (Figure 5.1); the regression was highly significant ($p<0.001$) and accounted for 55% of the variance in HT2+T2 concentration.

Analysis of variance in the first year showed that the choice of varieties was highly significant ($p < 0.001$) but the PGR application ($p = 0.068$), the interaction between variety and PGR treatment ($p = 0.392$) and height as covariate ($p = 0.828$) were

not significant as shown in Table 5.2. The same analysis in the second year did not show any significance (Table 5.3).

Table 5.2 ANOVA summary table for analysed oat grain samples in PGR experiment in 2012/2013

Variety	P < 0.001
Treatment (PGR)	P = 0.068
Variety x Treatment (PGR)	P = 0.392
Covariate (height)	P = 0.828
Minimum HT2+T2 (µg kg^{-1})	438
Mean HT2+T2 (µg kg^{-1})	929
Maximum HT2+T2 (µg kg^{-1})	6397
df	31
CV %	4

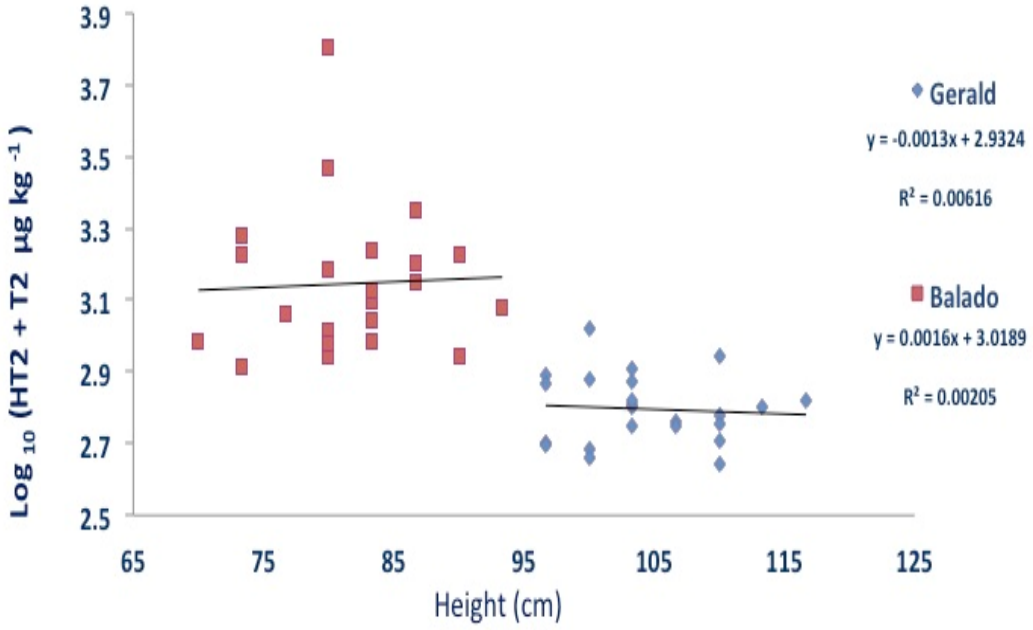

Figure 5.1 Log_{10} (HT2+T2 µg kg^{-1}) of harvested oat grain plotted against the plant height for the PGR experiment in 2012/2013

The plotted data from the second experimental year showed that variety Gerald was inconsistent with six plots with high HT2+T2, whereas all other plots showed low level of HT2+T2 irrespective of height. On the contrary, plots with Balado gave a weak negative relationship (Figure 5.2). In the second experimental year the regression was significant (p = 0.002) and was best fitted by a single line that accounted for only 17.5% of the variance in HT2+T2 concentration.

Table 5.3 ANOVA summary table for analysed oat grain samples in PGR experiment in 2013/2014

Variety	P = 0.263
Treatment (PGR)	P = 0.486
Variety x Treatment (PGR)	P = 0.299
Covariate (height)	P = 0.831
Minimum HT2+T2 (µg kg^{-1})	787
Mean HT2+T2 (µg kg^{-1})	1910
Maximum HT2+T2 (µg kg^{-1})	6383
df	32
CV %	7.7

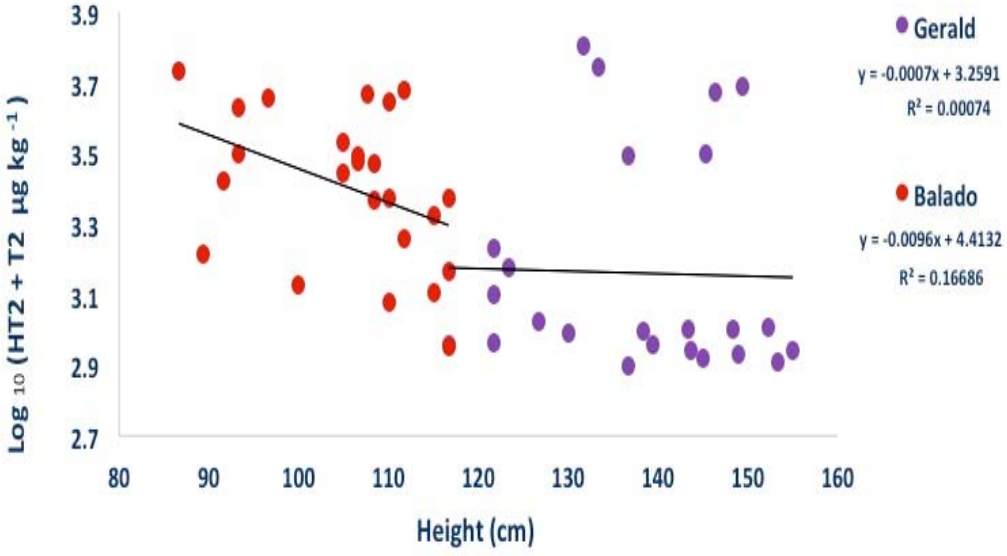

Figure 5.2 Log_{10} (HT2+T2 µg kg^{-1}) of harvested oat grain plotted against the plant height for the PGR experiment in 2013/2014

5.4 Discussion

In previous studies short-strawed oat varieties had a higher level of HT2+T2 (Edwards, 2007a, 2012b, 2015). It was hypothesised that this is due to genetic linkage between height and susceptibility to HT2+T2 producing *F. langsethiae* as for wheat (Hilton *et al.*, 1999; Draeger *et al.*, 2007; Srinivasachary *et al.*, 2009) or that it can be explained by the fact that dwarf varieties are shorter and thus closer to the ground where the predicted source of inoculum is (Yan *et al.*, 2011).

Based on the literature presented and the results from the experiments, height can be expected to be a form of morphological resistance but there is also evidence of a linkage as shown in wheat for *Rht-D1* locus. As debris is thought to be a source of inoculum, it is important to take into consideration the distance from the panicle to the ground. Also, another point for consideration is that due to the oat panicle structure a drier microclimate could be expected as the canopy structure is less dense with tall plants e.g. with long panicles. So it is possible that a combination of a genetic linkage and morphological resistance is a probable explanation.

An attempt to additionally manipulate plant height was made by applying different rates of plant growth regulators (PGR). The issue with using PGR is that it is not fully understood what other physiological effects, other than height reduction, they may have on different processes in plants as well as in sensitivity of the plant species when in stressed conditions (Rajala, 2004).

Varieties, rather than height, were significant after analysing grain samples in the first experimental year. In the second year, statistical analysis did not show any significance. This might be as the experiment relied on natural infection in the field

and fluctuations amongst plots are possible although cv% were relatively low for the field experiment (cv = 4% in the first experimental year and cv = 8% in the second experimental year). Another possible explanation was that even the varieties on a shorter end were higher than in the first year. The tallest Balado in the first years was 95 cm tall while the shortest Balado in the second year was 89 cm tall. The tallest Gerald in the first year was 115 cm tall and the shortest Gerald in second year was 120 cm tall. There is also an issue of not knowing the source of inoculum of *F. langsethiae* nor the dispersal mechanism. Different degrees of splash dispersal might occur between years.

Hilton *et al.* (1999) argued that the FHB severity on wheat is under genetic control and influenced by a number of genes as a quantitative trait. These findings were supported by the fact that plants were spray inoculated and that at similar height (around 90-95 cm), FHB severity was between 25-65%. The relationship between FHB sensitivity and height could be explained either by linkage between genes for resistance and height or pleiotropy where genes promoting one trait promote another trait as well (e.g. shorter height and susceptibility). Similarly to Hilton *et al.* (1999), in both experimental years presented in this Chapter, it was observed that at certain heights there was a range of HT2+T2 concentrations.

If testing for resistance, having an artificial method of inoculation would be of utmost importance. Without it and relaying on natural infection in the field and fluctuating weather conditions is unlikely to give conclusive results. If an artificial method of inoculation was available, then different methods of inoculation could be tested and field experiments as well as glasshouse experiments could be used.

Another challenge in understanding the relationship between height and HT2+T2 contamination on oats is that no QTL for resistance were found so far on oats for HT2+T2 mycotoxins. It would be interesting to see if QTL for height and HT2+T2 mycotoxin resistance co-localise as was shown for FHB on wheat and some wheat dwarfing genes. Evidence from the study on wheat of Srinivasachary *et al.* (2009) suggested that not all semi-dwarf alleles have a detrimental effect and the choice can be made so that desired height is achieved without compromising resistance to FHB. Opposite to findings by Hilton *et al.* (1999), Srinivasachary *et al.* (2009) found that the level of FHB depends on type of alleles (e.g. *Rht-B1b* or *Rht-D1b*). The review of Buerstmayr *et al.* (2003) showed that the resistance to initial infection and fungal spread (Type I and Type II respectively) in wheat can be controlled by different genes therefore an attempt to review the contribution to different dwarfing genes in oats should be made.

Chapter 6: Assessment of *Fusarium langsethiae* infection and mycotoxin production in a Buffalo x Tardis mapping population

6.1 Introduction

QTL (quantitative trait locus) mapping is used as a tool in dissecting complex traits such as resistance. When a number of minor genes are influencing a trait, then the score of that trait can be a range of values between two extremes. Those traits follow a continuous distribution, they have quantitative values and are called quantitative traits (Cell & Group, 1997). Resistance to FHB is thought to be a quantitative trait.

QTL studies rely on connecting phenotypic data such as disease resistance with genotypic data. Constructing linkage maps with markers can reveal the location of QTL related with traits of interest. QTL are parts of a genome which include genes controlling a trait of concern. In order to construct a linkage map, a mapping population is necessary where parents selected vary in a trait of interest. Prior to QTL analysis it is necessary to phenotypically characterise the mapping population (Collard *et al.*, 2005).

Markers represent the differences between individuals and they can be morphological, biochemical or molecular markers. Molecular markers are most commonly used and they show sites of variation at DNA level (Cell & Group, 1997). The disadvantage of morphological and biochemical markers is that they

are not present in great numbers but there are studies that used these markers in characterising differences between oat cultivars e.g. Souza & Sorrells (1991). However, morphological and biochemical markers can be useful when the genetics of the species examined is not well known.

Molecular markers are used to create linkage maps in order to show the estimated position and relative genetic distances in map units, or centimorgans (cM), of markers on chromosomes. Adjacent distances used are statistically showing the most probable marker position on a chromosome (Cell & Group, 1997). Of the different types of molecular markers, the most commonly used are microsatellite or simple sequence repeats known as SSRs markers (Li *et al.*, 2000a,b) and diversity array technology known as DArT markers (Jaccoud *et al.*, 2001; Tinker *et al.*, 2009; Oliver *et al.*, 2011). Relatively new, DArT markers were developed with the hope of lowering costs while offering a broad coverage of the genome (Jaccoud *et al.*, 2001). It is possible to use different types of markers in the same study and combined mapping by SSR and DArT markers has been used in barley (Hearnden *et al.*, 2007) and durum wheat (Mantovani *et al.*, 2008). More recently, DNA sequence analysis has identified many single nucleotide polymorphisms (SNPs) and these have been used to develop markers in a wide range of species including oats. The first oat consensus map was based on a set of 985 SNPs assayed in 390 recombinant inbred lines from six bi-parental populations (Oliver *et al.*, 2013).

Computer software is used to translate the frequency of recombination to centimorgans as they are not linearly related and neither is the distance calculated to the physical distance (Collard *et al.*, 2005). The process of mapping is based on the principles of meiosis where two alleles of a locus will separate with equal

frequencies into gametes (Cell & Group, 1997). The recombination frequency of markers tested can be calculated. If markers or genes are located far apart or not on the same chromosome they are considered as unlinked. Conversely, the lower the frequency of recombination amongst markers, reflects their position closer together on a chromosome. The linkage is estimated by the odds ratios that represent the ratio of linkage versus no linkage and often it is presented as the logarithmic value (logarithm of odds, LOD). Most commonly LOD value of 3 is used for the construction of linkage maps which suggests that the linkage is 1000 times more probable than no linkage (Collard *et al.*, 2005).

The relatively large genome size of hexaploid cultivated oats with 21 pairs of chromosomes (Leggett & Thomas, 1995) has probably resulted in the slow progress of oat mapping. The initial mapping work started with diploid oats (O'Donoughue *et al.*, 1992) with the first model map of cultivated oats published in 1995 by O'Donoughue *et al.* (1995) using the lines from a cross between varieties Kanote and Ogle. The same parents were used in a cross that was additionally mapped in 2003 by Wight *et al.* (2003) and the map of the same cross was improved with DArT markers in 2009 by Tinker *et al.* (2009).

The advantage of using QTL analysis is that it can lead to MAS (molecular marker - assisted selection) which significantly reduces the time needed for breeding new lines, as well as reducing the amount of mistakes related to incorrect phenotyping. Although very useful for simple traits, MAS still has to be improved for complex traits such as breeding for disease resistance due to its complexity and interactions with environment (Francia *et al.*, 2005).

Molecular mapping and identification of QTL associated with disease resistance has been used before in oats for crown rust (Barbosa *et al.*, 2006) and FHB and DON (He *et al.*, 2013). In 2013, (He *et al.*, 2013) detected QTL from two oat populations. To date this is the only study that identified QTL for FHB on oats. As it was conducted in Norway, plots were spawn inoculated with the most important toxigenic *Fusarium s*pecies in Norway, *F. graminearum*.

The 'Green revolution' has had a powerful impact on agriculture in the second half of the 20th century. The major change was the adoption and use of semi-dwarfing genes in wheat and rice. From that, the main result was an increase in yields as an outcome of the reduction in straw length (Milach & Federizzi, 2001). Much of the research on the influence of dwarfing genes was done on wheat. The dwarfing genes are not the same in all cereals and the impact of gibberellic acid differs depending on the type of cereal.

Dwarf phenotypes are found to result from either gibberellic acid (GA)- insensitive or GA-sensitive mutants. The dwarf phenotype of those lines insensitive to the application of GA is due to changes in the signal transduction pathway, while lines that are GA-sensitive are dwarfed as a result of changes in the gibberellin biosynthesis pathway. GA-sensitive mutants response to the application of the GA is stem elongation. Eighty percent of dwarf genes in wheat are GA-sensitive but the most commonly used *Rht1* (*Rht-B1b*) and *Rht2* (*Rht-D1b*) are GA-insensitive where the dwarfism is not a result of GA biosynthesis but rather of a transduction pathway (Milach & Federizzi, 2001). GA-insensitive mutants accumulate GA but there is a block in utilizing it, which would indicate that there are growth suppressors present (Peng *et al.*, 1999; Sun & Gubler, 2004). In oat, several dwarfing genes have been identified and all are GA-sensitive and involved in GA

metabolism. The characteristic of GA-sensitive mutants is that they are deficient in GA as a result of a block in GA biosynthesis (Milach & Federizzi, 2001).

From previous observational studies in the UK, it was shown that dwarf oat varieties had a higher concentration of HT2+T2 mycotoxins (Edwards, 2007a, 2012b, 2015). Yet the relationship between the plant height and the level of mycotoxins is not clear as differences in observed mycotoxin levels might be due to morphological traits of plant height or due to genetic linkages of the dwarfing gene used with other traits.

The high yield of dwarf wheat varieties encouraged research into dwarf oat lines. So far eight dwarfing genes have been identified in oats but only three are in use. Only *Dw6* is in use in the UK and winter oat variety Buffalo was the first dwarf variety to be included on the AHDB Recommended List of varieties in 2003. In addition to the total plant height being reduced by *Dw6,* the lengths of the upper internodes are also reduced (Marshall *et al.*, 2013). The height reduction in oats possessing the *Dw6* dwarfing gene is a consequence of the reduction of the length of the three top nodes whilst the number of nodes is the same. The *Dw7* dwarfing gene decreases the number of internodes while *Dw8* shortens all internodes without interfering with their number (Milacha *et al.*, 2002). Dwarfing can cause a negative effect as if not fully emerged, panicles can have sterile spikelets and a reduction in yield is likely. The dwarf variety Balado gave promising results where a reduction in height was combined with high yielding potential and this variety was included on the AHDB Recommended List in 2010 (Marshall *et al.*, 2013).

