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Identification of *Fusarium* resistance traits in UK oat varieties

Tijana Stancic[1]

[1] *Harper Adams University, Newport, Shropshire, TF10 8NB, UK*

[2]*IBERS, Aberystwyth University, Plas Gogerddan, Aberystwyth, SY23 3EB, UK*

Supervisors:

Simon Edwards[1] and Sandy Cowan[2]

Advisor:

Catherine Howarth[2]

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CONTENTS

1. Abstract

The aim of this project was to understand the variation in resistance of UK oat varieties to *Fusarium langsethiae*, which is a most common *Fusarium* species on oats in the UK.

The differences between varieties were identified and that those differences are due to different genetic backgrounds. Results from experiments conducted within this project indicate that some winter varieties such as Gerald and Balado had consistently higher HT2+T2 regardless of sowing date, and are therefore genetically more susceptible to *F. langsethiae* infection. It was also identified that some naked oat varieties such as winter Fusion and Grafton have high HT2+T2 levels before harvest and hence high susceptibility to *F. langsethiae*. Height *per se* was identified as a resistance factor, but as only one of many, or there is a close genetic linkage between the dwarf gene and susceptibility QTL. QTLs for resistance were identified as well and might find use in future breeding programs for high yielding, more resistant oat varieties. As it is currently not possible to successfully inoculate oat plants with *F. langsethiae* for the purpose of resistance screening, an experiment with a model plant was conducted and the results show that *Brachypodium distachyon* is a host for *F. langsethiae* and produces typical head blight symptoms after infection.

This PhD project provided several opportunities to improve existing knowledge that will extend the direction of *Fusarium* - oat research. 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. Based on results presented, it is possible to give better advice to growers, selecting for less susceptible oat varieties and recommending inclusion of spring oat varieties, as they tend to be less susceptible to *F. langsethiae* infection and subsequent HT2+T2 contamination.

2. Introduction

Oats have found a diverse use such as human food, animal feed and as a source of some components used in cosmetics and pharmaceutical industries. As a low input cereals they are grown worldwide with the main production in Europe and the American continent (Marshall *et al*., 2013). The oat groat is rich in antioxidants, proteins and essential amino acids and 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 NDA Panel (EFSA Panel on Dietetic Products Nutrition and Allergies), 2010). The consumption of oats is increasing and this is believed to be due to its health benefits (Marshall *et al*., 2013).

Fusarium langsethiae is the most common *Fusarium* species on UK oats, detected in 99% of oat samples collected in a survey from 2002-2004. 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 (Edwards *et al*., 2012).

In 2013, the recommendation to monitor the combined concentration of *Fusarium* mycotoxins, HT2 and T2 (HT2+T2) in food and feed was published by the European Commission. This recommendation is to be reviewed in the near future as the European Commission is planning on considering new legislative limits or guidelines for the joint concentration of HT2+T2 mycotoxins (European Commission, 2013).

In the field surveys conducted between 2002 and 2006, 16% of UK oats exceeded the proposed indicative levels of 1000 µg/kg HT2+T2 for unprocessed oats intended for human consumption (Edwards, 2007a). Analysing AHDB Recommended List oat trials, conducted from 2005-2011, for the level of HT2+T2, it was observed that there was a highly significant difference ($p<0.001$) for both spring and winter varieties. In general, all winter varieties had higher level of HT2+T2 mycotoxins compared with spring varieties, with Gerald and Balado having the highest level of HT2+T2 concentration. In addition to this, it is considered that the concentration of HT2+T2 in naked (huskless) oats is lower than in the conventional husked oats. The mycotoxin concentration of dwarf varieties (which are short-strawed) tends to be higher than of conventional varieties although the relationship between plant height and mycotoxin levels is not consistent. In addition to this, very few dwarf varieties were tested.

Prior to this study, the impact of agronomy on HT2+T2 accumulation on oats had not been clearly identified. It was not clear whether the difference observed between varieties was due to

agronomic (i.e. drilling date) or genetic differences. Differences may be due directly to the morphological trait of crop height or maybe due to genetic linkage.

This 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 producing *Fusarium* species.

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 with 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 *F. langsethiae*-cereal interaction

2.1. Evaluating resistance of UK oat varieties

To test the hypothesis that the difference observed were not due to agronomy, varietal experiments with selected conventional and naked winter and spring varieties were sown in the autumn and the spring at three sites in 2011/2012 and 2012/2013. Eight conventional (husked) and four naked (n) varieties were included, half of them being spring and half being winter oat varieties. Chosen locations were Edgmond (site of Harper Adams University, West Midlands), Cowlinge (site of Saaten Union, East of England) and Fife (site of Scottish Agronomy, Central Scotland). Spring varieties sown at each location were: Firth, Husky, Ascot, Canyon, Lennon (n; naked) and Zuton (n). Winter varieties sown at each location were: Gerald, Balado, Dalguise, Mascani, Grafton (n) and Fusion (n).

2.2. Susceptibility of naked oats compared with conventional oats

It is considered that the concentration of HT2+T2 in naked (huskless) oats is lower than in the conventional (husked) oats but these assumptions were made from studies comparing the grain samples after the harvest (Adler *et al*., 2003; Edwards, 2007a; Gagkaeva *et al*., 2011). It is important to note that naked and conventional oats differ at the point of harvest as the husk has been removed from naked oats while conventional husked varieties still have their husk after combining (Valentine, 1995). Researchers agree that the majority of mycotoxins are found in the husk (Edwards, 2007a; Scudamore *et al*., 2007; Pettersson *et al*., 2008) so when comparing husked and naked varieties the equivalent material can only be compared by analysing panicles before harvesting or by de-hulling husked oats after harvest.

It was not known how the HT2+T2 level of naked varieties compares to conventional varieties before harvest and how panicles of naked oats collected before harvest compared to the harvested grain. The Null hypothesis was that there is no difference in susceptibility to *Fusarium langsethiae* infection between naked oats and conventional oat varieties. The material analysed for this study was collected from the experiments described in Chapter 2.1 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. 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.

2.3. Height as a susceptibility trait of oats to *Fusarium* infection

To test whether a high concentration of HT2+T2 mycotoxins observed in dwarf (short-strawed) 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). This 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) and included six different concentrations of PGR treatments.

2.4. Assessment of *Fusarium langsethiae* infection and mycotoxin production in a Buffalo x Tardis mapping population

To test further the hypothesis of whether crop height is a susceptibility trait, another experiment was conducted from a field trial run by Aberystwyth University using plant lines developed from a

cross between short and tall winter oat varieties (Buffalo and Tardis). The Buffalo x Tardis cross was used for the identification of QTL for resistance and to determine genetic linkage with agronomic traits such as height. The experiment was sown in autumn 2011, 2012 and 2013 in field locations of University of Aberystwyth (Aberystwyth, Wales, UK).

2.5. Investigating the ability of *Fusarium langsethiae* to infect the model species *Brachypodium distachyon*

Due to difficulty with artificially inoculating oat plants in order to screen for resistance, experiments with a model plant species *Brachypodium distachyon* were conducted in order to see whether *Brachypodium* can serve as a model in plant - *Fusarium langsethiae* interaction experiments. Floral, detached leaf and root assays were performed.

3. Materials and methods

3.1. Field experiments

All field experiments conducted had a standard farm practice for milling oats applied, as recommended by agronomists at each site. The sites were Scottish Agronomy (Fife, Scotland), Saaten Union (Cowlinge, England), Harper Adams University (Edgmond, England) and the site of University of Aberystwyth (Aberystwyth, Wales, UK)

3.2. Infection with *Fusarium langsethiae*

As it is currently not possible to artificially inoculate plants with *F. langsethiae*, all field experiments depended on natural infection in the field.

3.3. Sample harvesting and milling

Prior to any analysis, thirty panicles were harvested randomly from each plot at approximately GS (growth stage) 85 and the crop was harvested as fully ripe at GS 92 (Zadoks *et al*., 1974) and grain sub-samples of 1 kg were milled. If necessary, samples were cleaned with a grain sample cleaner with a 2 mm screen and milling of grains was done with mills fitted with a 1 mm screen. Resulting samples were mixed thoroughly and ca. 200 g was kept for further analyses.

3.4. *Fusarium langsethiae* isolates, DNA extraction and PCR identification

Purified single spore isolates of *Fusarium langsethiae* from grain samples were used to produce spore suspensions used in inoculation experiments with *Barchypodium*. Isolates were grown on

PDA plates at room temperature and cultures that resembled *F. langsethiae* were harvested to be identified with PCR technology prior to purification. For this purpose, a crude DNA extraction with Chelex Carbon Buffer was used. In experiments where plant samples were used, DNA was extracted from milled plant samples as described by Opoku (2012) with modifications when necessary to account for sample size. *F. langsethiae* biomass was determined by quantitative PCR (qPCR) as described by Edwards *et al*.(2012) and primers used were reported by Wilson *et al*. (2004) to be specific for *F. langsethiae*.

3.5. Brachypodium distachyon

3.5.1. *Brachypodium distachyon* plants

Experiments were conducted with Bd 21, Bd 21-3 and Bd 3-1 lines. To prepare seeds for potting and growing a protocol developed by Vain *et al*. (2008) was used. Potted seedlings were grown in the Standard Fitotron® Growth Chamber (SGS 120, Fitotron®, Weiss Technic, UK) with temperature setting as outlined by Vain *et al*. (2008). In these conditions, plants were flowering after approximately four weeks.

3.5.2. Fungal inoculum

The purified single spore isolates of *F. langsethiae* were used. The concentration was determined using Improved Neubauer haemocytometer (Weber, England) and it was adjusted to 10^6 spores ml^{-1} sterile deionised water (SDW) and amended with 0.05% Tween20 (Sigma, UK).

3.5.3. Floral assay

Plants were sprayed with inoculum until run-off with a hand-held garden sprayer. The spraying was repeated three times with a break of approx. 15 min between spraying to allow inoculum on plants to dry before applying another spraying. Plants selected as non-inoculated control were treated with sterile water. The inoculated plants were bagged for 3 days to maintain a high humidity of 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 before milling and analysing for HT2+T2 mycotoxins and *F. langsethiae* DNA concentration as described earlier. This assay was performed twice.

3.5.4. Detached leaf assay

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 deionised water). 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. 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 *et al*., 2008). Brown necrotic lesions were measured by ruler 7 days after the inoculation with spore suspension of *F. langsethiae* (7 dai). The detached leaf assay was replicated four times.

3.5.5. 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 onto 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 and 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* 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.

3.6. Mycotoxin analysis

HT2 and T2 quantification was performed using Ridascreen® T-2 ELISA assay (R-Biopharm, AG, Germany) following manufacturer's instructions with minor changes due to the sample size. As HT2 and T2 occur together, in all results 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).

3.7. Statistical analysis

Results were analysed with GenStat (Version 13, VSN International Ltd). ANOVA (Analysis of Variance) was used to see whether there was a significant difference between treatments and Tukey's test (p=0.05) was used to distinguish significant differences between individual factor levels. When necessary to normalize the distribution of residuals prior to analysis, data were $\log_{10}$ transformed.

All experiments were analysed separately and where possible, experiments were combined, analysed together and blocked by repetition within experiment. Spring and winter trials were analysed separately.

4. Results

4.1. Evaluating resistance of UK oat varieties

The spring sown experiment at Harper Adams in 2012 was completely lodged and the plots were not harvested. The winter sown experiment at Scottish Agronomy in 2012 had very low levels of infection with no significant differences in HT2+T2 and *F. langsethiae* DNA across the experiment. These two trials were excluded and the remaining experiments analysed together. 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 1) and spring sown trials (Figure 2) and *F. langsethiae* biomass within trials sown in winter (Figure 3) and trials sown in spring (Figure 4).

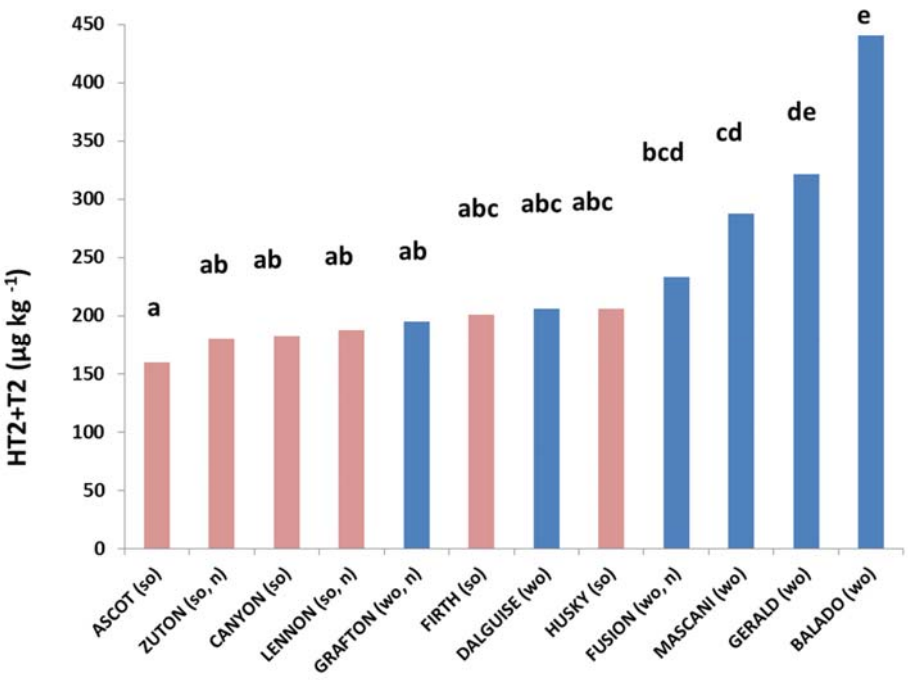

Figure 1 Concentration of HT2 and T2 mycotoxins (µg kg $^{-1}$) in winter sown winter and spring oat varieties. Two winter trials (Saaten Union and Harper Adams) from 2011/2012 and three winter trials (Scottish Agronomy, Saaten Union and Harper Adams) from 2012/2013 were analysed together blocked by 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 2) was 222 µg kg $^{-1}$ and 258 µg kg $^{-1}$ in winter sown experiments (Figure 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.