It is difficult to establish the homology between oat genes and dwarfing genes of other cereals as hexaploid oat has various chromosomal translocations,

duplications and rearrangements (Milach & Federizzi, 2001). Milach *et al.* (1997) mapped *Dw6* gene on the chromosome 18 that is considered to be the smallest while *Dw7* was mapped on the longest satellite chromosome 19 and *Dw8* to the linkage group 3. It is worth mentioning that *Dw6* and *Dw7* are mapped on the region considered to have other important genes such as genes for rust resistance (Milach & Federizzi, 2001).

A number or research experiments were conducted to determine if the differences in cereal infection with mycotoxins is due to height per se or is it the result of genetic differences. Although researchers agree that there is a negative relationship between the plant height and the level of mycotoxins in wheat genotypes (Graham & Browne, 2009), the cause of this disparity is not clear. Some work was done on identifying QTL associated with FHB and those associated with plant height. The co-localising QTL can be due to linkage or pleiotropy (Draeger *et al.*, 2007; Srinivasachary *et al.*, 2009).

Buerstmayr *et al.* (2003) showed that different genes control fungal penetration (Type I) and fungal spread (Type II) resistance. Height could be contributing to the Type I resistance. Klahr *et al.* (2007) found a correlation between FHB and plant height ($r = -0.42$, $p = 0.01$) and identified seven QTL on wheat chromosomes 1BS, 1DS, 3B, 3DL, 5BL, 7BS and 7 AL explaining 57% of the phenotypic difference involved in FHB infection. Draeger *et al.* (2007) identified FHB resistance QTL on wheat chromosome 4D that co-localised with *Rht-D1* reduced height locus. That QTL accounted for 24% of the phenotypic variance for FHB, so might indicate that the higher infection rate of the dwarfing varieties is due to the gene linkage or that there is a pleiotropic effect rather than the differences being the result of height. Likewise, Srinivasachary *et al.* (2009) identified the major QTL in the region of *Rht-*

D1 locus but not the other well exploited dwarfing allele, *Rht-B1b*. Lines carrying *Rht-B1b* allele had an increase in resistance to fungal spread (Type II). The results from this study showed that breeding for short varieties without compromising resistance is possible when selecting the right dwarfing alleles.

In another study, Hilton *et al.* (1999) found that although the negative relationship between the height and the level of FHB was observed ($p < 0.001$), there was not a difference in the level of FHB infection between lines carrying different dwarfing alleles. FHB might be under genetic control as a quantitative trait and linkage or pleiotropy are possible. Another observation from their experiments is that at certain heights the FHB severity varied in different lines, suggesting genetic control of it rather than height per se.

Yan *et al.* (2011) argued that the differences in FHB susceptibility of wheat lines are the result of height as a morphological trait. An experiment supporting this view was done by physically raising dwarf plants to the level of tall lines. There was no difference in FHB severity except in the double line with *Rht8* and *Rht9* that was more susceptible. Interestingly, dwarf lines showed higher resistance to fungal spread (Type II resistance) which led authors to suggest that a number of morphological and structural changes might exist under the control of *Rht* genes.

When testing wheat NILs with *F. culmorum* it was observed that the increase in disease was not statistically significant ($p<0.05$) between the dwarf and tall lines (Miedaner & Voss, 2008). They noted that one of the dwarfing alleles tested, *Rht8c* from chromosome 2D is closely associated with photoperiod insensitive *Ppd1* allele that affects heading by shortening of the life cycle and earlier

flowering. The authors recommended further exploiting *Rht8c* as it can be separated from *Ppd1*.

Some similar work on barley was conducted by Zhu *et al.* (1999). QTL for plant height were found on chromosomes 1, 2, 3 and 4 to correspond with QTL for FHB. One of the largest effects was on chromosome 2 and it could explain 33% of the phenotypic variance.

The aim of this study was to get an insight into the genetic control of resistance to *F. langsethiae* and the influence of height. A recombinant inbred line population (RILs) derived from a cross between two hexaploid winter oats, Buffalo and Tardis, were used and lines were screened for HT2+T2 mycotoxin levels, *F. langsethiae* DNA and plant height. QTL analysis was conducted on all traits in order to identify genomic regions related to resistance traits and to determine if QTL for different traits co-located in the same regions of the genome. This study aimed to discover links between phenotypic with genotypic data by combining them together in QTL analysis.

6.2 Materials and methods

A mapping population developed at University of Aberystwyth was used for this study. Lines from a F7 population developed from a cross between two conventional husked hexaploid oat varieties Buffalo and Tardis were selected for this experiment. Parents of the population were chosen due to their differences in height. Tardis is of a conventional height and Buffalo contains the dwarfing gene

Dw6. Lines were sown in autumn 2011, 2012 and 2013 in field locations of University of Aberystwyth (Aberystwyth, Wales, UK) at different sites each year, but in close proximity to each other. In all three experimental years, lines were planted in a randomized block design with three replicates but due to the lack of viable seeds some lines were only present in two blocks. Plots were approximately $1m^2$.

Panicle emergence was scored when the panicle was 50% emerged from 50% of the plants in each plot. This was used as an estimate of flowering time. The height measurements of mature crop were taken in each plot from ground to panicle tip. Harvesting was done with a plot harvester when the crop was ripe (growth stage (GS) 92). Samples were stored at room temperature until approximately 100-200 g of samples were sub-sampled. This was then milled using a laboratory mill fitted with a 1 mm screen for subsequent analysis of DNA and HT2+T2 content as described in Chapter 2.

In the first experimental year (2011/2012) all three reps of each of 31 lines plus the parents were analysed. In the second year this was expanded to include a total of 116 lines, whilst in the third experimental year 85 lines were included. Lines were selected based on differences on height and based on the genetic information available. As in previous experiments and due to an inability to artificially inoculate plants, the experiment relied on natural infection at each site.

DNA data and HT2+T2 level of mycotoxins were $\log_{10}$ transformed to normalize the distribution prior to statistical analysis. Analyses of variance and regression were performed using Genstat as detailed in Chapter 2.

The genetic map used in QTL analysis was constructed at University of Aberystwyth and supplied by Dr Catherine Howarth. It consisted of 495 loci from a combination of microsatellite, DaRT and SNP markers and covered a total of 35 linkage groups. Each linkage group was aligned to the most recent consensus map in oats. QTL analysis was conducted on the individual rep data as well as the mean data for each year. Traits used in analysis were height, *F. langsethiae* DNA, HT2+T2 concentration and time to panicle emergence. QTL analysis were performed using simple interval mapping in PLABQTL (Version 1.2, University of Hohenheim, Germany).

Simple interval mapping (SIM) was chosen for QTL analysis. SIM analyses intervals between adjacent sets of markers along chromosomes at the same time. The greater the LOD score, the better chances of QTL being placed correctly. A logarithm of odds score (LOD) at or above 2 was set to be considered as significant in this study. The higher percentage of variance explained indicates how much of the phenotypic variation was explained by the QTL and is another measure of the importance of the QTL (Collard *et al.*, 2005). For each QTL it was also possible to determine which parent provided the positive allele by an examination of the additive effect.

6.3 Results

6.3.1 Field trials 2011/2012

In the first experimental year (2011/2012 growing season) 31 lines were included. There was a low level of HT2+T2 infection and no further lines were analysed. Lines were chosen based on their height with an aim to include a range of heights.

Unbalanced ANOVA was performed blocked by repetition. When analysing *F. langsethiae* DNA, the influence of repetition ($p < 0.001$) and the influence of different line ($p < 0.001$) were highly significant. While in HT2+T2 analysis repetition was highly significant ($p < 0.001$) and the line was significant ($p = 0.026$). Significance of repetition can be explained as the experiments relied on natural infection and spatial variations within the field are possible.

In this first experimental year the average height of Buffalo was 104 cm and of Tardis was 121 cm. HT2+T2 concentration for Buffalo had a back-transformed mean of 245 μg kg^{-1} and Tardis of 200 μg kg^{-1}. Concentration of HT2+T2 in lines was between 114 and 283 μg kg^{-1} (Figure 6.1).

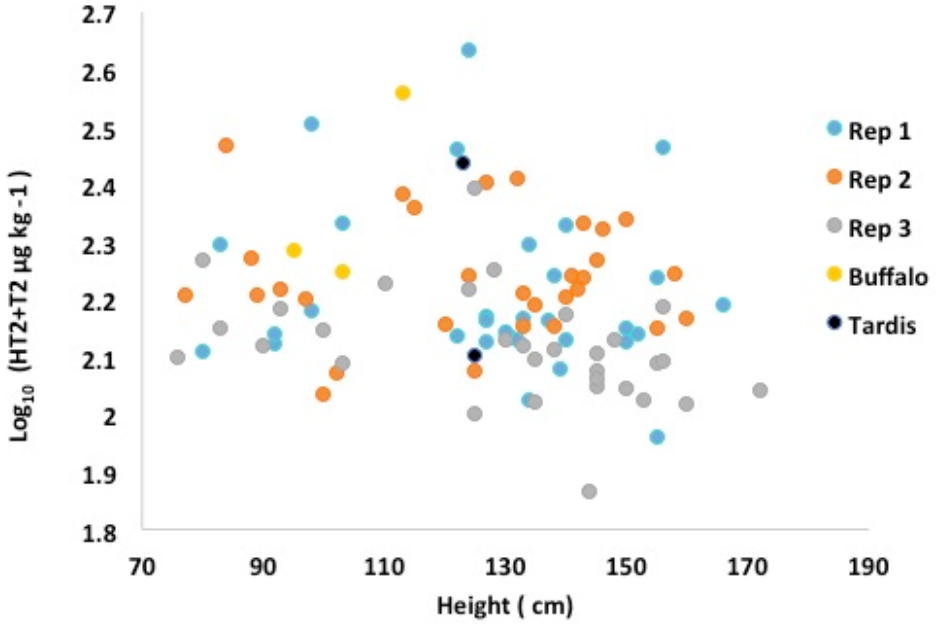

Figure 6.1 Relationship between HT2+T2 mycotoxins (µg kg^{-1}) and height of 31 lines from a cross between winter oat varieties Buffalo and Tardis from the first preliminary experimental year 2011/2012. Three repetitions (Rep1, Rep2, Rep3) are presented separately as well as the parents.

Fusarium langsethiae DNA concentration for Buffalo had a back-transformed mean of 0.043 pg ng $^{-1}$ and Tardis of 0.0098 pg ng $^{-1}$. Generally DNA concentration of Buffalo x Tardis lines was between 0.0056 pg ng $^{-1}$ and 0.078 pg ng $^{-1}$ (Figure 6.2).

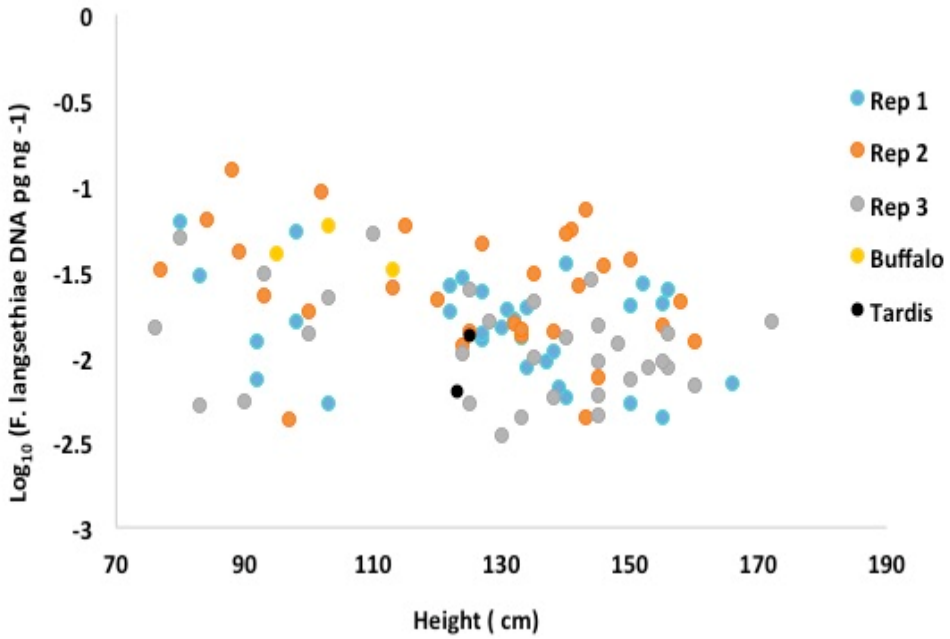

Figure 6.2 Relationship between Fusarium langsethiae DNA (pg ng $^{-1}$) and height of 31 lines from a cross between winter oat varieties Buffalo and Tardis from the first preliminary experimental year 2011/2012. Three repetitions (Rep1, Rep2, Rep3) are presented separ separately as well as the parents

6.3.2 Field trials 2012/2013

In the second experimental year, a total of 116 lines from a Buffalo x Tardis cross were used. Lines were chosen based on height that was between 60 cm to 131 cm with height of Buffalo at 73 cm and height of Tardis at 94 cm. All lines were grown in triplicate but not all plots emerged sufficiently well to be harvested.

Analysis of unbalanced ANOVA revealed repetition and line to be highly significant ($p < 0.001$) for both HT2+T2 and *F. langsethiae* DNA concentrations. Mean back-transformed HT2+T2 concentration of Buffalo was 2039 µg kg^{-1} and of Tardis was

1354 µg kg^{-1}. Concentration of HT2+T2 was between 585 µg kg^{-1} and 5170 µg kg^{-1} (Figure 6.3).

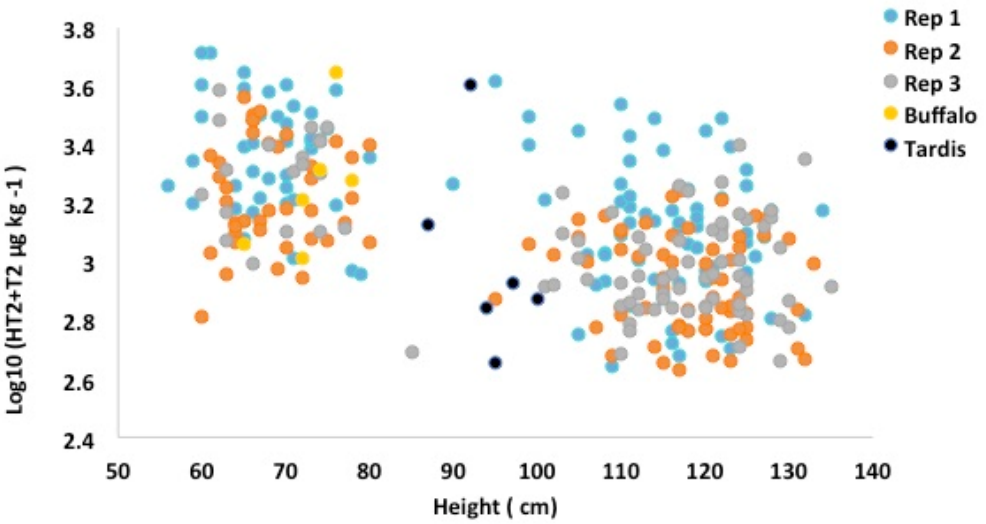

Figure 6.3 Relationship between HT2+T2 mycotoxins (µg kg^{-1}) and height of 116 lines from a cross between winter oat varieties Buffalo and Tardis from the second experimental year 2012/2013. Three repetitions (Rep1, Rep2, Rep3) are presented separately as well as the parents

Mean back-transformed *F. langsethiae* DNA concentration in Buffalo was 0.052 pg ng $^{-1}$and of Tardis it was 0.019 pg ng $^{-1}$. The concentration of *F. langsethiae* DNA among all analysed lines was between 0.0036 pg ng $^{-1}$ and 0.88 pg ng $^{-1}$ (Figure 6.4).

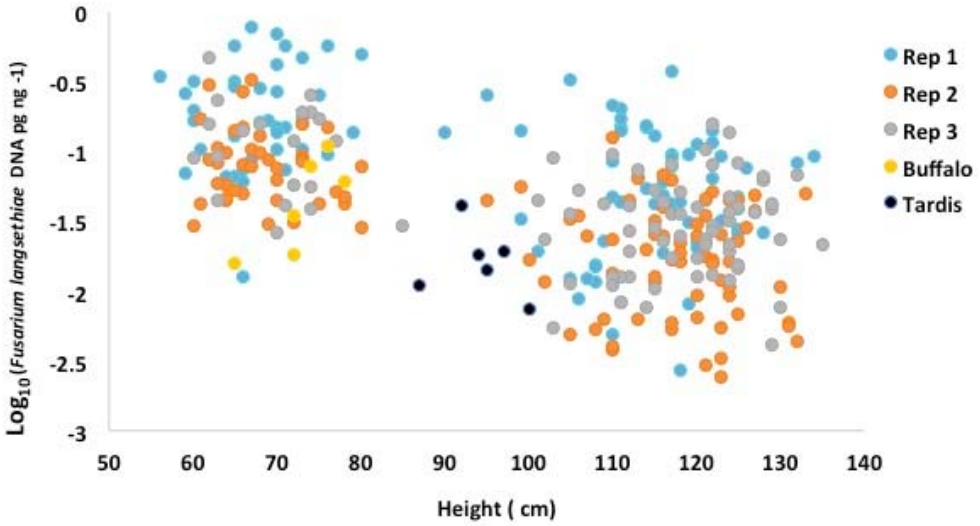

Figure 6.4 Relationship between Fusarium langsethiae DNA (pg ng $^{-1}$) and height of 116 lines from a cross between winter oat varieties Buffalo and Tardis from the second experimental year 2012/2013. Three repetitions (Rep1, Rep2, Rep3) are presented separately as well as the parents.