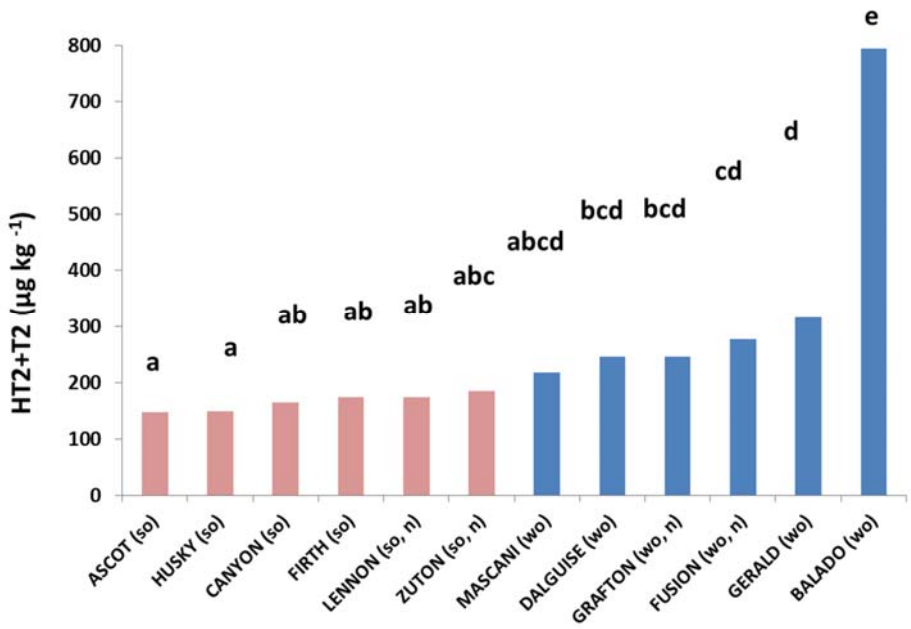

Figure 2 Concentration of HT2 and T2 mycotoxins (µg kg $^{-1}$) in spring sown winter and spring oat varieties. Two spring trials (Scottish Agronomy and Saaten Union) from 2011/2012 and three spring trials (Scottish Agronomy, Saaten Union and Harper Adams) from 2012/2013 were analysed together blocked 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.

Similar results were obtained for *F. langsethiae* DNA (Fig 3 and 4) with generally low levels detected on spring varieties and significantly higher DNA levels detected on Gerald and Balado irrespective of sowing date.

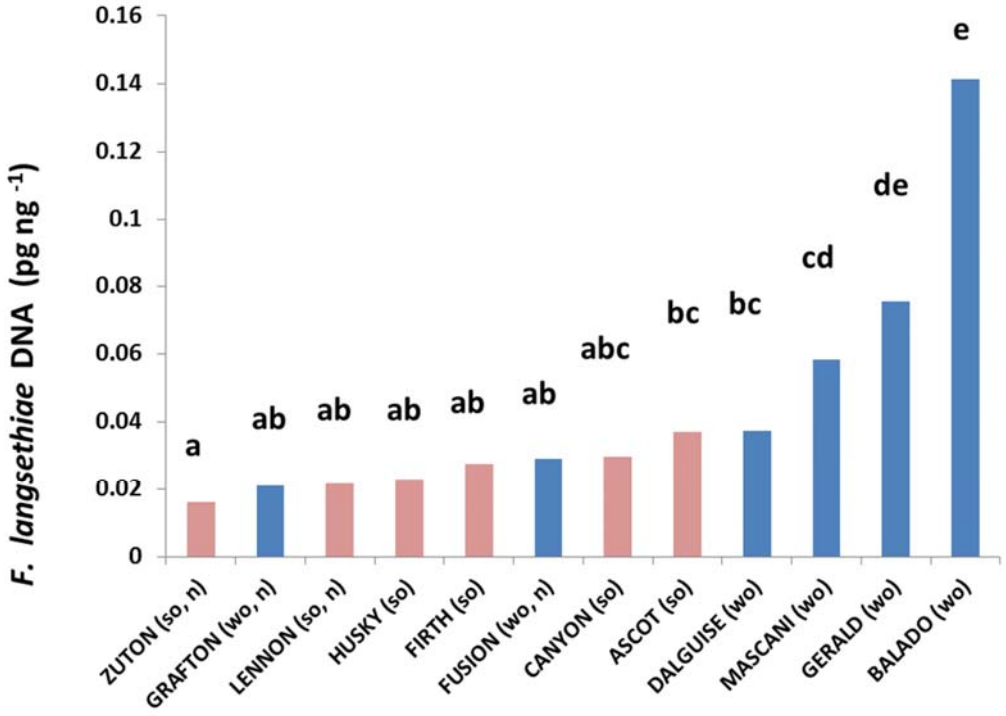

Figure 3 *Fusarium langsethiae* DNA (pg ng $^{-1}$) in winter sown winter and spring oat varieties. Two winter trials (Saaten Union and Harper Adams) from 2011/2012 and three winter sown trials (Scottish Agronomy, Saaten Union and Harper Adams) from 2012/2013 were analysed together blocked by block within 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.

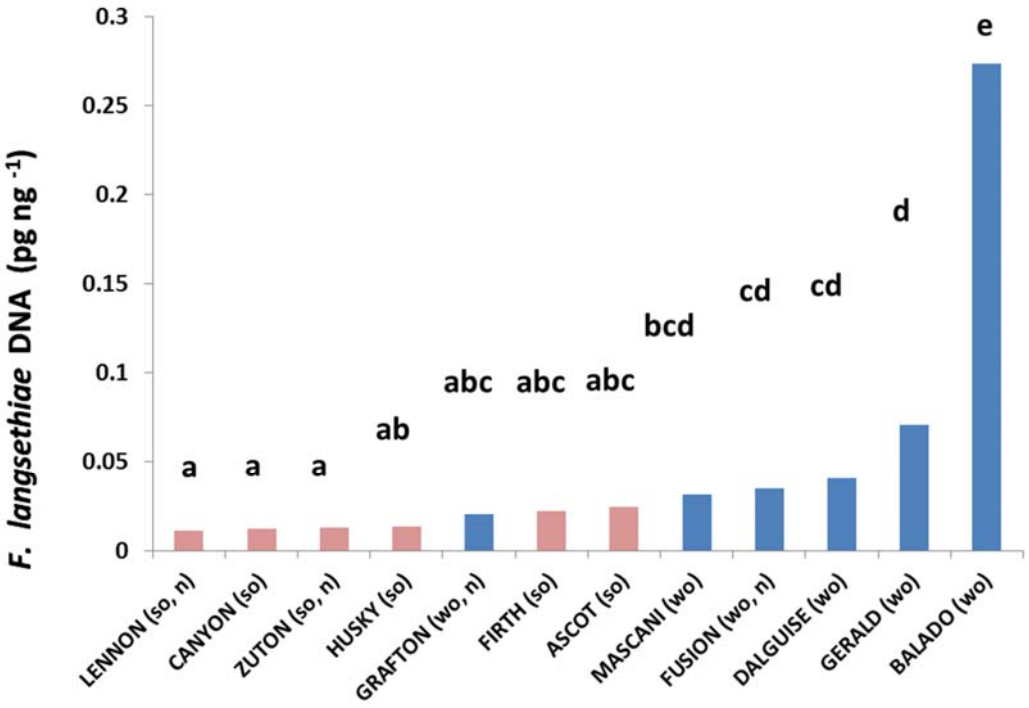

Figure 4 *Fusarium langsethiae* DNA (pg ng $^{-1}$) in spring sown winter and spring oat varieties. Two winter trials (Saaten Union and Harper Adams) from 2011/2012 and three winter trials (Scottish Agronomy, Saaten Union and Harper Adams) from 2012/2013 were analysed together blocked by block within 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.

4.2. Susceptibility of naked oats compared with conventional oats

The concentration of HT2+T2 mycotoxins in UK oat varieties prior to harvest was conducted by collecting and analysing panicles before the harvest of the experiment detailed in Chapter 4.1. It was identified that naked varieties had higher level of HT2+T2 mycotoxins. This was especially noticeable for winter naked varieties Grafton and Fusion that when previously analysed as grain samples (section 4.1) showed a lower level of contamination. Panicles of variety Fusion showed the greatest difference having had five times more HT2+T2 then grain samples in the spring sown experiments.

Results from panicles collected in this study were compared with results from Chapter 4.1 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 5). 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 6).

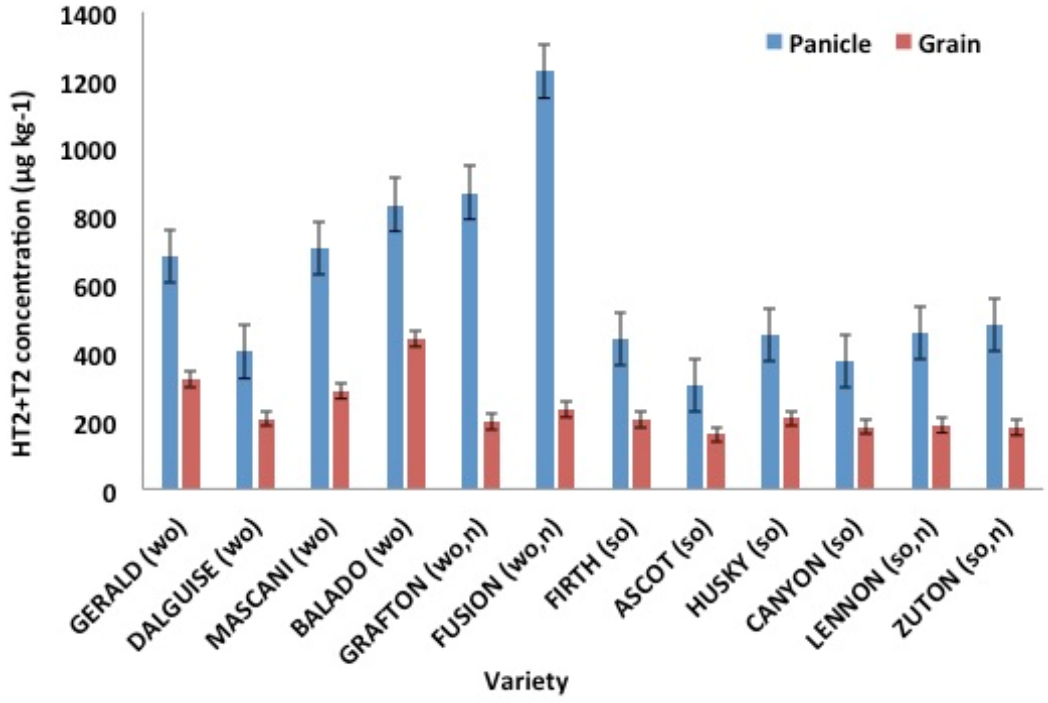

Figure 5 Concentration of HT2 and T2 mycotoxins (µg kg $^{-1}$) in panicle and grain samples from winter sown oat varieties. Error bars represent standard errors. After variety names; (so) spring oat variety, (wo) winter oat variety, (n) naked oat variety.

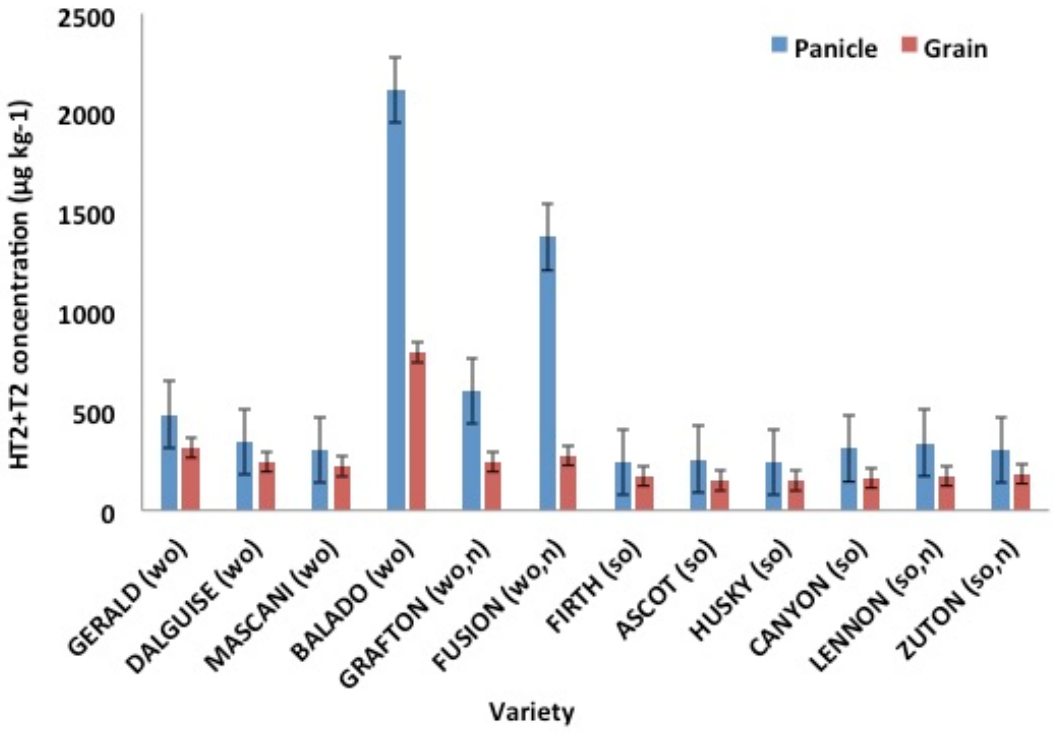

Figure 6 Concentration of HT2 and T2 mycotoxins (µg kg $^{-1}$) in panicles and grain samples from spring sown oat varieties. Error bars represent standard errors. After variety names; (so) spring oat variety, (wo) winter oat variety, (n) naked oat variety.