6.3.3 Field trials 2013/2014

In the third experimental year, a total of 85 lines were analysed. As in the previous year, repetition and line were highly significant ($p < 0.001$) in analysis of unbalanced ANOVA of both HT2+T2 and *F. langsethiae* DNA concentrations. The height of the lines ranged between 67 cm to 159 cm with mean Buffalo height of 102 cm and Tardis of 115 cm. Where possible, and in most of cases, lines were grown in triplicate. Mean back-transformed HT2+T2 concentration of Buffalo was 612 µg kg^{-1} and for Tardis it was 312 µg kg^{-1}. The overall HT2+T2 concentration was between 272 µg kg^{-1} and 814 µg kg^{-1} (Figure 6.5).

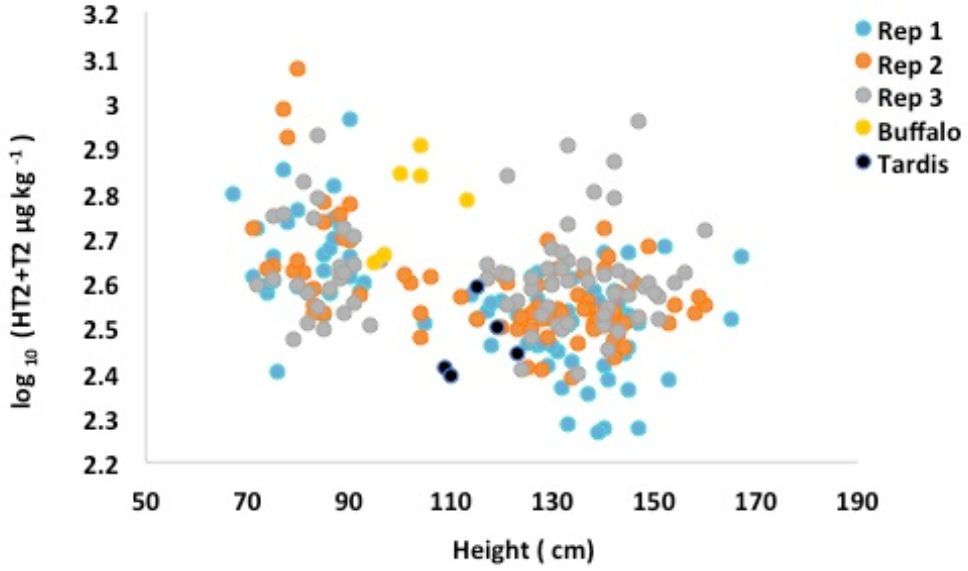

Figure 6.5 Relationship between HT2+T2 mycotoxins (µg kg^{-1}) and height of 85 lines from a cross between winter oat varieties Buffalo and Tardis from the third experimental year 2013/2014. Three repetitions (Rep1, Rep2, Rep3) are presented separately as well as the parents.

Mean back-transformed *F. langsethiae* DNA concentration of Buffalo was 0.19 pg ng $^{-1}$and for Tardis it was 0.015 pg ng $^{-1}$. Concentration of *F. langsethiae* DNA among all analysed lines was between 0.0021 pg ng $^{-1}$ and 0.29 pg ng $^{-1}$ (Figure 6.6).

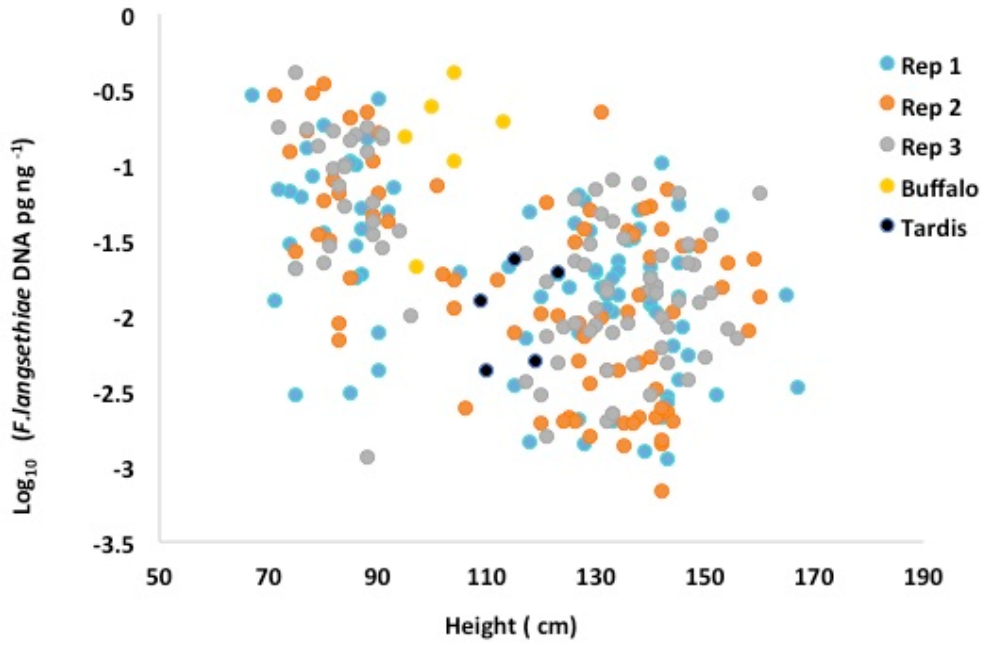

Figure 6.6 Relationship between Fusarium langsethiae DNA (pg ng $^{-1}$) and height of 85 lines from a cross between winter oat varieties Buffalo and Tardis from the third experimental year 2013/2014. Three repetitions (Rep1, Rep2, Rep3) are presented separately as well as the parents.

6.3.4 Combined analysis of three years

Due to similar variance between individual experiments, all experiments were analysed together blocked by block within year. In individual years, data of three repetitions form each trial was presented separately but for the combined analysis, mean data from repetitions is presented. In individual years, HT2+T2 data and DNA data was presented but as it is shown in Chapter 3 by using regression analysis that the relationship between the concentration of HT-2 and T-2 mycotoxins and the level of *F. langsethiae* is significantly related ($p < 0.001$), in this chapter for linear regression with groups only mycotoxin data is presented.

Simple linear regression analysis with groups was performed to investigate the relationship between the level of HT2+T2 mycotoxin concentration in lines from a Buffalo x Tardis cross and height of the lines.

Mean height across all three years ranged between 60 cm to 159 cm. Mean height of Buffalo was 90 cm and mean height of Tardis was 107 cm. Log_{10} transformed HT2+T2 concentration (μg kg^{-1}) across all three years was between 2.055 (back transformed 113 μg kg^{-1}) and maximum of 3.713 (back transformed 5164 μg kg^{-1}). HT2+T2 mycotoxin mean level for Buffalo was 1171 μg kg^{-1} and for Tardis was 743 μg kg^{-1}.

The relationship between the mycotoxin concentration and height proved to be significant ($p < 0.001$). The relationship was best fitted to three separate non-parallel lines. Fitting by three parallel lines was also significant and accounted for 87.6% of the variance, whereas three separate lines accounted for only an additional 1% of the variance. Accordingly three lines could be fitted, one for first year data ($R^2 = 0.057$, $p = 0.098$), another for second year data ($R^2 = 0.43$, $p < 0.001$) and the final line for the third year data ($R^2 = 0.50$, $p < 0.001$) as shown in Figure 6.7.

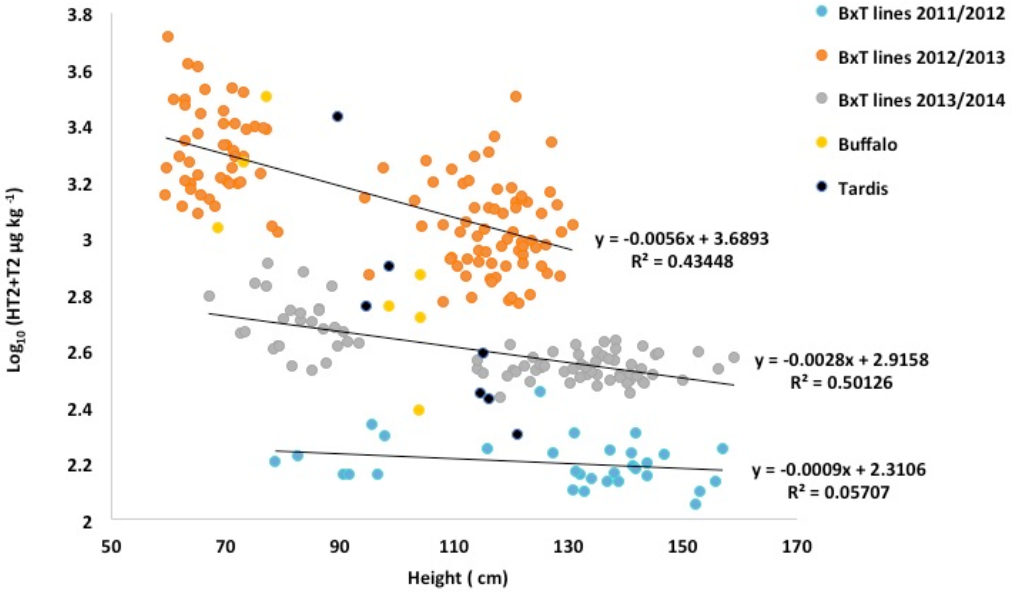

Figure 6.7 Log_{10} transformed HT2+T2 concentration (µg kg^{-1}) of lines from a cross between winter oat varieties Buffalo and Tardis plotted against the plant height. Three trials from 2011/2012, 2012/2013 and 2013 were analysed together grouped by year. The mean of three repetitions from each year was used. In the legend; (BxT lines 2011/2012) lines from first experimental year, (BxT lines 2012 2013) lines from second experimental year, (BxT lines 2013/2014) lines from third experimental year

6.3.5 QTL analysis

A total number of 60 traits related to HT2+T2 concentration and *F. langsethiae* DNA concentration was used in QTL analysis. This involved all lines being analysed separately for each replicate and each experimental year. Log_{10} transformed data was also included alongside back-transformed data. This is because transformed data was used for all other statistical analysis. In addition, traits scored at University of Aberystwyth that related to flowering time and height

were included. A total of 252 significant QTL were identified (LOD>2). The complete list of QTL detected is included in Appendix Table A8.

Generally where QTL for HT2+T2 were identified, QTL for *F. langsethiae* were identified on the same linkage group. Linkage group 29 had the highest concentration of QTL with 24 detected whose LOD score ranged from 21 to 90 and 43 to 86% of variance could be explained by them (Appendix A8). Those QTL with the highest LOD score were detected for height and flowering time. This is in support that the effect of flowering and height is often hard to separate with taller oats having earlier flowering times in this population. Linkage group 29 is equivalent to chromosome 18D where previously the dwarfing gene, *Dw6* has been mapped (Molnar *et al.*, 2012).

QTL for *F. langsethiae* DNA were detected on linkage groups 1, 6, 9, 11, 16, 29, 30, 37 and 39. The ones with the highest LOD score and the percentage of variance explained were in close proximity to QTL identified for height or flowering time on linkage groups 29 and 30 (Figure 6.9).

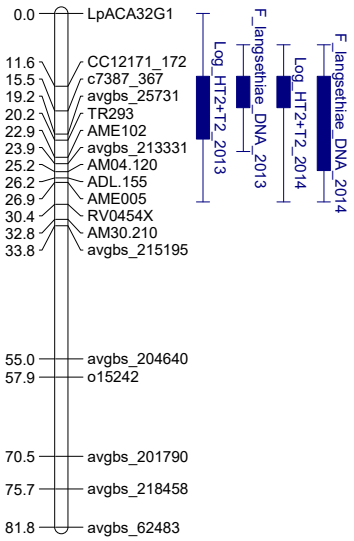

Figure 6.8 Significant QTL identified on linkage group 1 (BT1_Mrg11). Genetic distances in centimorgans are included to the left of the chromosomes followed by markers. QTL identified are on the right side of the chromosome. (Figure provided by Dr Catherine Howarth, IBERS, University of Aberystwyth)

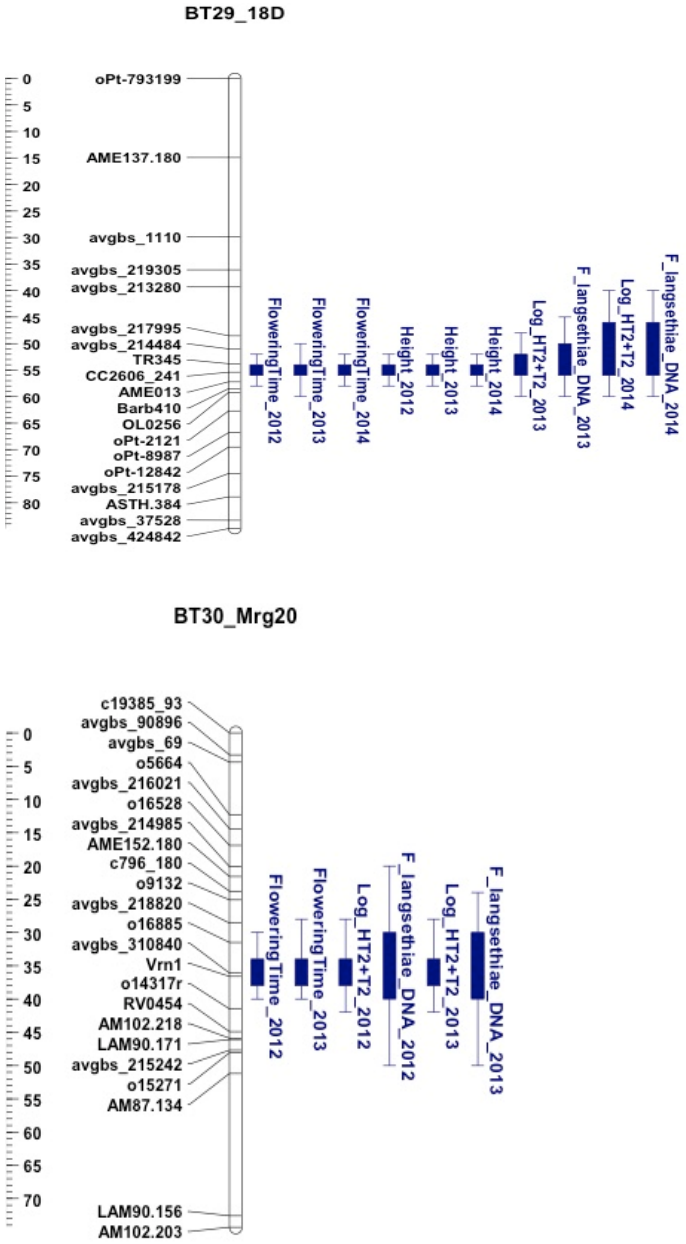

Figure 6.9 Significant QTL identified on linkage group 29 (BT29_18D) and linkage group 30 (BT30_Mrg20). Genetic distances in centimorgans are included to the left of the chromosomes followed by markers. QTL identified are on the right side of the chromosome. (Figure provided by Dr Catherine Howarth, IBERS, University of Aberystwyth)

QTL for HT2+T2 concentration were found on similar linkage groups as 1, 8, 12, 16, 18, 22, 25, 29, 30 and 37. Again as for *F. langsethiae* DNA, those QTL that had the higher LOD and a higher percentage of variance explained were found in close proximity to height and flowering QTL. For the QTL found on linkage groups 29 and 30, the Tardis allele provided resistance to infection and accumulation of mycotoxin as well as promoting taller plants.

Some of these QTL were only identified in individual rep data from one year. For example, linkage group 6 revealed few QTL associated only with the level of mycotoxins but their effect was not stable across repetitions and years. The same was true for linkage group 8. Similarly, linkage groups 11, 12, 18, 22, 37 and 39 all had QTL detected for both *F. langsethiae* and the level of HT2+T2 mycotoxins but they were also not repeated in all repetitions or years.

It would be interesting to investigate further into linkage group 1 (Figure 6.8). That group did not have any QTL identified for height and flowering associated with it but did have some QTL identified for both *F. langsethiae* DNA and the concentration of HT2+T2 in both the 2012/2013 and 2013/2014 field seasons. The LOD score ranged between 2.5 to 4 for HT2+T2 and accounted for 16% of the variance.

6.4 Discussion

This is the first study that identified QTL for *F. langsethiae* resistance and HT2+T2 concentration in oats. Some of the QTL were found in close proximity with the QTLs for height and flowering. However, QTL for *F. langsethiae* resistance were identified on linkage group 1 which were not associated with height or flowering.

Across the three years there was a range of HT2+T2 observed with high levels and a greater range in the second and third year. The DNA and mycotoxin analysis showed significant differences in the level of contamination between different lines from a Buffalo x Tardis cross particularly in the second and third year. In low HT2+T2 situations height had little influence but as risk increased the importance of height increased. In all three experimental years there was a distinctive cluster dividing data sets into two groups based on height. Taller lines tended to have lower levels of HT2+T2 mycotoxins but on the shorter end of heights there was a range of HT2+T2 levels. This might indicate that it is still possible to breed for shorter height while not losing *Fusarium* resistance. This is supported by the findings of Hilton *et al.* (1999) who found that wheat lines of similar height had different FHB levels suggesting it is not only the straw height per se affecting FHB severity. In that study a plant height at around 90-95 cm resulted in FHB severity varying between 25-65% in different lines and in addition to that, the lines of similar height had a significant difference in disease severity.

In the first experimental year there was little difference between lines and as only 31 lines were included, this experiment was used only as a preliminary study. This is because the entire study depended on natural infection in the field and the first

experimental year was used to determine if there is a difference in the level of HT2+T2 mycotoxins between different lines of a Buffalo x Tardis cross. A wet summer in 2012 might have contributed to the lower level of HT2+T2 concentration. With a lower level of mycotoxin levels it is harder to statistically determine the differences between different lines. Despite this, it is possible to see consistent trends where there was a negative relationship between height and HT2+T2 mycotoxins.

The noticeable differences in the level of HT2+T2 mycotoxins between the three experimental years can be explained by lack of artificial inoculation and sensitivity to natural conditions such as rainfall. Due to an inability to artificially inoculate plant, the experiment depended on natural infection in the field. It is essential to develop methods for artificial inoculation and test different populations of hexaploid oats for *F. langsethiae* resistance. QTL can then be compared across populations and environments for a more stable prediction of the position of resistance traits along the chromosomes. This could be used further by breeders in future breeding programs.

QTL for HT2+T2 and *F. langsethiae* were identified in close proximity, on the same linkage groups. This strengthens the earlier findings that *F. langsethiae* is the predominate producer of HT2+T2 mycotoxins in UK oats (Edwards *et al.*, 2012; Opoku *et al.*, 2013).

The highest LOD score and variance that can be explained was associated with height and flowering QTL. They were often found on the same genomic region or linkage group. Often the same linkage group had QTL for *F. langsethiae* and HT2+T2 and for height. It is hard to dissect the genetic influence of height or

flowering on resistance and susceptibility. Having QTL found together might indicate linkage but it is not necessary that height exclusively influence the level of HT2+T2 mycotoxins. Some QTL were detected only in one season. It would be worth including more lines from the same population and testing them for *F. langsethiae* resistance.

Few QTL for either HT2 +T2 concentration or for *F. langsethiae* DNA were detected in the 2011/2012 field season. Not only was the level of infection low in this year but also only 31 lines of the population were analysed and this reduced greatly the power of the QTL analysis. Increasing the number of lines analysed from the subsequent two years of field screening could potentially increase the accuracy and resolution of the QTL identified in this analysis.

Results have shown that parts of the genome responsible for *F. langsethiae* disease resistance are either in close proximity with QTL for height (on linkage groups 29 and 30) or that they are also placed in different linkage groups (such as linkage group 1) therefore it is possible to breed for resistance while keeping the desired reduced plant height. QTL which are stable across years with high LOD score might find use in marker-assisted breeding.

The only other study of QTL for resistance to FHB in oats was done by He *et al.* (2013). The markers reported were not common to this study and as a different cross was used it was hard to compare the QTL identified. Moreover, in a study by He *et al.* (2013), a different *Fusarium* species was used which resulted in the accumulation of DON rather than T2+HT2. Only one of the crosses used (Hurdal x Z595-7) had QTL identified in the same region as in the study presented in this Chapter. QTL identified were for plant height, days to maturity and days to heading, all found on chromosome 18D (QTL region 45-48 cM). On the same

chromosome there were no QTL for either DON or FHB identified. Analysis of QTL in this chapter identified flowering time, height, *F. langsethiae* DNA and HT2+T2 on the similar region of the 18D chromosome.

The manipulation and improvement of traits such as disease resistance would benefit society as mycotoxins produced by *Fusarium* species are harmful and not desirable in neither food nor feed. QTL mapping could help in breeding for more resistant varieties. It can provide valuable information when genomic regions involved in resistance are mapped. The advantage of QTL analysis is in combining phenotypic with genotypic data. The number of QTL identified, their position and effect are important but so is the environment. As not all QTL are stable across different environments (Snape *et al.*, 2006) it is worth checking their stability but also checking them in different populations for a more accurate estimation of an effect.

Visual assessment of *F. langsethiae* infection in oats is not possible as to date no visible symptoms of *F. langsethiae* on oats have been documented. Another limiting factor is the lack of methods for artificial inoculation. When relying on natural infection if a disease pressure is low, it is likely that subtle differences in resistance or susceptibility between different varieties will not be observed. To successfully score for disease resistance, methods of artificial inoculation need to be developed or adapted from already existing methods of inoculation with different plant pathogenic species. Future work might include testing near isogenic lines (NILs) that only differ in a small genomic region of interest in a wide range of environments.

Chapter 7: Investigating the ability of *Fusarium langsethiae* to infect the model species *Brachypodium distachyon*

7.1. Introduction

Fusarium langsethiae is the predominant *Fusarium* species on UK oats. Damage is not direct as there is no yield reduction but there is a great potential for economic loss due to contamination of grains with HT2+T2 mycotoxins (Edwards, 2012b; Opoku *et al.*, 2013). Symptoms of *F. langsethiae* on cereals are not reported yet but as oat grain can be contaminated with harmful mycotoxins it is important to investigate differences in resistance of different oat varieties. To do so a reliable method of artificial inoculation is needed.

In a glasshouse study by Divon *et al.* (2011), brown lesions, light in colour were reported on spikelets as a result of artificial inoculation with microconidial suspension of *F. langsethiae* and an extended period of high humidity. Previous efforts to cause symptoms on oats by artificially inoculating with *F. langsethiae* were not successful as reported by Imathiu (2008) and Opoku (2012). Repeated experiments with artificial inoculation of oats as part of this project failed to show any symptoms (results not shown).

A wealth of information on using *Arabidopsis thaliana* as a model species in cereal-pathogen interactions and especially interaction with *Fusarium* species is documented (Brewer & Hammond-Kosack, 2015). Although information obtained

using *Arabidopsis thaliana* as a model species in FHB (Fusarium Head Blight disease complex) is valuable, *Arabidoposis* has some obvious differences to cereals, the main one being that it is a dicotyledon. Rice, maize, sorghum and different biomass grasses were at one point seen as potentially good candidates for model species but there were a number of practical reasons for not using them such as their difference to cereals photosynthetic pathway, lifecycle, plant height and intellectual property agreements (Brkljacic *et al.*, 2011).

Brachypodium distachyon was for the first time proposed as a model species for genomics in grasses in 2011. It is a diploid plant with five chromosomes and described as having the simplest genome of all grasses. It is a self fertile, inbreeding annual plant without demanding growing requirements. Its popularity is increasing due to a relatively fast life cycle (from seed to seed in less than 4 months) and a small size of approximately 20 cm (Draper *et al.*, 2001). There are between 15 and 18 *Brachypodium* species with different geographical distribution and ploidy but the only annual diploid *B. distachyon* was suggested as a model species. Molecular markers derived from *B. distachyon* are available which make identifying loci for traits of interest and translating that knowledge to cereals possible (Mur *et al.*, 2011).

Previously there were attempts of challenging *B. distachyon* with various plant pathogenic species. Chosen *Brachypodium* lines were all resistant to *Blumeria graminis* (powdery mildew) and *Puccinia recondita* (brown rust). Opposite to that, *Brachypodium* lines showed symptoms of diseases when inoculated with *Magnaporthe grisea* (rice blast) and *Puccinia striformis* (yellow stripe rust) (Draper *et al.*, 2001). Successful inoculation of *B. distachyon* lines with *F. graminearum* and *F. culmorum* was also reported (Peraldi *et al.*, 2011). The aim of this chapter

was to investigate whether selected *Brachypodium* lines are susceptible to the infection with *F. langsethiae* and if infection results in visible symptoms.

7.2 Materials and methods

7.2.1. Fungal inoculum

Inoculation experiments with *B. distachyon* required isolated cultures of *F. langsethiae* to be purified to single spore and spores produced adjusted to known concentration. Four isolates of *F. langsethiae* from grain originating at different locations (isolate codes as follow: D5, R2, B1 and G1) were used to produce inoculum as detailed in Chapter 2. Single spore isolates from stored PDA slopes were cultured onto PDA plated at room temperature. Approximately 3-week-old cultures were flooded with 5 ml SDW and the surface was scraped with a glass rod. Harvested spore suspension was filtered through two layers of sterile muslin cloth and the concentration was determined using Improved Neubauer haemocytometer (Weber, England). The concentration was adjusted to 10^6 spores ml^{-1} SDW and amended with 0.05% Tween20 (Sigma, UK) L^{-1} SDW.

7.2.2. *Brachypodium distachyon*

A preliminary experiment was conducted with Bd 21 line and as infection with *F. langsethiae* was successful, the decision was made to include Bd 21-3 and Bd 3-1 lines in all *B. distachyon* studies such as detached leaf, root and floral assay with spikes at anthesis.

Seeds of Bd 21 and Bd 3-1 were obtained from Dr Antoine Peraldi, Department of Crop Genetics, John Innes Centre. Seeds of Bd line 21-3 was obtained from Dr Luis Murr, Institute of Biological, Environmental and Rural Sciences, University of Aberystwyth. These lines were chosen as they are well known and exploited in *Brachypodium* research community (Mur *et al.*, 2011).

To prepare seeds for potting they were soaked in sterile water for approximately 2 hours for the easier removal of the back lemma. After removal of the lemma, seeds were immersed in 70% ethanol for 30 sec, rinsed with sterile deionised water (SDW) and soaked in sodium hypochlorite (1% available chlorine) for 4 min with gentle shaking. Disinfected seeds were rinsed three times with SDW and placed on a wet sterile filter paper in Petri dishes for incubation. Initial incubation was in darkness at 5°C for 3 days which was followed with seed exposure to 16 h light and 8 h dark period and at 25°C for 3 days to allow the germination. Germinated seeds were potted in pots (13 cm diameter x 9.9 cm depth; Desch Plantpak, Netherlands) if used for flowering experiment or 24-cell tray (50 x 48mm) in case of the detached leaf experiment. The potting mixture was made by mixing a volume of 50% John Innes compost Number 2 with 25% peat and 25% grit. The mixture was mixed in a cement mixer.

Potted seedlings were grown in the Standard Fitotron® Growth Chamber (SGS 120, Fitotron®, Weiss Technic, UK). Temperature settings differed depending on the assay and it is detailed in the relevant subsections of this chapter. Humidity was set to 60% which is considered to be a standard practice at Harper Adams glasshouses. They were watered twice a week. In these conditions plants were flowering after approximately four weeks.

7.2.3. Floral assay

Potted plants for inoculation at anthesis were grown at 22°C under 20 h light and 4 h dark photoperiod. There were18 pots of each line (as the shelf space allowed for). Each pot had 3 plants but in some pots there were only two plants as germination was not equally successful.
The Fitotron growing cabinet used in this experiment had two shelves and each shelf was randomised with pots of each *B. distachyon* line.

Plants were sprayed with inoculum until run-off (i.e. until the first droplets were collected on the leaf and dripped from the leaf edge). A hand-held garden sprayer was used for spraying. Plants on each shelf were sprayed three times. There was a break of approx. 15 min between spraying to allow inoculum on plants to dry before applying another spraying. Plants selected as uninoculated control were treated with sterile water. The inoculated plants were bagged for 3 days to maintain a high humidity. Data loggers placed inside the bags showed RH up to 90%. When bags were removed, symptoms were recorded on a daily basis. *Brachypodium distachyon* heads were harvested 30 days after the inoculation to allow plants to fully ripen. All heads were harvested, frozen and then freeze dried in a Modulyo® Freeze Dryer (Edwards, UK). Harvested spikes were kept at -20°C until subsequent milling and analysing for HT2+T2 mycotoxins and *F. langsethiae* DNA concentration. DNA extraction and qPCR were performed as described in Chapter 2 adjusted for low sample weight of 0.250 g and 1.08 ml CTAB buffer. HT2+T2 extraction and quantification were performed as described in Chapter 2 adjusted for low sample weight of 0.250 g and 0.9 ml of 70% Methanol.

The floral assay was performed twice. Data was $\log_{10}$ transformed to normalise distribution prior to analysis of variance. Analysis of variance was performed blocked by individual experiments and repetitions within.

7.2.4. Detached leaf assay

Seeds were prepared as described previously in this chapter, potted in 24-cell tray with the soil mixture described earlier and grown under 16 h light and 8 h dark period and at 20°C. For this assay a Sanyo environmental test chamber was used. Eighteen days after potting, the third leaf was cut (approx. 55 mm long piece) and placed in prepared water agar plates (20 g agar/L dH_2O). Cut pieces of leaves were placed on the agar surface with top leaf surface facing up. The ends of the leaves were pushed down into the agar with a sterile narrow spatula so that the leaves were fixed into their position. Each Petri dish had three randomised leaves. Inoculum made of single spore cultures was made as previously described. A 5 µl drop of inoculum was placed in the middle of the leaf with a 10 µl pipette tip. Care was taken not to wound the leaves. Leaves were incubated and kept at 20°C as that is shown to be favourable for *F. langsethiae* growth and HT2+T2 toxin production (Infantino *et al.*, 2007; Imathiu, 2008).

The detached leaf assay was replicated four times. Brown necrotic lesions were measured by ruler 7 days after the inoculation with spore suspension of *F. langsethiae* (7 dai). Four independent experiments were analysed together by ANOVA, blocked by experiment and repetitions within.

7.2.5. Root assay

Seeds were treated as previously described. For this assay a Panasonic MIR -154 cooled incubator was used. After 3 days in darkness at 5°C seeds were transferred into a new filter paper in a Petri dish, wetted with SDW and incubated at 20°C for 16/8 light period to allow germination. After 3 days, germinated seedlings were transferred onto a new Petri dish with 3 seeds per Petri dish, inoculated with 5 mm mycelium plug using the reverse of a pipette (1000 µl) tip from the edge of a 3-week-old colony of *F. langsethiae* (isolate D5) grown on PDA. The mycelium plug was placed upside down on the root making a direct contact between the root and the sporulating side of the agar, positioned at equal distance from either end of the root. Plates were incubated for 3 days at 20°C for 16/8 light period. Three days after the inoculation brown lesion on *B. distachyon* roots were scored for symptom extension (SE) and their length was measured in mm. The protocol and scale for screening roots was adapted from Covarelli *et al.* (2013). The root assay was repeated five times.

7.3. Results

In all assays, there was no development of symptoms on control plants, inoculated with sterile water and incubated in the same way as treated plants. On inoculated spikes, leaves and roots brown necrotic lesions developed (Figure 7.1). Results of measurements from individual assays were analysed by ANOVA.

7.3.1. Floral assay

Statistical analysis showed that *B. distachyon* line (Bd) was significant for the HT2+T2 concentration ($p = 0.015$). The range of HT2+T2 was between 119 µg kg^{-1} to 1596 µg kg^{-1}. Tukey's test showed that that there was a significant difference between Bd 21 (back transformed mean 615 µg kg^{-1}) with the highest concentration of HT2+T2 and between Bd 3-1 that had the lowest concentration of HT2+T2 mycotoxins (back transformed mean 440 µg kg^{-1}). In the middle was Bd 21-3 that was not significantly different from other lines (back transformed mean 470 µg kg^{-1}), as shown on Figure 7.2.