4.3. Height as a susceptibility trait of oats to *Fusarium* infection

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 7) and for the second year (Figure 8).

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 7). 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 8). 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 7). 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 8). There was not a strong relationship between

plant height of either variety and HT2+T2 mycotoxins in either of the years (Figure 7 and Figure 8). In the first experimental year, there were distinct differences between varieties but no indication of an effect of height (Figure 7).

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 7); 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 1. The same analysis in the second year did not show any significance (Table 2).

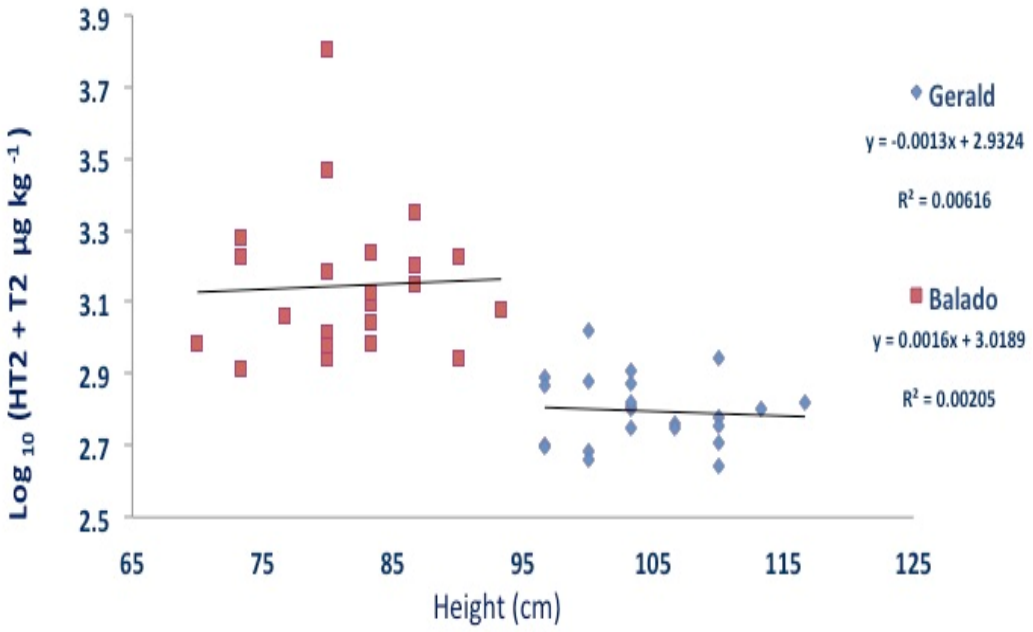

Figure 7 Log_{10} (HT2+T2 µg kg^{-1}) of harvested oat grain plotted against the plant height for the PGR experiment in 2012/2013.

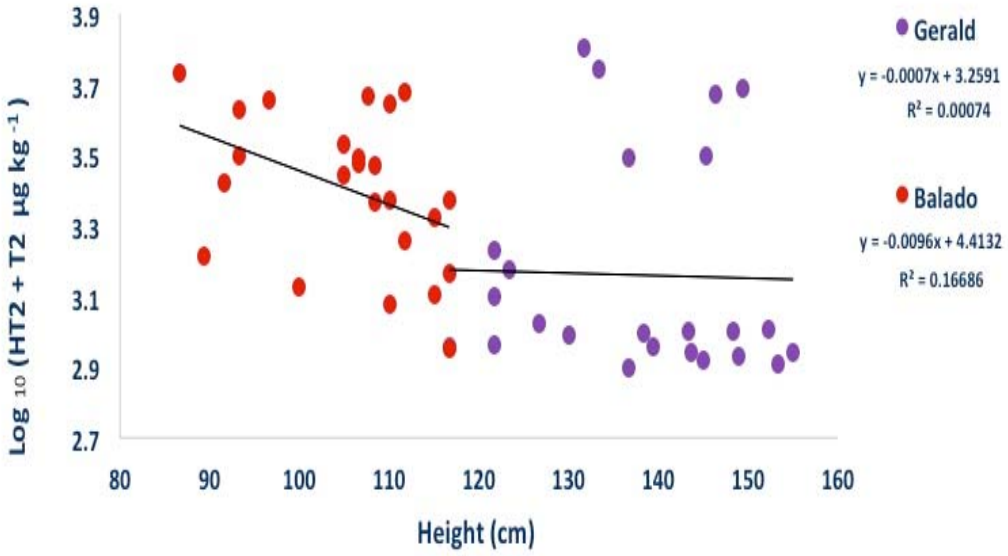

Figure 8 Log_{10} (HT2+T2 µg kg^{-1}) of harvested oat grain plotted against the plant height for the PGR experiment in 2013/2014.

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 8). 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 1 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

Table 2 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

4.4. Assessment of Fusarium langsethiae infection and mycotoxin production in a Buffalo x Tardis mapping population

Due to similar variance between individual experiments, all experiments were analysed together, block by block within year. Mean data for HT2+T2 mycotoxin concentration 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. Accordingly three separate lines were fitted as shown in Figure 9.

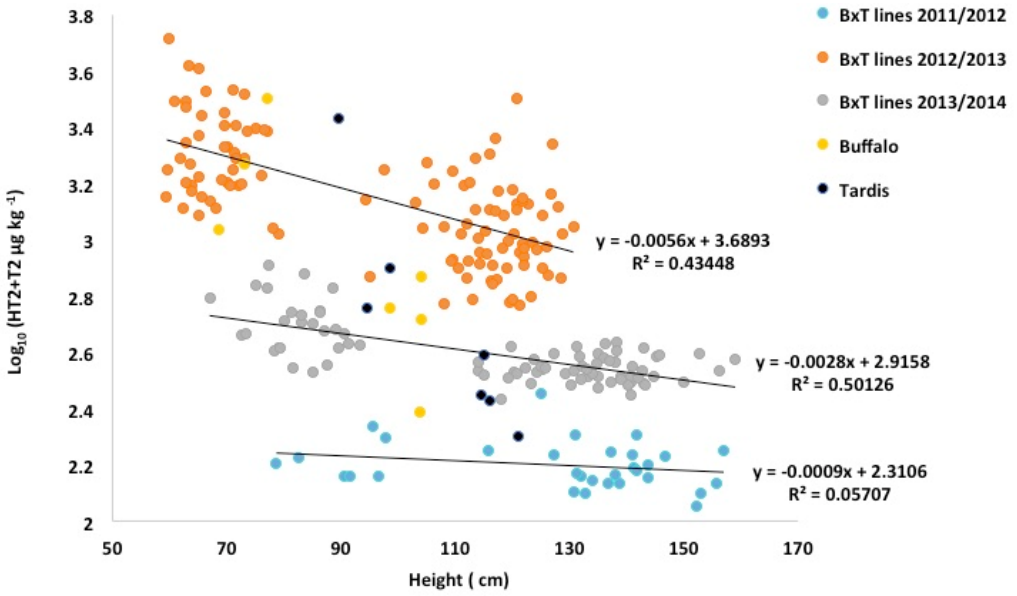

Figure 9 Log_{10} transformed HT2+T2 concentration (µg kg^{-1}) of lines from a cross between winter oat varieties Buffalo and Tardis plotted against the plant height. Three trials from 2011/2012, 2012/2013 and 2013 were analysed together grouped by year. The mean of three repetitions from each year 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.

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 (logarithm of the odds, LOD>2).

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. 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 10).

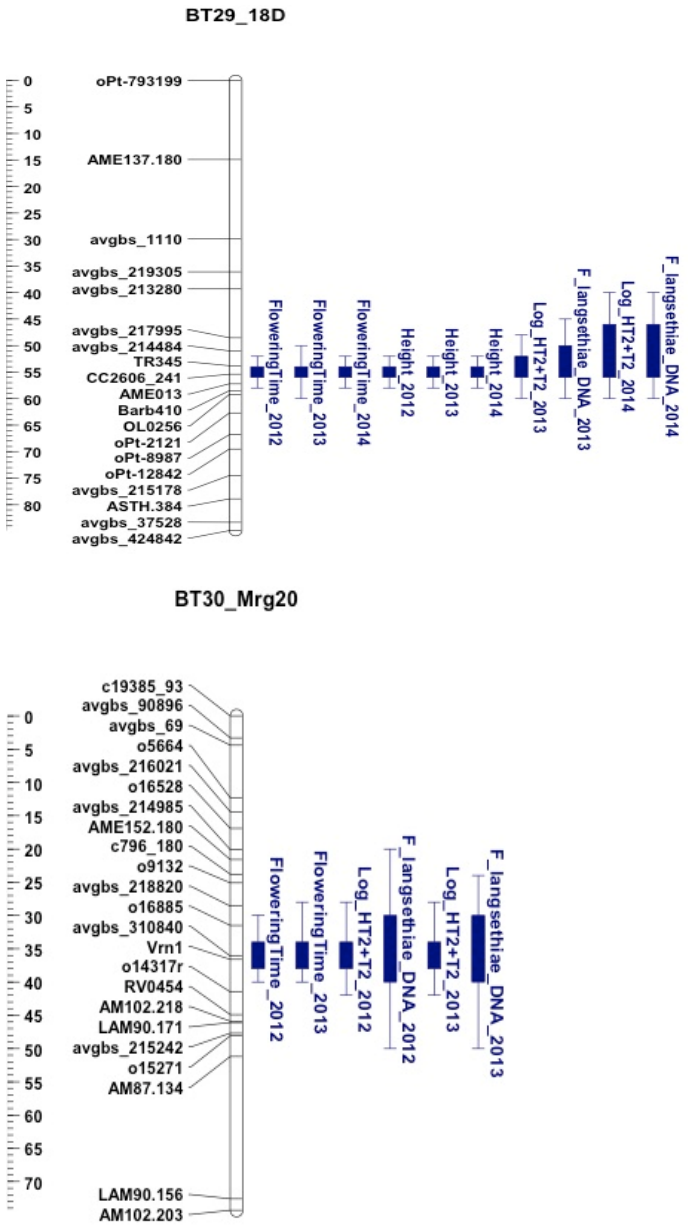

Figure 10 Significant QTL identified on linkage group 29 (BT29_18D) and linkage group 30 (BT30_Mrg20). Genetic distances in centimorgans are included to the left of the chromosomes followed by markers. QTL identified are on the right side of the chromosome. (Figure provided by Dr Catherine Howarth, IBERS, University of Aberystwyth).

Linkage group 1 (Figure 11) did not have any QTL identified for height and flowering associated with it but it 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.

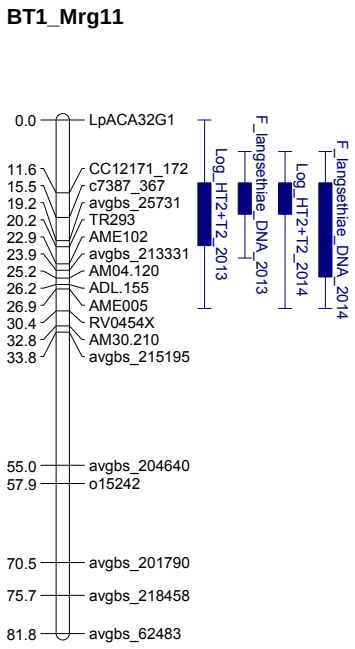

Figure 11 Significant QTL identified on linkage group 1 (BT1_Mrg11). Genetic distances in centimorgans are included to the left of the chromosomes followed by markers. QTL identified are on the right side of the chromosome. (Figure provided by Dr Catherine Howarth, IBERS, University of Aberystwyth).

4.5. Investigating the ability of *Fusarium langsethiae* to infect the model species *Brachypodium distachyon*

4.5.1. Floral assay

On inoculated spikes, necrotic lesions developed as shown in Figure 12. 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 in Figure 13.

Figure 12 Symptoms of *Fusarium langsethiae* infection on spikes of *Brachypodium distachyon* line Bd21. Top row are inoculated plants, bottom right are uninoculated controls.

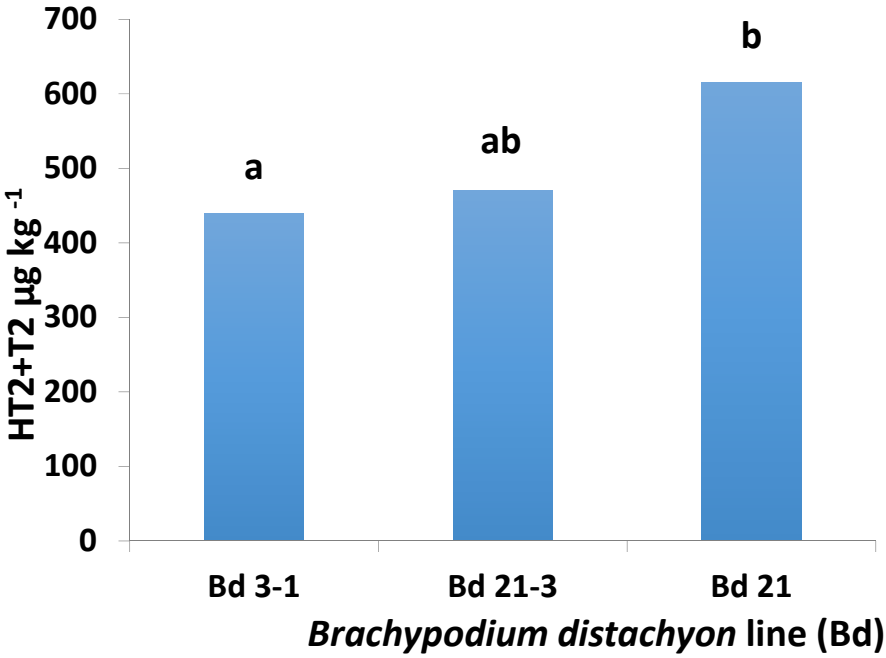

Figure 13 Back-transformed concentration of HT2+T2 mycotoxins (µg kg $^{-1}$) in three different *B. distachyon lines* (Bd 3-1, Bd 21-3, Bd 21). Lines with the same letter were not significantly different according to Tukey's test (p=0.05).