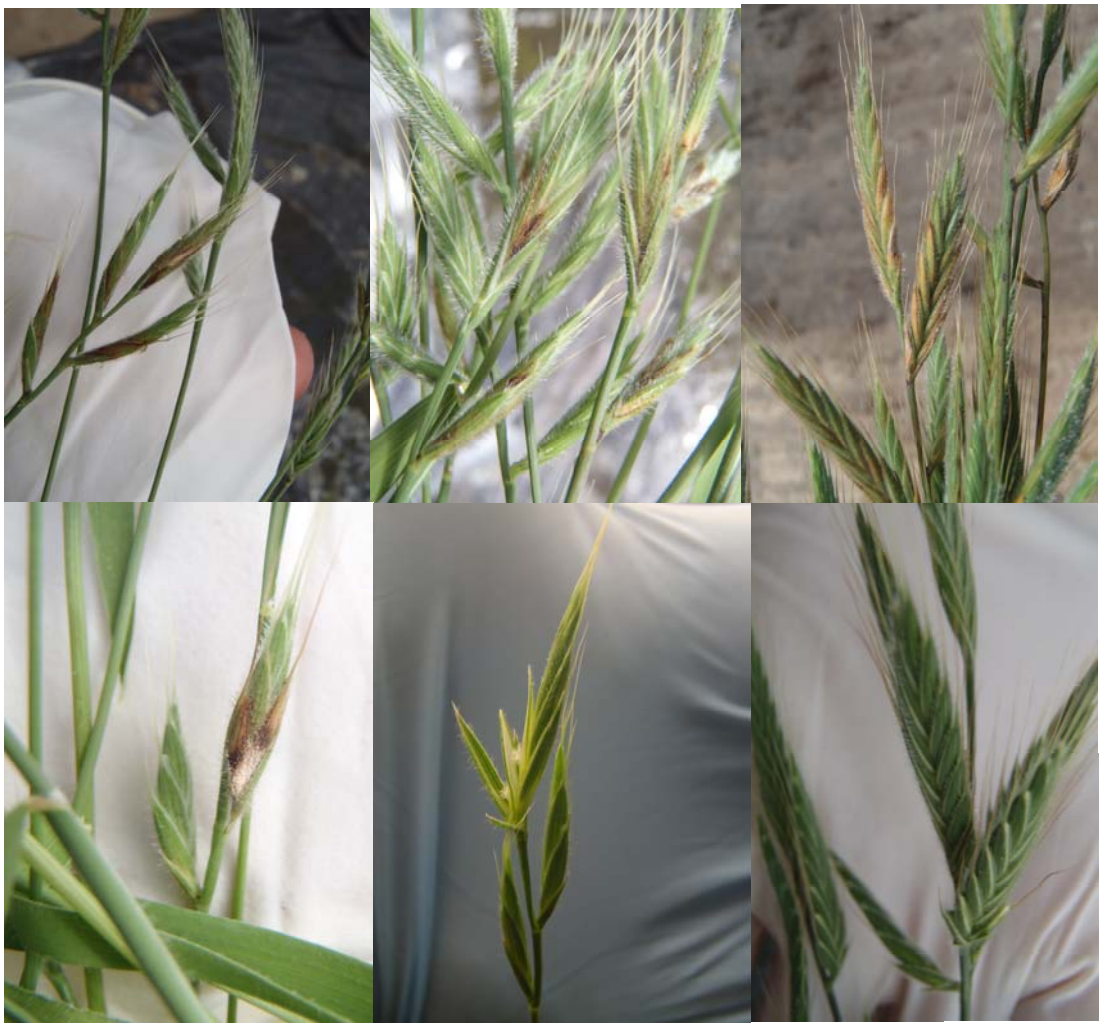

Figure 7.1 Symptoms of Fusarium langsethiae infection on spikes of Brachypodium distachyon line Bd21. Top row and bottom left are inoculated plants, bottom middle and right are uninoculated controls

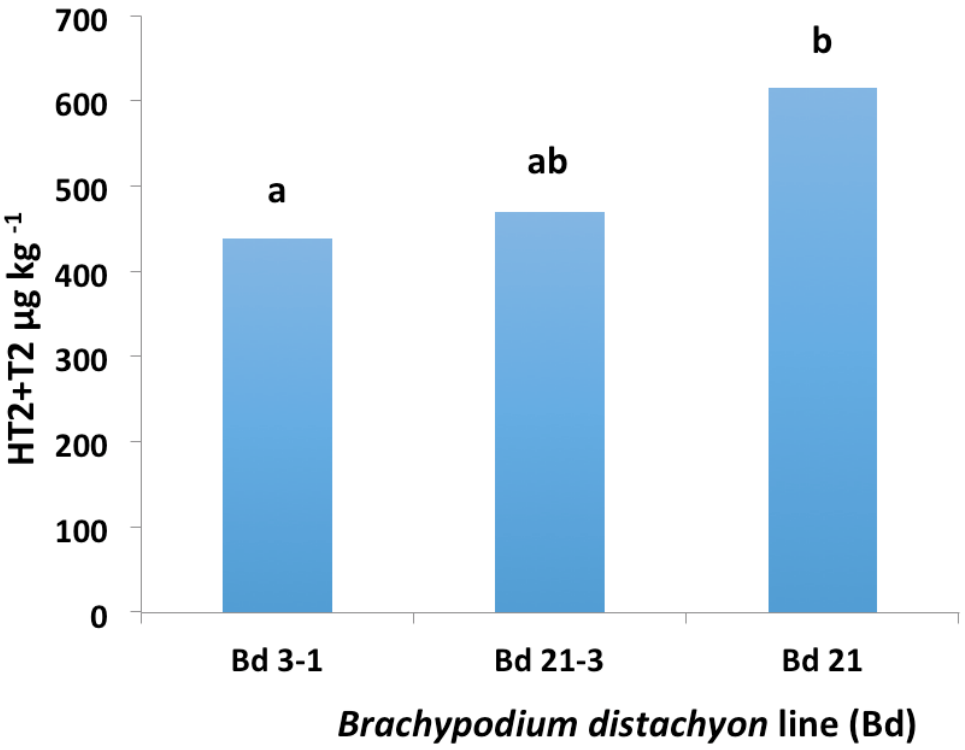

Figure 7.2 Back-transformed concentration of HT2+T2 mycotoxins (µg kg $^{-1}$) in three different B. distachyon lines (Bd 3-1, Bd 21-3, Bd 21). Lines with the same letter were not significantly different, according to Tukey's test (p=0.05).

When analysing *F. langsethiae* DNA concentration, Bd line was significant (p = 0.009) and the differences between lines were as for HT2+T2 mycotoxins. The range of *F. langsethiae* DNA was between 0.023 pg ng $^{-1}$ and 13.90 pg ng $^{-1}$. Tukey's test showed that that there was a significant difference between Bd 21 that had the highest level of DNA (back transformed mean of 1.54 pg ng $^{-1}$) and

between Bd 3-1 that had the lowest concentration of DNA (back transformed mean of 0.51 pg ng $^{-1}$). In the middle was Bd 21-3 that was not significantly different from other lines (back transformed mean of 0.97 pg ng $^{-1}$), as shown in Figure 7.3.

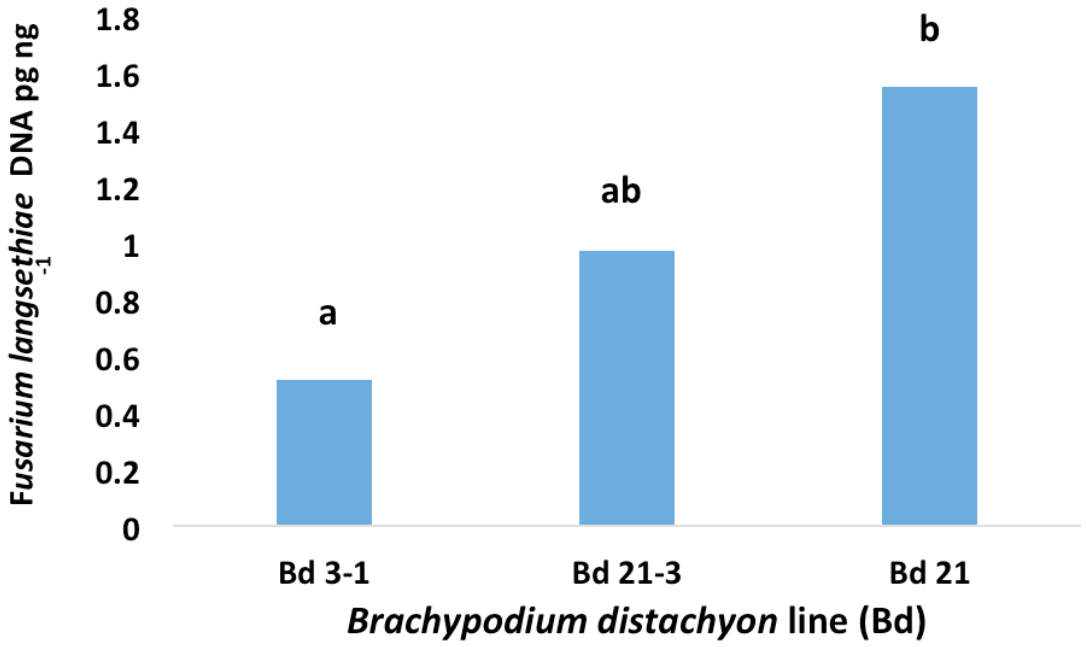

Figure 7.3 Back-transformed concentration of Fusarium langsethiae DNA (pg ng $^{-1}$) in three different Brachypodium distachyon lines (Bd 3-1, Bd 21-3, Bd 21). Lines with the same letter were not significantly different, according to Tukey's test (p=0.05).

7.3.2. Detached leaf assay

Analysis of variance showed that the impact *of B. distachyon* (Bd) line was significant (p = 0.015). Lesions measured on leaves ranged between 2 mm to 9 mm. Tukey's test identified that there was a significant difference between Bd 21-3 that had the smallest lesion size (mean 5 mm) and the other two lines i.e. Bd 21 (mean 6 mm) and Bd 3-1 (mean 6 mm), as shown in Figure 7.4.

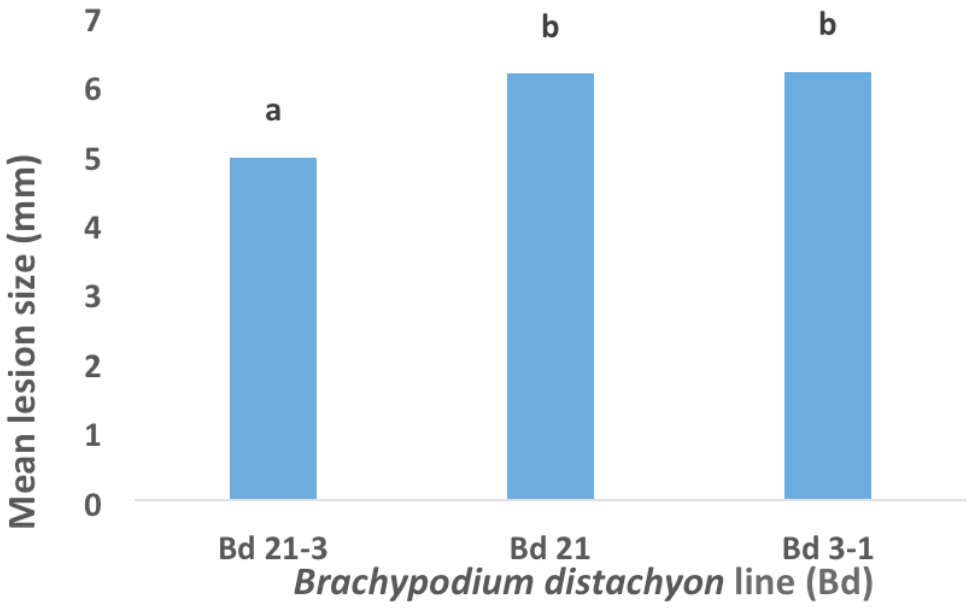

Figure 7.4 Mean size of lesions measured (mm) on leaves of three different Brachypodium distachyon lines (Bd 3-1, Bd 21-3, Bd 21). Four independent experiments were analysed together blocked by experiment and repetitions within. Lines with the same letter were not significantly different, according to Tukey's test (p=0.05).

7.3.3. Root assay

ANOVA was performed on results blocked by experiment. Results did not show any significance and that the *B.distachyon* line had a significant influence (p = 0.084), with a general mean of 15 mm. Due to this insignificance, results are not shown.

7.4. Discussion

This is the first study to successfully inoculate *B. distachyon* lines with *Fusarium langsethiae*.

The European Commission is currently considering putting new legislative limits or guidelines for the joint concentration of HT2+T2 mycotoxins in cereals and cereal products (European Commission, 2013b). *Fusarium langsethiae* is the predominant species on UK oats, detected in 99% of oats samples collected in a survey from 2002-2004 (Edwards *et al.*, 2012). A highly significant relationship between *F. langsethiae* DNA from oat samples and the level of HT2+T2 mycotoxins in the same samples indicates that *F. langsethiae* is the main producer of these toxins on UK oats (Opoku *et al.*, 2013). When testing for varietal response to different pathogens, a method of artificial inoculation is needed. This project has demonstrated the difficulties of relying on natural infection in the field when testing oat varieties for *F. langsethiae* disease resistance. The only report outlining a method of artificial inoculation in controlled conditions and symptoms of *F. langsethiae* on oat panicles did not manage to produce easily distinguishable

symptoms (Divon *et al.*, 2012). One additional difficulty is that there are no reports of *F. langsethiae* symptoms in the field (Opoku *et al.*, 2013).

Due to the inability to artificially inoculate cereals with *F. langsethiae*, the inability of *F. langsethiae* to cause visual symptoms on cereals and given the recent successful inoculation of *B. distachyon* with other *Fusarium* species (Peraldi *et al.*, 2011), this chapter's aim was to investigate possibilities of infecting *B. distachyon* with *F. langsethiae*.

Some promising results were obtained. All two testing assays (detached leaf and flowering assay) showed successful inoculation with *F. langsethiae*. Dark brown lesions were recorded in all three assays. In addition to that, *F. langsethiae* DNA was isolated from all samples and HT2+T2 mycotoxins quantified. This is important as mycotoxin contamination is one of the most important challenges for food safety.

The root assay did not show significant difference between different lines. This might be due to an inadequate scoring method where browning was scored and expressed as length of lesions. Often lesions were not well defined. In addition, when handling roots and leaving them for the incubation they naturally start changing colour from white to cream which in older roots can become brown. For that reason the existing protocol was modified and lesions scored 3 days after the inoculation.

Preliminary experiments with wounded and unwounded leaves (results not shown) showed similar results and it was decided that the unwounded leaves assay mimics better the natural infection. The lesions were oval in shape thus it was

decided that measuring length with a ruler was adequate. The ability of *F. langsethiae* to cause symptoms on unwounded leaves of *B. distachyon* is in accordance to the previous work by Imathiu *et al.* (2009) where wounding was necessary for infection of wheat leaves but unnecessary for infection and development of symptoms on oat leaves. Results of a detached leaf assay showed that there was a difference between different *B. distachyon* lines. Line Bd 21-3 was shown to be less susceptible to the infection with *F. langsethiae*.

The floral assay was the most laborious method but might be the most accurate as it did not depend on subjective observation of colour or on a measure of lesions that was easily confused with natural discolouration. Spikes of all the lines tested gave symptoms of infection but for the more accurate testing of susceptibility, all heads were harvested and *F. langsethiae* DNA as well as concentration of HT2+T2 was quantified. The floral assay is also more likely to be a closer mimic of the natural infection of oat panicles.

Apart from the root assay that did not give any differences in *B. distachyon* lines, applying statistical analysis on the floral and leaf assay it was possible to compare responses in three lines. Whilst Bd 3-1 was the most resistant in the floral assay it showed greater susceptibility in the leaf assay. This is not surprising as one would expect there is a different selection of genes acting on resistance for floral, leaves and roots where some genes are different and some are the same (Broekaert *et al.*, 2000).

The method of *B. distachyon* inoculation itself can be improved. The observed disadvantage of this method for the leaf assay is that narrow leaves as those from *B. distachyon* can twist and inoculum placed on the side can slip. If the drop takes

longer when slipping, the inoculum leaves traces of spores over a wider area than that covered with only 5 µl of inoculum, thus when measuring lesions it is hard to compare which lesions were made by droplet of inoculum and which lesions are wider because the inoculum did not stay in place. A method with a thickening agent such as guar gum could help standardise the inoculum droplet size.

Results from this study suggest that out of three tested *B. distachyon* lines Bd 21 showed significant susceptibility to *F. langsethiae* infection and production of HT2+T2 mycotoxins where Bd 3-1 had lowest level of recovered *F. langsethiae* DNA and HT2+T2 mycotoxins. Findings from this study are opposite to findings of the only other study investigating susceptibility of *B. distachyon* to *Fusarium* species (Peraldi *et al.*, 2011). In that study, Bd line 3-1 showed the greatest susceptibility where symptoms on *Brachypodium* spikes and leaves developed faster when inoculated with *F. graminearum,* although there was not a difference between different Bd lines in the size of lesion developed on leaves and in the production of DON mycotoxin. Differences between *B. distachyon* response to inoculation *with F. graminearum* and *F. langsethiae* are to be expected as the two pathogens have many differences as outlined in Chapter 1 such as different metabolic profile (Parry *et al.*, 1995; Thrane *et al.*, 2004), not responding to the same fungicides (Pettersson *et al.*, 2008) and other agronomic differences (Edwards, 2007a,b, 2009, 2011). This would mean that breeding for resistance to one *Fusarium* species does not necessary give resistance towards other *Fusarium* species.

The value of using model species is in the ability to compare them with crops. Conservation of gene order within the grasses makes it possible to associate genetic information from species to species (Moore *et al.*, 1995). As molecular

markers developed for one species can be transferred to other species, marker mapping of those species allows for maps to be compared. This comparative mapping of grasses showed that gene order is hugely conserved and there is a considerable co-linearity between *Poaceae* family that allows for their comparison and comparison of QTL analysis (Gale & Devos, 1998; Devos & Gale, 2000). Both oats and *Brachypodium* belong to Poaceae family.

An Affymetrix microarray has been developed using data from the *Brachypodium* genome and that has opened new possibilities for gene expression research (Brkljacic *et al.*, 2011). The oat genome is not sequenced yet but the information obtained from sequencing *B. distachyon* will help to assemble larger genomes such as that of oats. Thus *Brachypodium* can greatly facilitate and accelerate research in cereals (Vain, 2011).

With the *B. distachyon* genome sequenced, its similarity to cereals (The International Brachypodium Initiative, 2010) and ability of *F. langsethiae* to cause symptoms on selected lines, the *B. distachyon-F. langsethiae* appears to be a suitable model system to aid investigation of interactions of *F. langs*ethiae with cereals. Evidence of susceptibility of *B. distachyon* were presented. Only three lines were tested for this preliminary study but future work should include more lines. It was beyond this study to investigate alternative methods of inoculation but that should be part of future research as well, methods investigated could be those used for other plant pathogens. Those methods will need adapting to *F. langsethiae* so a range of plant responses are achieved.

To sum up, results from this chapter suggest that *B. distachyon* has the potential to be used as a model host for oat-*F. langsethiae* interactions. *Brachypodium*

distachyon is susceptible to *F. langsethiae*, has a genome that can be compared to other cereals, has a short life cycle and small stature that is easy to work with in laboratory conditions. Nevertheless, model plant species cannot replace cereals in the field especially given their complex interaction with different microorganisms and the environment. Therefore models species can be considered as valuable tools helping understand complex plant-pathogen interactions but the findings need to be tested on a crop species in the field.

Chapter 8: General discussion and recommendation for future work

8.1. General discussion

Oats are cereals used for human food, animal feed and as a source of components used in other sectors such as cosmetics and pharmaceutical industries. They are low input cereals grown worldwide with the main production in Europe and the American continent (Marshall *et al.*, 2013). The oat groat is highly rated with regards to its nutritional composition, antioxidants, proteins and essential amino acids it contains. It is also very rich in dietary fibre β-glucan (Olson & Frey, 1987). The European Food Safety Authority has agreed that there is a link between regular consumption of β-glucan and certain health benefits such as lowering blood cholesterol and reducing the risk of heart diseases (EFSA Panel on Dietetic Products Nutrition and Allergies, 2010). The consumption of oats is increasing and this is believed to be due to the health benefits detailed above (Marshall *et al.*, 2013).

The *Fusarium* genus is diverse and rich in species that are plant pathogens although it contains opportunistic pathogens as well (Parry *et al.*, 1995; Desjardins, 2006; Nucci & Anaissie, 2007). Fusarium head blight (FHB) is a disease complex of different *Fusarium* species on cereals and considered to cause great economic losses as a result of reduction in cereal yield (Nganje *et al.*, 2004). Even if reduction in yield does not happen in every case, there is a great economic loss due to grain contamination with mycotoxins produced by *Fusarium*

species (Parry *et al.*, 1995; Desjardins, 2006). Some sources of resistance are identified (Buerstmayr *et al.*, 2009) but there is no absolute resistance to plant pathogenic *Fusarium* species.

Oat, like other cereals, is susceptible to different *Fusarium* species but in the UK the predominant *Fusarium* pathogen is *F. langsethiae* (Edwards *et al.*, 2012; Opoku *et al.*, 2013). *F. langsethiae* is a relatively newly discovered *Fusarium* species (Torp & Nirenberg, 2004). It produces a range of toxins, but HT2 and T2 mycotoxins are the predominant ones (Thrane *et al.*, 2004). HT2 and T2 belong to the trichothecene mycotoxins which inhibit protein synthesis in eukaryotes (Kimura *et al.*, 2001; Brown *et al.*, 2001).

The European Commission issued a recommendation in 2013 and is currently considering legislative limits or guidelines for HT2 and T2 (European Commission, 2013b). The issued recommendation included an indicative level of 1000 μg kg^{-1} for the sum concentration of HT2 and T2 mycotoxins (HT2+T2) as they always occur together and have equivalent toxicity after consumption. In observational studies across the UK, between 2002-2005, around 20% of harvested oat samples exceeded 1000 μg kg^{-1} HT2+T2 over 4 years of studies and in 2005 there were 30% of samples that exceeded this level (Edwards, 2007a). In that and subsequent observational studies that followed, it was shown that winter varieties had higher levels of HT2+T2 and that there was a range of levels between different varieties where spring varieties did not show much of a difference between them and all had lower levels of HT2+T2 when compared with winter varieties (Edwards, 2012b, 2015). It was not, however, clear why there was a difference between spring and winter varieties.

The aim of this PhD project was to better understand resistance to *F. langsethiae* in UK oats. To achieve this a set of objectives was established:

- To conduct experiments with different sowing dates for winter and spring varieties
- To test naked and conventional varieties comparing level of HT2+T2 mycotoxins before the harvest in panicles and after the harvest in grain samples
- To test the relationship of height with *F. langsethiae* infection and HT2+T2 mycotoxin contamination
- To test a mapping population made from a cross between a dwarf (Buffalo) and a tall (Tardis) oat variety and identify QTL for resistance and mycotoxin contamination
- To test whether *B. distachyon* can be infected with *F. langsethiae* and whether HT2+T2 mycotoxins can be produced in order to investigate appropriateness of *B. distachyon* as a model species for *Fusarium langsethiae*-cereal interaction

To test whether the observed differences in earlier studies (Edwards, 2007a, 2012b, 2015) where winter varieties had higher level of HT2+T2 mycotoxins compared to spring varieties were due to genetics or sowing date the experiment described in Chapter 3 was conducted. The conclusion was that regardless of the sowing date some winter varieties such as Gerald and Balado always had higher levels of mycotoxins. The experiment was repeated at three different locations and during two growing seasons and the trends were stable.

The same experimental setup for Chapter 3 was used for samples in Chapter 4. This was desired as comparison between grain samples (analysed in Chapter 3)

and panicles (analysed in Chapter 4) was necessary to determine if the varieties before the harvest had the same relative level of mycotoxins after the harvest. The particular focus was on naked varieties as in earlier studies their concentration of HT2+T2 mycotoxins was low after the harvest when grain samples were analysed (Edwards, 2007a, 2012b, 2015). Results from Chapter 3 suggested that naked varieties are not necessary more resistant to HT2+T2 concentration. It is rather that their loose husk, which is easily removed during the harvest, is the reason for the lower level of HT2+T2 when grain samples were analysed. Spring naked varieties such as Lennon and Zuton and winter naked varieties such as Grafton and Fusion had lower level of HT2+T2 when grain samples were anlaysed in Chapter 3 but when panicles were collected and analysed in Chapter 4 the same varieties had higher level of HT2+T2 mycotoxins. In fact, results indicate that the winter naked oat varieties Grafton and Fusion are highly susceptible to *Fusarium langsethiae*.

Lower level of HT2+T2 in grain samples of naked varieties were observed earlier in studies by Adler *et al.* (2003) and Edwards (2007, 2012, 2015) but no other studies compared panicles with grain samples. The results suggest that the comparison after the harvest is not equal, knowing that naked varieties easily loose their husk at harvest and that a reduction of 90% can be achieved by de-hulling (Scudamore *et al.*, 2007; Pettersson *et al.*, 2008).

Another observation made in earlier studies was that short strawed varieties had higher levels of HT2+T2 in comparison with varieties of conventional height (Edwards, 2007a, 2012b, 2015). To test whether this observed difference is due to genetics or morphological traits such as height, the experiments in Chapter 5 were conducted. The results from two consecutive experimental years were different.

One of the reasons might be dependency on natural infection where there are no methods of artificial inoculation available. Testing two varieties and additionally altering their height with PGR, in the first year showed that there were highly significant ($p < 0.001$) differences between varieties whilst in the second year differences were not significant ($p = 0.263$). Despite a non-consistent result there was a certain trend suggesting that shorter plants had a range of HT2+T2 values which after certain height results were more consistently lower suggesting taller lines had a generally lower level of HT2+T2 with some exceptions. The difficulty with this study was relying on natural infection in the field. Also, in the second year all plants were taller than in the first year so it was hard to compare the results.

To additionally test the relationship between height and the level of HT2+T2 contamination, a mapping population between dwarf (Buffalo) and tall (Tardis) oat varieties was used for experiments in Chapter 6. Thirty-one lines in the first year, 116 lines in the second year and 85 lines in the third year were used. Taller lines from the mapping population had consistently lower levels of HT2+T2 where with shorter lines there was a range of HT2+T2 levels. QTLs analysis was conducted and a significant number of QTLs were found for HT2+T2 contamination and *F. langsethiae* DNA. This is the first study of such kind to be conducted on oats, identifying QTLs for *F. langsethiae* resistance and HT2+T2 mycotoxin contamination. Linkage group 29 had a number of significant QTLs detected that were associated with height and flowering. This supports previous findings that the effect of flowering and height are often hard to separate. Interesting is the linkage group 1 where QTLs for *F. langsethiae* DNA and HT2+T2 accumulation were found but there was no indication of height or flowering QTLs present in this region. This is worth investigating in greater detail. Height and flowering might be traits of susceptibility or there may be genetic linkage between these traits and

traits of susceptibility. This study could not distinguish between a direct effect of height and genetic linkage but the wide range of responses at specific heights would indicate there are other factors involved.

Finally, to overcome the gap in knowledge and inability to artificially inoculate oat plants with *F. langsethiae,* which proved to be challenging during this project, experiments with the model species *Brachypodium distachyon* were conducted and described in Chapter 7. Three lines of *B. distachyon* were inoculated in root, leaf and floral assays. All three types of inoculation were shown to be successful and this is the first report of *B. distachyon* inoculation with *F. langsethiae* it is also the first report of symptoms after inoculation on a plant species with *F. langsethiae* as the fungus is considered to be symptomless and only limited symptoms of any kind were reported previously (Infantino *et al.*, 2014). Similar experiments were previously successfully conducted with *F. graminearum* by Peraldi *et al.* (2011). The results of the study described in Chapter 7 were different to those presented in a work by Peraldi *et al.* (2011) as tested *B. distachyon* lines did not show the same level of susceptibility and resistance. This would underline the need to include *F. langsethiae* in experiments when testing for resistance as resistance to one *Fusarium* species does not necessary mean resistance to the other species of the same genus. Further work in optimizing inoculation methods is needed but successful inoculation of *B. distachyon* can be further exploited and used in studies to help in the understanding of *F.langsethiae*-plant interaction.

Research questions tested and proved in this PhD project were:

1) Winter oats are genetically more susceptible to *F. langsethiae* than spring oats
2) Naked oats are genetically more susceptible to *F. langsethiae* than husked oats
3) Dwarf oats are genetically more susceptible to *F. langsethiae* than conventional oats

8.2. Recommendations for future work

Throughout this study difficulties encountered were mainly related to uncertainty due to dependence on natural infection. It is therefore of greatest importance to develop a system of artificial inoculation so that screening for resistance does not need to depend on natural infection. Artificial inoculation will not only give more reliable and repetitive results but will also enable the use of the glasshouse for preliminary screenings. Future work might include looking for ways of artificial inoculation not only using methods commonly employed when screening for FHB but seeking examples from work with different pathogens.

What might work as a method of artificial inoculation will be easier to establish if more is known about biology and lifecycle of *F. langsethiae*. Since the first suggestion from the European Commission that legislative limits might be proposed for the level of HT2+T2 mycotoxins in cereals, many of the studies related to *F. langsethiae* were focused on HT2+T2 mycotoxins. Very few studies focused on better understanding of the mycotoxin producer or *F. langsethiae* in the

case of UK oats. If we want to understand the interaction between the toxin producer and the different oat varieties, much of the attention should be focused on *Fusarium langsethiae*. Having a spore trap in the field might give some useful answers as it is still not clear where does the spore inoculum come from and when spores are dispersed. To date it was reported that *F. langsethiae* had only microconidia thus it is challenging to collect spores using a traditional volumetric Burkard trap. As ascospores are not reported, but are the type of spores dispersed by wind and for which a Burkard trap can be useful for monitoring, it might be worth trying to set up spore traps collecting rainfall. Rainfall could be filtered with microbiological filters and catchment could be analysed with species-specific primers using PCR technology. Another possibility is setting up Petri plates with selective media in the field.

As well as the lack of knowledge on the biology of *Fusarium langsethiae*, the role of HT2+T2 mycotoxins is not known. If methods of artificial inoculation are to be established it would be worth testing the impact of inoculation with *Fusarium langsethiae* and in combination with HT2+T2 toxins as well as toxins alone in different concentrations. It is worth further testing collected isolates for their HT2+T2 production.

As shown in Chapter 7, it is possible to inoculate model species *B. distachyon* with *F. langsethiae* and the next step would be developing inoculation method with HT2+T2 mycotoxins on roots, leaves and flowers of *B. distachyon*. Using *B. distachyon* as a model species can help better understand the very complex *Fusarium langsethiae*-oat interactions. Thanks to its sequenced, small genome (The International Brachypodium Initiative, 2010) and ease of use in laboratories (Draper *et al.*, 2001) there is a potential for using *B. distachyon* as a model

species. In Chapter 7 only three *B. distachyon* lines were included but future work should look at testing more lines. Other work with *B. distachyon* could include studies of infected plant tissue examined with confocal microscopy so that the pathway and mechanism of infection with *F. langsethiae* are better understood.

For the first time QTLs for *F. langsethiae* resistance and HT+T2 mycotoxin production were detected in a Buffalo x Tardis population. It would be useful to test different populations or to include more lines to confirm QTLs found or to test near isogenic lines that differ only in a small genomic region of interest.

Anther extrusion and flowering is often mentioned in studies of wheat-*Fusarium* interactions. There are reports suggesting anther extrusion can be a successful tool breeders can use in their programs for *Fusarium* disease resistance (Skinnes *et al.*, 2010; Lu *et al.*, 2012). It is suggested as one of the avoidance mechanisms and should be further explored in oats. Due to its panicles structure, it is much harder to score anther extrusion but it is worth exploring further. When differences can be observed in the field sometimes it is hard to distinguish whether anthers are extruding or if it is the developing grain pushing them. To overcome this, panicles need to be collected and dissected rather than scored in the field as with wheat.

It was described before that where other *Fusarium* species are found, less *F. langsethiae* was present (Edwards, 2007a). This might be the result of different environmental requirements but it is not known how *F. langsethiae* competes with other plant pathogenic species from the *Fusarium* genus. Studies of challenging *Fusarium langsethiae* with other species from the FHB complex might help understand the interactions between pathogens when found in nature. This can

also give an indication of how potent different mycotoxins are to other plant pathogens. In its early years of discovery, *F. langsethiae* was considered as a potential endophyte due to not causing symptons on a host plant. Some endophytes are sensitive to drought and produce secondary metabolites as a result of stress (Schulz *et al.*, 1999; Porras-Alfaro & Bayman, 2011). It was observed that more HT2+T2 mycotoxins are produced in dry weather opposite to what we know about other toxins like DON produced by different *Fusarium* species. If we could establish a method of artificial inoculation, the influence of drought stress on HT2+T2 production could be tested.

Recent developments of genomics and sequencing (Lysøe *et al.*, 2016b) should help to better understand *F. langsethiae* and its life cycle which will consequently lead to a better pathogen control. The sustainable control would include the development of resistant oat varieties. This project identified differences between different varieties and that those differences are due to different genetic backgrounds. QTLs for resistance are identified as well and might find use in future breeding programs for high yielding, more resistant oat varieties.

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Appendix

Table A.1 Concentration of HT2+T2 (µg kg $^{-1}$) mycotoxins in winter and spring sown experiments at different locations. For each year trials were analysed together blocked by block within the trial. Spring trials were analysed separately from winter trials. After variety names; (so) spring oat variety, (wo) winter oat variety, (n) naked oat variety.

	HT2+T2 µg kg $^{-1}$ (Mean Log_{10} (HT2+T2 µg kg^{-1}))			
	Year 2011/2012 (2 sites)		Year 2012/2013 (3 sites)	
Variety	Winter sown	Spring sown	Winter sown	Spring sown
Gerald (wo)	188 (2.27)	307 (2.49)	548 (2.74)	322 (2.51)
Dalguise (wo)	116 (2.06)	149 (2.17)	368 (2.57)	342 (2.53)
Mascani (wo)	169 (2.23)	146 (2.16)	490 (2.69)	281 (2.45)
Balado (wo)	176 (2.25)	746 (2.87)	1101 (3.04)	830 (2.92)
Grafton (wo, n)	112 (2.05)	185 (2.27)	342 (2.53)	296 (2.47)
Fusion (wo, n)	116 (2.06)	180 (2.26)	463 (2.67)	374 (2.57)
Firth (so)	115 (2.06)	168 (2.22)	353 (2.55)	182 (2.26)
Ascot (so)	110 (2.04)	109 (2.04)	232 (2.36)	182 (2.26)
Husky (so)	115 (2.05)	129 (2.11)	373 (2.57)	163 (2.21)
Canyon (so)	126 (2.10)	136 (2.13)	264 (2.42)	185 (2.27)
Lennon (so, n)	123 (2.09)	129 (2.11)	287 (2.46)	213 (2.33)
Zuton (so, n)	115 (2.06)	132 (2.12)	283 (2.45)	233 (2.37)
Mean value	129 (2.11)	176 (2.25)	387 (2.59)	268 (2.43)
P value	<0.001	<0.001	<0.001	<0.001
SEM (df)	0.0479 (119)	0.0707 (75)	0.0445 (121)	0.0396 (120)
CV %	7.9	8.9	6.0	5.6

Table A.2 Fusarium langsethiae DNA (pg ng $^{-1}$) in winter and spring sown experiments at different locations. For each year trials were analysed together blocked by block within the trial. Spring trials were analysed separately from winter trials. After variety names; (so) spring oat variety, (wo) winter oat variety, (n) naked oat variety.