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 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 14.

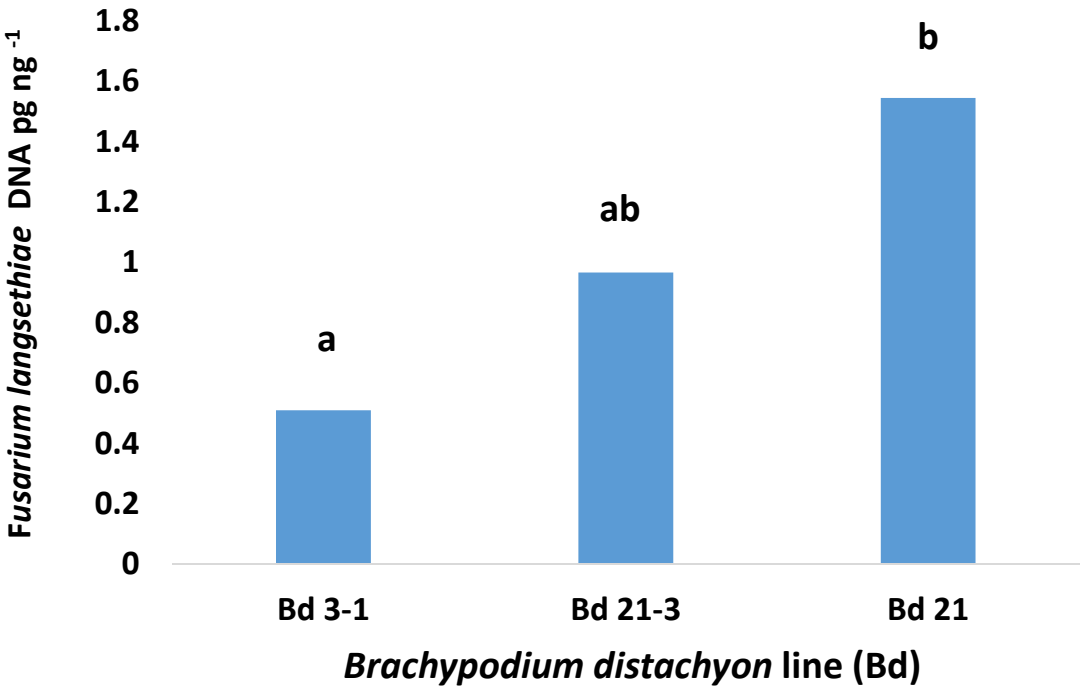

Figure 14 Back-transformed concentration of *Fusarium langsethiae* DNA (pg ng $^{-1}$) in three different Brachypodium distachyon lines (Bd 3-1, Bd 21-3, Bd 21). Lines with the same letter were not significantly different according to Tukey's test (p=0.05).

4.5.2. 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 15.

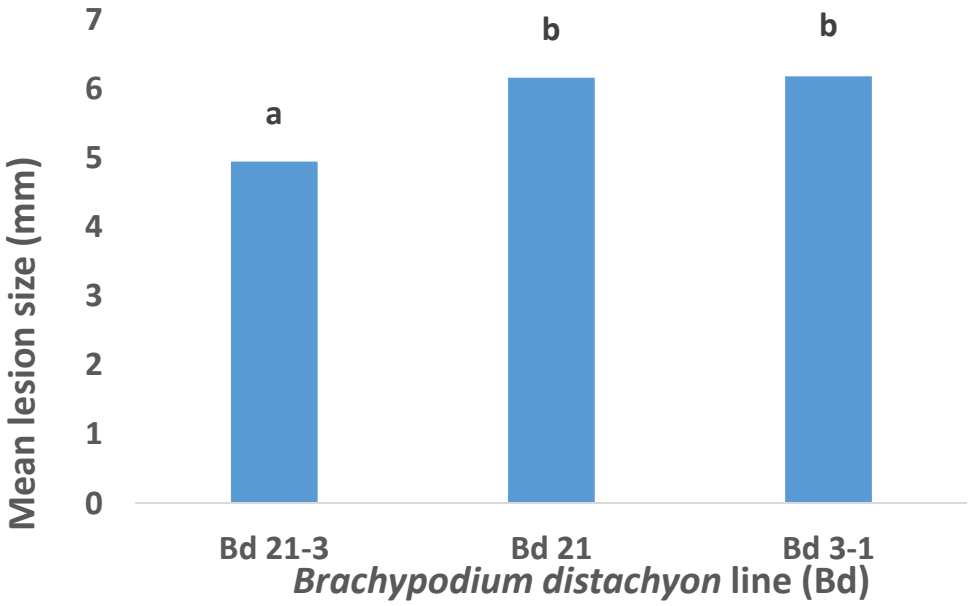

Figure 15 Mean size of lesions measured (mm) on leaves of three different *Brachypodium distachyon* lines (Bd 3-1, Bd 21-3, Bd 21). Four independent experiments were analysed together blocked by experiment and repetitions within. Lines with the same letter were not significantly different according to Tukey's test (p=0.05).

4.5.3. Root assay

ANOVA performed on results from the root assay did not show any significance although brown lesions developed with a general mean of 15 mm. Due to statistical insignificance, results are not shown.

5. Discussion

5.1. Evaluating resistance of UK oat varieties

The resistance of UK oat varieties to *F. langsethiae* and HT2+T2 mycotoxin contamination was evaluated in the first experiment. Results showed that the sowing date might not be of major importance as some varieties such as Gerald and Balado had significantly higher levels of HT2+T2, regardless of the sowing date, when infection was detected. As the experiment was repeated across sites and time, this showed that the observed differences are stable phenotypes. Given this, genetic resistance should be investigated in greater detail. This can be achieved by phenotyping and subsequently, QTL analysis of a mapping population constructed from parent varieties that differ in their level of susceptibility to *F. langsethiae* and HT2+T2 mycotoxins. These types of analysis are used for complex quantitative traits such as disease resistance and they rely on the link between genotypic and phenotypic data but they require a method of artificial inoculation to be developed.

In some trials the overall mean of HT2+T2 was low, resulting in no quantifiable differences between different oat varieties. This is due to uncertainty of weather conditions (i.e. wet summer in 2012) and due to dependency on natural infection with *F. langsethiae*. The same trend was noticeable across trials with winter oat varieties such as Gerald and Balado having the highest level of HT2+T2 when moderate to high levels of infections occurred.

Results are in accordance with differences detected in observational studies from the AHDB Recommended List trials where HT2+T2 has been quantified from winter and spring variety trials (Edwards, 2007a, 2012b, 2015). However, this is not in agreement with results from France 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.

A significant relationship between *F. langsethiae* DNA and the concentration of HT2+T2 mycotoxins was detected in all except the spring trials in the second year. The discrepancy observed might be due to oat growth stage and the weather conditions especially around flowering time. This is important given that anther extrusion is weather dependent (De Vries, 1971) and flower opening has been previously suggested as a mechanism of disease avoidance (Skinnes *et al*., 2010; Lu *et al*., 2012; Kubo *et al*., 2013). However, there is a current lack of understanding of how and when infection with *F. langsethiae* happens in the field.

5.2. Susceptibility of naked oats compared with 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 showed that naked varieties are not genetically more resistant to HT2+T2 contamination but that the reduction of HT2+T2 mycotoxins is rather the result of their loose hull, removed during harvest. This is in agreement with studies by Edwards (2012a)

Previous results showed that regardless of the sowing date, grain samples of Gerald and Balado had the highest concentration HT2+T2. Results from experiments presented here 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*.

This 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; Edwards, 2007a). Differences such as these, repeated across environments, would suggest strong genetic control. Thus, when comparing the level of resistance between different varieties, it is important that equivalent material is analysed.

5.3. Height as a susceptibility trait of oats to Fusarium infection

It was hypothesised that short-strawed oat varieties having a higher level of HT2+T2 (Edwards, 2007a, 2012b, 2015) may be due to genetic linkage between height and susceptibility to HT2+T2 producing *F. langsethiae*, as suggested 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 being shorter are closer to the ground where the predicted source of inoculum is (Yan *et al*., 2011).

An attempt to additionally manipulate plant height was made by applying different rates of plant growth regulators (PGR). The experiment was repeated in two consecutive seasons. The analysis in the first year showed that varieties, rather than height, were significant. However, in the second year there were no statistical differences. This can be explained as the source of inoculum of *F. langsethiae* and its dispersal mechanism is not known, as well as the fact that the experiment relied on natural infection in the field and fluctuations amongst plots are possible. Another possible explanation was that, even the varieties on a shorter end were higher than in the first year, so there were no true short-strawed varieties. But certain trends were noted in both years, where it was observed that, at certain heights, there was a range of HT2+T2 concentrations. This is in

agreement with Hilton *et al*. (1999) who argued that the FHB severity on wheat is under genetic control and influenced by a number of genes as a quantitative trait.

Results from the experiments indicate that height can be expected to be a form of morphological resistance, but there is also a possibility of a linkage, as shown in wheat. Important points to consider are the distance from the panicle to the ground. And 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.

The recommendation for further studies of resistance would include developing an artificial method of inoculation. Currently, relying 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.

5.4. Assessment of *Fusarium langsethiae* infection and mycotoxin production in a Buffalo x Tardis mapping population

This was the first study that identified QTL for *F. langsethiae* resistance and HT2+T2 concentration in oats. Results show 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). QTL which are stable across years with high LOD score might find use in marker-assisted breeding. The results 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 the experiment conducted, the plant height and flowering QTL were often found on the same genomic region or linkage group and the highest LOD score and variance was associated with them. Often, the same linkage group had QTL for *F. langsethiae* and HT2+T2 and for height. Thus, 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.

Srinivasachary *et al*. (2009) suggested that not all semi-dwarf alleles of wheat have a detrimental effect and the choice can be made so that desired height is achieved without compromising resistance to FHB suggesting that the level of FHB depends on type of alleles (e.g. Rht-B1b or

Rht-D1b). Furthermore, 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.

As with previous experiments described, due to an inability to artificially inoculate plants, the experiment depended on natural infection in the field that resulted in noticeable differences in the level of HT2+T2 mycotoxins between the three experimental years. Relying on natural infection in conditions where a disease pressure is low and without visible symptoms is likely to produce subtle differences that are hard to detect. To successfully score for disease resistance, methods of artificial inoculation need to be developed.

It is highly recommended for future studies to test different populations of hexaploid oats for *F. langsethiae* resistance. This will allow for QTL comparison across populations and environments for a more stable prediction of the position of resistance traits along the chromosomes. Breeders in future breeding programmes could use those results further. Future studies might include more lines from the same population to be tested for *F. langsethiae* resistance or testing near isogenic lines (NILs) that only differ in a small genomic region of interest in a wide range of environments. QTL mapping could help in breeding for more resistant varieties by providing valuable information when genomic regions involved in resistance are mapped. The advantage of this method is in combining genotypic with phenotypic data as it is necessary to check for QTL stability different environments and populations for a more accurate estimation of an effect.

5.5. Investigating the ability of *Fusarium langsethiae* to infect the model species *Brachypodium distachyon*

Due to the already underlined problem with inability to artificially inoculate oat plants with *F. langsethiae*, which results in unreliable level of response, and due to the lack of visible symptoms in the field when infection occurs naturally, an experiment with *B. distachyon* was conducted
This is the first study to successfully inoculate *B. distachyon* lines with *F. langsethiae*. Previously, a successful inoculation of *B. distachyon* with other *Fusarium* species was reported by Peraldi *et al*. (2011).

Some promising results were obtained. The detached leaf and flowering assay showed successful inoculation with *F. langsethiae*. Dark brown lesions were recorded and *F. langsethiae* DNA was isolated from all samples and HT2+T2 mycotoxins quantified. 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 might be the most accurate as all heads were harvested and *F. langsethiae* DNA, as well as concentration of HT2+T2, was quantified. This assay is also more likely to be a closer mimic of the natural infection of oat panicles. Whilst Bd 3-1 was the most resistant in the floral assay, it showed greater susceptibility in the leaf assay. This can be explained by different selection of genes acting on resistance for floral heads, leaves and roots where some genes are different and some are the same (Broekaert *et al*., 2000).

The root assay did not show significant difference between different lines. This might be due to an inadequate scoring method where browning naturally occurred as a result of aging and handling of roots.

Findings from experiments presented are opposite to findings of the study investigating susceptibility of *B. distachyon* to *Fusarium graminearum* (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*. These differences are to be expected as the two pathogens have many differences (Parry *et al*., 1995; Thrane *et al*., 2004; Pettersson *et al*., 2008; Edwards, 2007a,b, 2009; Edwards & Anderson, 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 and associate genetic information from species to species (Moore *et al*., 1995). 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 thus *Brachypodium* can greatly assist and accelerate research in cereals (Vain, 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. Given all this and ability of *F. langsethiae* to cause symptoms on selected lines, *B. distachyon* appears to be a suitable model host for oat - *F. langsethiae* interactions. Future work should include more *Brachypodium* lines, as well as investigate alternative methods of inoculation.

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