	F. langsethiae DNA pg ng $^{-1}$ (Log_{10} (*F.langsethiae* DNA pg ng^{-1}))			
	Year 2011/2012 (2 sites)		Year 2012/2013 (3 sites)	
Variety	Winter sown	Spring sown	Winter sown	Spring sown
Gerald (wo)	0.0356 (-1.45)	0.0406 (-1.39)	0.4273 (-0.37)	0.1030 (-0.99)
Dalguise (wo)	0.0191 (-1.72)	0.0172 (-1.76)	0.1417 (-0.85)	0.0718 (-1.14)
Mascani (wo)	0.0230 (-1.64)	0.0109 (-1.96)	0.2688 (-0.57)	0.0640 (-1.19)
Balado (wo)	0.0475 (-1.32)	0.1482 (-0.83)	1.2342(0.091)	0.4121 (-0.38)
Grafton (wo, n)	0.0084 (-2.08)	0.0063 (-2.20)	0.0715 (-1.15)	0.0431 (-1.36)
Fusion (wo, n)	0.0055 (-2.26)	0.0142 (-1.85)	0.1724 (-0.76)	0.0634 (-1.20)
Firth (so)	0.0104 (-1.98)	0.0078(-2.105)	0.1263 (-0.90)	0.0429 (-1.37)
Ascot (so)	0.0152 (-1.82)	0.0111 (-1.95)	0.1816 (-0.74)	0.0417 (-1.38)
Husky (so)	0.0096 (-2.02)	0.0050 (-2.3)	0.0871(-1.06)	0.0265 (-1.58)
Canyon (so)	0.01762(-1.75)	0.0085 (-2.07)	0.1026 (-0.99)	0.0152 (-1.82)
Lennon (so, n)	0.0092 (-2.03)	0.0034 (-2.46)	0.0665 (-1.18)	0.0242 (-1.62)
Zuton (so, n)	0.0051 (-2.29)	0.0061 (-2.21)	0.0672 (-1.17)	0.0216 (-1.66)
Mean value	0.0137 (-1.86)	0.0119 (-1.92)	0.1570 (-0.80)	0.0492 (-1.31)
P value	<0.001	<0.001	<0.001	<0.001
SEM (df)	0.0808 (74)	0.1173 (76)	0.0751 (121)	0.104 (121)
CV %	12.3	17.2	32.4	27.5

Table A.3 Sorted sensitivity estimates after Finlay Wilkinson regression for trails sown in winter. All trials were analysed together and Log_{10} (HT2+T2 μg kg^{-1}) was presented

Ranking	Variety	Sensitivity	Mean Log_{10} (HT2+T2 μg kg^{-1})	Mean HT2+T2 μg kg^{-1}
1)	Lennon	0.6707	2.274	188
2)	Ascot	0.7225	2.203	160
3)	Canyon	0.7239	2.261	182
4)	Zuton	0.8156	2.256	180
5)	Grafton	0.9051	2.291	195
6)	Firth	0.9952	2.304	201
7)	Fusion	1.0179	2.368	233
8)	Mascani	1.1095	2.457	286
9)	Dalguise	1.1473	2.314	206
10)	Gerald	1.1744	2.507	321
11)	Husky	1.1929	2.315	207
12)	Balado	1.5239	2.644	441

Table A.4 Sorted sensitivity estimates after Finlay Wilkinson regression for trials sown in spring. All remaining trials were analysed together and Log_{10} (HT2+T2 μg kg^{-1}) was presented

Ranking	Variety	Sensitivity	Mean Log_{10} (HT2+T2 μg kg^{-1})	Mean HT2+T2 μg kg^{-1}
1)	Gerald	-0.0188	2.5	316
2)	Balado	0.3301	2.9	794
3)	Firth	0.3775	2.253	179
4)	Husky	0.7835	2.172	149
5)	Canyon	0.7914	2.215	164
6)	Grafton	1.0301	2.39	245
7)	Mascani	1.0787	2.344	221
8)	Ascot	1.1861	2.17	148
9)	Lennon	1.28	2.241	174
10)	Zuton	1.4432	2.268	185
11)	Fusion	1.8233	2.445	279
12)	Dalguise	1.8479	2.39	245

Table A.5 Concentration of HT2+T2 (µg kg^{-1}) mycotoxins of harvested panicles in winter and spring sown experiments at different locations. For each year trials were analysed together blocked by trial and block (repetition within the trial). Spring trials were analysed separately from winter trials.

	HT2+T2 µg kg^{-1} (Mean Log_{10} (HT2+T2 µg kg^{-1}))			
	Year 2011/2012 (3 sites)		Year 2012/2013 (2 sites for winter and 3 sites for spring sown)	
Variety	Winter sown	Spring sown	Winter sown	Spring sown
Gerald (wo)	546 (2.74)	544 (2.74)	955 (2.98)	426 (2.63)
Dalguise (wo)	357 (2.55)	386 (2.59)	479 (2.68)	309 (2.49)
Mascani (wo)	458 (2.66)	363 (2.56)	1349 (3.13)	257 (2.41)
Balado(wo)	578 (2.76)	2582 (3.41)	1439 (3.16)	1726 (3.24)
Grafton (wo, n)	434 (2.64)	682 (2.83)	2455 (3.39)	530 (2.72)
Fusion (wo, n)	487 (2.69)	1778 (3.25)	4875 (3.69)	1062 (3.03)
Firth (so)	353 (2.55)	285 (2.45)	609 (2.78)	203 (2.31)
Ascot (so)	285 (2.45)	331 (2.52)	329 (2.52)	196 (2.29)
Husky (so)	310 (2.49)	342 (2.53)	803 (2.90)	171 (2.23)
Canyon (so)	341 (2.53)	386 (2.59)	433 (2.64)	235 (2.37)
Lennon (so, n)	378 (2.58)	447 (2.65)	608 (2.78)	252 (2.40)
Zuton (so, n)	336 (2.53)	406 (2.61)	822 (2.91)	231 (2.36)
Mean value	405 (2.61)	711 (2.85)	1263 (3.10)	466 (2.67)
P value	<0.001	<0.001	<0.001	<0.001
SEM (df)	0.1554 (121)	0.2014(118)	0.2291 (77)	0.1615 (121)
CV %	6.0	7.4	7.7	6.4

Table A.6 Sorted sensitivity estimates after Finlay Wilkinson regression for panicles harvested in winter sown experiments. Log_{10} (HT2+T2 μg kg^{-1}) and mean HT2+T2 T2 μg kg^{-1} was presented

Ranking	Variety	Sensitivity	Mean Log_{10} (HT2+T2 μg kg^{-1})	Mean HT2+T2 μg kg^{-1}
1)	Canyon	0.8181	2.574	375
2)	Ascot	0.8212	2.48	302
3)	Dalguise	0.829	2.604	402
4)	Lennon	0.9224	2.66	457
5)	Zuton	0.9606	2.682	481
6)	Husky	0.969	2.656	453
7)	Firth	1.0515	2.643	439
8)	Mascani	1.0618	2.848	705
9)	Grafton	1.0859	2.939	869
10)	Balado	1.1323	2.921	834
11)	Gerald	1.1324	2.834	682
12)	Fusion	1.2124	3.088	1225

Table A.7 Sorted sensitivity estimates after Finlay Wilkinson regression for trails sown in spring. Harvested panicles were analysed together. Log_{10} (HT2+T2 μg kg^{-1}) and mean HT2+T2 μg kg^{-1} was presented

Ranking	Variety	Sensitivity	Mean Log_{10} (HT2+T2 μg kg^{-1})	Mean HT2+T2 μg kg^{-1}
1)	Dalguise	0.656	2.538	345
2)	Balado	0.737	3.325	2113
3)	Mascani	0.767	2.485	305
4)	Firth	0.783	2.381	240
5)	Ascot	0.919	2.401	252
6)	Zuton	1	2.486	306
7)	Grafton	1.028	2.779	601
8)	Canyon	1.199	2.477	300
9)	Fusion	1.205	3.138	1374
10)	Husky	1.209	2.384	242
11)	Gearld	1.226	2.682	481
12)	Lennon	1.306	2.526	336

Table A.8 List of QTL identified from the analysis of B x T population

Year	Repetition or mean value	Trait	Chrom	Left Marker (name)	LOD	R^2 %
2013/2014	rep 1	F lang/ total DNA (pg/ng)	CHROM1	AME102	3	15.3
2012/2013	rep 1	F lang/total DNA (pg/ng)	CHROM1	TR293	2.52	10
2012/2013	rep 1	LOG_ F.lang/total DNA (pg/ng)	CHROM1	TR293	2.92	11.5
2013/2014	rep 1	LOG_ F.lang/total DNA (pg/ng)	CHROM1	AME102	3.76	18.8
2013/2014	mean	Log_Mean Flang /total DNA	CHROM1	AME102	2.28	11.6
2012/2013	mean	Log_Mean Flang/total DNA	CHROM1	AME102	2.86	10.8
2012/2013	mean	Mean Flang/total DNA (pg/ng)	CHROM1	AME102	2.5	9.5
2012/2013	rep 2	HT2+T2	CHROM1	LpACA32G	2.33	10
2012/2013	rep 1	HT2+T2	CHROM1	LpACA32G	3.16	12.5
2012/2013	rep 1	HT2+T2	CHROM1	TR293	3.35	13.1
2013/2014	rep 3	LOG_ HT2/T2	CHROM1	LpACA32G	2.79	14.7
2013/2014	rep 3	LOG_ HT2/T2	CHROM1	avgbs_21	2.86	15
2012/2013	rep 2	Log_HT2/T2	CHROM1	LpACA32G	2.61	11.1
2012/2013	rep 1	Log_HT2/T2	CHROM1	TR293	4.08	15.7
2013/2014	mean	Log_Mean HT2/T2	CHROM1	avgbs_21	2.49	12.6
2012/2013	mean	Log_Mean HT2/T2	CHROM1	TR293	3.96	14.7
2013/2014	mean	Mean HT2/T2	CHROM1	avgbs_21	2.52	12.8
2012/2013	mean	Mean HT2/T2	CHROM1	LpACA32G	3.52	13.3
2012/2013	mean	Mean HT2/T2	CHROM1	AME102	3.54	13.2
2013/2014	rep 3	T2/HT2	CHROM1	LpACA32G	2.34	12.5
2013/2014	rep 3	T2/HT2	CHROM1	avgbs_21	2.73	14.4
2013/2014	rep 1	LOG_ F.lang/total DNA (pg/ng)	CHROM11	o10692	2.1	11
2013/2014	rep 3	LOG_ F.lang/total DNA (pg/ng)	CHROM11	o10692	2.48	13.3
2011/2012	mean	Flowering	CHROM11	avgbs_21	2.27	4.4
2013/2014	rep 1	LOG_ HT2/T2	CHROM11	avgbs_21	2.18	11.4
2013/2014	mean	Log_Mean HT2/T2	CHROM11	TR151.18	2.27	11.6
2013/2014	mean	Mean HT2/T2	CHROM11	o11857	2.07	10.8
2013/2014	mean	Mean HT2/T2	CHROM11	TR151.18	2.08	10.6
2011/2012	mean	Flowering	CHROM12	o13547	2.45	9
2012/2013	mean	Flowering	CHROM12	o13547	2.63	9.7
2012/2013	rep 1	HT2+T2	CHROM12	o9496	2.12	9.8

Table A.8 List of QTL identified from the analysis of B x T population

Year	Repetition or mean value	Trait	Chrom	Left Marker (name)	LOD	R^2 %
2012/2013	mean	Log_Mean HT2/T2	CHROM12	o9496	2.93	12.6
2012/2013	mean	Mean HT2/T2	CHROM12	o9496	3.24	13.9
2012/2013	mean	Dwarf or Tall	CHROM16	avgbs_88	2.12	10
2011/2012	mean	Dwarf or Tall	CHROM16	avgbs_88	2.13	10.1
2012/2013	rep 2	LOG_ F.lang/total DNA (pg/ng)	CHROM16	avgbs_88	2.53	13.2
2011/2012	rep 1	LOG_Flang/total DNA (pg/ng)	CHROM16	avgbs_88	2.05	34.9
2012/2013	rep 2	Height (cm)	CHROM16	avgbs_88	2.24	11.8
2012/2013	mean	Mean height (cm)	CHROM16	avgbs_20	2.18	10.6
2011/2012	mean	Log_Mean HT2/T2	CHROM16	avgbs_88	2.16	36.4
2011/2012	mean	Mean HT2/T2	CHROM16	avgbs_88	2.03	34.6
2013/2014	rep 1	LOG_ HT2/T2	CHROM18	o11382	2.09	11.8
2011/2012	rep 2	Log_HT2+T2	CHROM18	o477	2.15	27.3
2011/2012	rep 2	T2+HT2	CHROM18	o477	2.39	29.9
2011/2012	rep 2	Log_HT2+T2	CHROM22	c12578_3	2.38	39.2
2011/2012	mean	Log_Mean HT2/T2	CHROM22	c12578_3	2.1	35.5
2011/2012	mean	Mean HT2/T2	CHROM22	c12578_3	2.19	36.8
2011/2012	rep 2	T2+HT2	CHROM22	c12578_3	2.61	42.1
2011/2012	rep 3	Log_HT2+T2	CHROM25	AME010	2.21	28
2011/2012	rep 3	T2+HT2	CHROM25	AME010	2.21	28
2011/2012	mean	Flowering	CHROM27	AME152.1	7.82	14.6
2011/2012	rep 1	Height (cm)	CHROM27	c19227_1	2.13	40.3
2012/2013	mean	Dwarf or Tall	CHROM29	avgbs_42	12.99	42.8
2013/2014	mean	Dwarf or Tall	CHROM29	avgbs_42	13.38	44.1
2011/2012	mean	Dwarf or Tall	CHROM29	avgbs_42	13.95	39.5
2011/2012	mean	Dwarf or Tall	CHROM29	ASTB.384	22.11	48.4
2012/2013	mean	Dwarf or Tall	CHROM29	ASTB.384	23.06	55
2013/2014	mean	Dwarf or Tall	CHROM29	ASTB.384	23.63	56.2
2012/2013	mean	Dwarf or Tall	CHROM29	o793199	23.84	76
2013/2014	mean	Dwarf or Tall	CHROM29	o793199	25.52	78.7
2011/2012	mean	Dwarf or Tall	CHROM29	o793199	27.54	81.2
2012/2013	mean	Dwarf or Tall	CHROM29	m27/049.6	66.99	86
2013/2014	mean	Dwarf or Tall	CHROM29	m27/049.6	68.94	86.9

Table A.8 List of QTL identified from the analysis of B x T population

Year	Repetition or mean value	Trait	Chrom	Left Marker (name)	LOD	R^2 %
2011/2012	mean	Dwarf or Tall	CHROM29	m27/049.6	90.04	84.3
2013/2014	rep 2	F lang/ total DNA (pg/ng)	CHROM29	ASTB.384	2.77	14.9
2013/2014	rep 3	F lang/ total DNA (pg/ng)	CHROM29	ASTB.384	3.29	17.4
2013/2014	rep 1	F lang/ total DNA (pg/ng)	CHROM29	o15157	3.45	17.8
2013/2014	rep 1	F lang/ total DNA (pg/ng)	CHROM29	ASTB.384	4.44	22.1
2013/2014	rep 3	F lang/ total DNA (pg/ng)	CHROM29	o15157	4.71	24.3
2013/2014	rep 3	F lang/ total DNA (pg/ng)	CHROM29	avgbs_42	4.94	33.9
2013/2014	rep 1	F lang/ total DNA (pg/ng)	CHROM29	avgbs_21	4.95	24.6
2013/2014	rep 2	F lang/ total DNA (pg/ng)	CHROM29	avgbs_21	5.42	27.4
2013/2014	rep 2	F lang/ total DNA (pg/ng)	CHROM29	avgbs_21	5.42	27.4
2013/2014	rep 3	F lang/ total DNA (pg/ng)	CHROM29	avgbs_21	8.79	40.5
2012/2013	rep 2	F lang/total DNA (pg/ng)	CHROM29	o793199	3.94	30
2012/2013	rep 3	F lang/total DNA (pg/ng)	CHROM29	avgbs_21	5.56	29.3
2012/2013	rep 1	F lang/total DNA (pg/ng)	CHROM29	TR345	5.65	21.1
2012/2013	rep 2	F lang/total DNA (pg/ng)	CHROM29	avgbs_21	8.98	36.5
2012/2013	rep 2	F lang/total DNA (pg/ng)	CHROM29	TR345	8.99	32.8
2013/2014	rep 2	LOG_ F.lang/total DNA (pg/ng)	CHROM29	o793199	2.21	22.5
2012/2013	rep 1	LOG_ F.lang/total DNA (pg/ng)	CHROM29	o15157	2.74	12.3
2013/2014	rep 2	LOG_ F.lang/total DNA (pg/ng)	CHROM29	ASTB.384	2.76	14.9
2013/2014	rep 1	LOG_ F.lang/total DNA (pg/ng)	CHROM29	o793199	3.26	30.7
2013/2014	rep 1	LOG_ F.lang/total DNA (pg/ng)	CHROM29	TR345	3.67	18.4
2012/2013	rep 2	LOG_ F.lang/total DNA (pg/ng)	CHROM29	o793199	4.33	32.4

Table A.8 List of QTL identified from the analysis of B x T population

Year	Repetition or mean value	Trait	Chrom	Left Marker (name)	LOD	R^2 %
2013/2014	rep 1	LOG_ F.lang/total DNA (pg/ng)	CHROM29	avgbs_21	4.55	22.8
2013/2014	rep 3	LOG_ F.lang/total DNA (pg/ng)	CHROM29	avgbs_42	4.93	33.8
2012/2013	rep 3	LOG_ F.lang/total DNA (pg/ng)	CHROM29	TR345	4.97	24.4
2012/2013	rep 3	LOG_ F.lang/total DNA (pg/ng)	CHROM29	avgbs_21	5	26.7
2013/2014	rep 3	LOG_ F.lang/total DNA (pg/ng)	CHROM29	ASTB.384	5.8	28.7
2013/2014	rep 3	LOG_ F.lang/total DNA (pg/ng)	CHROM29	o15157	6.05	30
2013/2014	rep 2	LOG_ F.lang/total DNA (pg/ng)	CHROM29	avgbs_21	6.43	31.6
2012/2013	rep 1	LOG_ F.lang/total DNA (pg/ng)	CHROM29	TR345	9.29	32.2
2012/2013	rep 2	LOG_ F.lang/total DNA (pg/ng)	CHROM29	TR345	12.37	42.2
2013/2014	rep 3	LOG_ F.lang/total DNA (pg/ng)	CHROM29	avgbs_21	12.85	53.2
2013/2014	mean	Log_Mean Flang /total DNA	CHROM29	avgbs_42	2.12	15.1
2013/2014	mean	Log_Mean Flang /total DNA	CHROM29	o15157	4.19	20.8
2013/2014	mean	Log_Mean Flang /total DNA	CHROM29	avgbs_21	11.56	47.4
2012/2013	mean	Log_Mean Flang/total DNA	CHROM29	o793199	4.46	29.4
2012/2013	mean	Log_Mean Flang/total DNA	CHROM29	TR345	12.88	40.3
2013/2014	mean	Mean Flang /total DNA	CHROM29	o15157	5.09	24.6
2013/2014	mean	Mean Flang /total DNA	CHROM29	ASTB.384	5.26	25.1
2013/2014	mean	Mean Flang /total DNA	CHROM29	avgbs_21	9.85	42.1

Table A.8 List of QTL identified from the analysis of B x T population

Year	Repetition or mean value	Trait	Chrom	Left Marker (name)	LOD	R^2 %
2012/2013	mean	Mean Flang/total DNA (pg/ng)	CHROM29	o793199	2.73	19.2
2012/2013	mean	Mean Flang/total DNA (pg/ng)	CHROM29	TR345	7.66	26.4
2013/2014	mean	Flowering	CHROM29	o793199	5.11	34.8
2011/2012	mean	Flowering	CHROM29	o793199	8.46	39.7
2013/2014	mean	Flowering	CHROM29	ASTB.384	8.72	32.8
2012/2013	mean	Flowering	CHROM29	o793199	11.15	48.7
2011/2012	mean	Flowering	CHROM29	ASTB.384	11.19	28.3
2012/2013	mean	Flowering	CHROM29	ASTB.384	13.59	37.8
2013/2014	mean	Flowering	CHROM29	m27/049.6	15.41	44.7
2012/2013	mean	Flowering	CHROM29	m27/049.6	25.86	53.4
2011/2012	mean	Flowering	CHROM29	m27/049.6	27.7	43.1
2012/2013	mean	Height (cm)	CHROM29	avgbs_42	12.21	41.5
2011/2012	mean	Height (cm)	CHROM29	ASTB.384	17.13	40.5
2011/2012	mean	Height (cm)	CHROM29	o793199	17.65	66.2
2013/2014	mean	Height (cm)	CHROM29	ASTB.384	19.81	50.2
2013/2014	mean	Height (cm)	CHROM29	o793199	21.26	72.4
2012/2013	mean	Height (cm)	CHROM29	o793199	21.38	73.1
2012/2013	mean	Height (cm)	CHROM29	ASTB.384	22.38	54.5
2013/2014	mean	Height (cm)	CHROM29	m27/049.6	43.94	73.1
2011/2012	mean	Height (cm)	CHROM29	m27/049.6	59.57	71.1
2012/2013	mean	Height (cm)	CHROM29	m27/049.6	65.66	86.1
2013/2014	rep 3	Height (cm)	CHROM29	avgbs_42	3.39	24.4
2013/2014	rep 2	Height (cm)	CHROM29	avgbs_42	3.67	25.7
2013/2014	rep 3	Height (cm)	CHROM29	o793199	3.87	36
2013/2014	rep 1	Height (cm)	CHROM29	avgbs_42	4.16	28.2
2013/2014	rep 2	Height (cm)	CHROM29	o793199	4.52	40.6
2013/2014	rep 1	Height (cm)	CHROM29	o793199	5.2	44.2
2012/2013	rep 3	Height (cm)	CHROM29	o793199	5.25	44.6
2012/2013	rep 1	Height (cm)	CHROM29	avgbs_42	6.57	31.8
2012/2013	rep 2	Height (cm)	CHROM29	avgbs_42	6.75	34.7
2013/2014	rep 2	Height (cm)	CHROM29	ASTB.384	7.37	34.9
2012/2013	rep 3	Height (cm)	CHROM29	avgbs_42	7.94	46.2

Table A.8 List of QTL identified from the analysis of B x T population

Year	Repetition or mean value	Trait	Chrom	Left Marker (name)	LOD	R^2 %
2013/2014	rep 3	Height (cm)	CHROM29	ASTB.384	9.89	43.4
2012/2013	rep 3	Height (cm)	CHROM29	ASTB.384	10.52	45.8
2013/2014	rep 1	Height (cm)	CHROM29	ASTB.384	10.82	45.5
2012/2013	rep 2	Height (cm)	CHROM29	o793199	12.05	66.3
2012/2013	rep 1	Height (cm)	CHROM29	ASTB.384	13.46	45.2
2013/2014	rep 3	Height (cm)	CHROM29	TR345	13.65	54
2012/2013	rep 2	Height (cm)	CHROM29	ASTB.384	13.98	48.5
2012/2013	rep 1	Height (cm)	CHROM29	o793199	14.06	68.5
2013/2014	rep 2	Height (cm)	CHROM29	avgbs_21	18.69	66.8
2013/2014	rep 3	Height (cm)	CHROM29	avgbs_21	21.74	71.8
2012/2013	rep 3	Height (cm)	CHROM29	avgbs_21	22.39	75.2
2013/2014	rep 1	Height (cm)	CHROM29	avgbs_21	24.34	74.9
2012/2013	rep 1	Height (cm)	CHROM29	avgbs_21	37.55	83.5
2012/2013	rep 2	Height (cm)	CHROM29	m27/049.	39.1	82.3
2011/2012	rep 1	Height (cm)	CHROM29	avgbs_42	2.19	35.4
2011/2012	rep 1	Height (cm)	CHROM29	TR345	2.55	31.5
2011/2012	rep 1	Height (cm)	CHROM29	avgbs_21	3.48	46.1
2011/2012	rep 3	Height (cm)	CHROM29	TR345	4.09	45.5
2011/2012	rep 2	Height (cm)	CHROM29	avgbs_21	4.48	54.8
2011/2012	rep 3	Height (cm)	CHROM29	avgbs_21	5.93	65
2011/2012	mean	Mean height (cm)	CHROM29	TR345	4.1	45.6
2013/2014	mean	Mean height (cm)	CHROM29	avgbs_42	4.21	27.6
2011/2012	mean	Mean height (cm)	CHROM29	avgbs_21	5.53	62.5
2013/2014	mean	Mean height (cm)	CHROM29	o793199	5.85	46.5
2013/2014	mean	Mean height (cm)	CHROM29	ASTB.384	10.97	45.2
2013/2014	mean	Mean height (cm)	CHROM29	avgbs_21	25.94	76.3
2012/2013	mean	Mean height (cm)	CHROM29	avgbs_42	8.24	37
2012/2013	mean	Mean height (cm)	CHROM29	o793199	14.41	67.5
2012/2013	mean	Mean height (cm)	CHROM29	ASTB.384	16.16	49.8

Table A.8 List of QTL identified from the analysis of B x T population

Year	Repetition or mean value	Trait	Chrom	Left Marker (name)	LOD	R^2 %
2012/2013	mean	Mean height (cm)	CHROM29	avgbs_21	42.45	85.6
2012/2013	rep 3	HT2+T2	CHROM29	o793199	2.33	23
2012/2013	rep 1	HT2+T2	CHROM29	o15157	2.44	11
2012/2013	rep 2	HT2+T2	CHROM29	o793199	3.7	28.4
2012/2013	rep 1	HT2+T2	CHROM29	TR345	4.65	17.7
2012/2013	rep 3	HT2+T2	CHROM29	avgbs_21	6.42	33
2012/2013	rep 2	HT2+T2	CHROM29	ASTB.384	6.49	26.8
2012/2013	rep 2	HT2+T2	CHROM29	m27/049.	9.65	35.1
2013/2014	rep 2	LOG_ HT2/T2	CHROM29	avgbs_42	2.37	17.4
2013/2014	rep 1	LOG_ HT2/T2	CHROM29	o15157	2.99	15.6
2013/2014	rep 2	LOG_ HT2/T2	CHROM29	o793199	3.06	29.7
2013/2014	rep 2	LOG_ HT2/T2	CHROM29	ASTB.384	3.42	18.1
2013/2014	rep 3	LOG_ HT2/T2	CHROM29	o15157	3.75	19.6
2013/2014	rep 3	LOG_ HT2/T2	CHROM29	ASTB.384	4.89	24.5
2013/2014	rep 2	LOG_ HT2/T2	CHROM29	TR345	5.13	25.6
2013/2014	rep 2	LOG_ HT2/T2	CHROM29	avgbs_21	6.92	33.5
2013/2014	rep 1	LOG_ HT2/T2	CHROM29	m27/049.	7.8	35.5
2013/2014	rep 3	LOG_ HT2/T2	CHROM29	avgbs_21	8.71	39.8
2012/2013	rep 3	Log_HT2/T2	CHROM29	o793199	2.23	22.2
2012/2013	rep 1	Log_HT2/T2	CHROM29	o15157	2.46	11.1
2012/2013	rep 2	Log_HT2/T2	CHROM29	o793199	3.82	29.2
2012/2013	rep 3	Log_HT2/T2	CHROM29	avgbs_21	5.87	30.6
2012/2013	rep 1	Log_HT2/T2	CHROM29	TR345	6.11	22.6
2012/2013	rep 2	Log_HT2/T2	CHROM29	ASTB.384	6.78	27.8
2012/2013	rep 2	Log_HT2/T2	CHROM29	m27/049.	11.55	40.4
2013/2014	mean	Log_Mean HT2/T2	CHROM29	o15157	5.09	24.6
2012/2013	mean	Log_Mean HT2/T2	CHROM29	o793199	5.64	35.6
2013/2014	mean	Log_Mean HT2/T2	CHROM29	ASTB.384	7.44	33.5
2013/2014	mean	Log_Mean HT2/T2	CHROM29	TR345	8.58	37.2
2012/2013	mean	Log_Mean HT2/T2	CHROM29	m27/049.	11.56	37.3
2013/2014	mean	Log_Mean HT2/T2	CHROM29	avgbs_21	12.09	48.9
2012/2013	mean	Mean HT2/T2	CHROM29	o793199	4.61	30.2
2013/2014	mean	Mean HT2/T2	CHROM29	o15157	4.77	23.2
2013/2014	mean	Mean HT2/T2	CHROM29	TR345	7.95	35
2012/2013	mean	Mean HT2/T2	CHROM29	m27/049.	9.43	31.7
2013/2014	mean	Mean HT2/T2	CHROM29	avgbs_21	10.75	44.9
2013/2014	rep 2	T2/HT2	CHROM29	o793199	2.74	27.1
2013/2014	rep 1	T2/HT2	CHROM29	o15157	3.41	17.6
2013/2014	rep 3	T2/HT2	CHROM29	o15157	3.77	19.7
2013/2014	rep 2	T2/HT2	CHROM29	avgbs_21	5.24	26.6

Table A.8 List of QTL identified from the analysis of B x T population

Year	Repetition or mean value	Trait	Chrom	Left Marker (name)	LOD	R^2 %
2013/2014	rep 1	T2/HT2	CHROM29	ASTB.384	5.57	26.9
2013/2014	rep 3	T2/HT2	CHROM29	ASTB.384	5.59	27.5
2013/2014	rep 1	T2/HT2	CHROM29	m27/049.	7.45	34.2
2013/2014	rep 3	T2/HT2	CHROM29	avgbs_21	8.59	39.4
2013/2014	mean	Dwarf or Tall	CHROM30	o16528	2.24	7
2012/2013	mean	Dwarf or Tall	CHROM30	o16528	2.39	7.4
2011/2012	mean	Dwarf or Tall	CHROM30	avgbs_90	3.61	9.1
2013/2014	rep 2	LOG_ F.lang/total DNA (pg/ng)	CHROM30	avgbs_90	2.02	11.8
2013/2014	rep 1	LOG_ F.lang/total DNA (pg/ng)	CHROM30	o16528	2.69	13.9
2013/2014	mean	Log_Mean Flang /total DNA	CHROM30	o16528	3.27	16.2
2013/2014	mean	Mean Flang /total DNA	CHROM30	avgbs_90	2.27	12.6
2012/2013	mean	Flowering	CHROM30	o16528	2.97	9.1
2011/2012	mean	Flowering	CHROM30	o16528	4.26	10.1
2011/2012	mean	Height (cm)	CHROM30	o16528	2.37	5.9
2013/2014	mean	Height (cm)	CHROM30	o16528	2.4	7.6
2011/2012	rep 1	Height (cm)	CHROM30	m28/000.	2.23	43.5
2011/2012	rep 2	Height (cm)	CHROM30	m28/000.	2.59	48.5
2011/2012	rep 3	Height (cm)	CHROM30	avgbs_90	2.8	38
2011/2012	mean	Mean height	CHROM30	avgbs_90	2.43	33.9
2011/2012	mean	Mean height	CHROM30	m28/000.	2.89	52.3
2012/2013	rep 3	HT2+T2	CHROM30	o16885	2.62	13.7
2013/2014	rep 1	LOG_ HT2/T2	CHROM30	o15271	3.06	15.6
2012/2013	rep 2	Log_HT2/T2	CHROM30	o16528	2.08	9.3
2012/2013	rep 3	Log_HT2/T2	CHROM30	o16885	2.84	14.8
2013/2014	mean	Log_Mean HT2/T2	CHROM30	avgbs_90	2.16	12
2013/2014	rep 1	T2/HT2	CHROM30	o15271	2.43	12.6
2013/2014	mean	Flowering	CHROM33	m30/005.8	2.01	7.5
2012/2013	rep 2	LOG_ F.lang/total DNA (pg/ng)	CHROM37	AME066	2.4	10.3
2012/2013	rep 2	Log_HT2/T2	CHROM37	AME066	2.04	8.9
2011/2012	rep 2	F lang/total DNA (pg/ng)	CHROM39	AM01	2.45	35.2
2011/2012	rep 2	LOG_Flang/total DNA (pg/ng)	CHROM39	AM01	2.64	37.4
2011/2012	mean	Log_Mean Flang/total DNA	CHROM39	AM01	2.05	30.4
2013/2014	mean	Dwarf or Tall	CHROM5	c841_728	2.48	8.2
2012/2013	mean	Dwarf or Tall	CHROM5	c841_728	2.63	8.6
2012/2013	rep 2	Height (cm)	CHROM5	c841_728	2.86	13.1

Table A.8 List of QTL identified from the analysis of B x T population

Year	Repetition or mean value	Trait	Chrom	Left Marker (name)	LOD	R^2 %
2011/2012	rep 1	F lang/total DNA (pg/ng)	CHROM6	AB_AM829	2.13	27.1
2011/2012	rep 1	LOG_Flang/total DNA (pg/ng)	CHROM6	AB_AM829	2.14	27.2
2011/2012	mean	Log_Mean Flang/total DNA	CHROM6	o794612	2.32	29.1
2011/2012	mean	Mean Flang/total DNA (pg/ng)	CHROM6	o794612	2.14	27.3
2011/2012	mean	Flowering	CHROM6	o794289	2.14	12.8
2011/2012	rep 3	Log_HT2+T2	CHROM8	avgbs_20	2.77	33.8
2011/2012	mean	Log_Mean HT2/T2	CHROM8	TR072X	2.51	31.1
2011/2012	mean	Mean HT2/T2	CHROM8	TR072X	2.51	31.1
2011/2012	rep 3	T2+HT2	CHROM8	avgbs_20	2.55	31.6
2013/2014	rep 2	LOG_ F.lang/total DNA (pg/ng)	CHROM9	o795192	2.85	25.3
2011/2012	rep 3	F lang/total DNA (pg/ng)	NONE FOUND			
2011/2012	rep 3	LOG_Flang/total DNA (pg/ng)	NONE FOUND			
2011/2012	rep 1	Log_HT2+T2	NONE FOUND			
2011/2012	rep 1	T2+HT2	NONE FOUND			