Student Report No. 23 **July 2013**

Study of *Fusarium langsethiae* infection in UK cereals

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Study of *Fusarium langsethiae* infection in UK cereals

by

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CONTENTS

1. ABSTRACT

This research sought to generate novel information on the epidemiology and life-cycle of *Fusarium langsethiae* to aid the development of control strategies to minimize T-2 and HT-2 mycotoxin content in cereals.

A field survey was performed to study the infection and development of *F. langsethiae* in the growing season of wheat, barley, oats and triticale under commercial production (2009 – 2011). Plants sampled (from tillering to harvest) were divided into roots, leaves, lower stem, upper stem and inflorescence/head sub-samples, depending on the growth stage of the cereal. DNA was extracted and *F. langsethiae* DNA quantified using real-time PCR. Fusarium mycotoxins HT-2 and T-2 were quantified in harvested samples. The data showed oat to contain the highest levels of both *F. langsethiae* biomass and HT-2+T-2 mycotoxins in harvested heads of the cereals studied. Head infection, if it occurred, was at head emergence but before flowering, a deviation from other *Fusarium* species. This information is very important for the design of a control strategy against *F. langsethiae* infection. Seemingly, symptomless heads had high levels of *F. langsethiae* DNA and HT-2+T-2, confirming previous suggestions that *F. langsethiae* is a symptomless pathogen of oats. Four field experiments where winter and spring varieties of wheat, barley and oats were cultivated under identical field and agronomic conditions at two sites again showed oats to have the highest *F. langsethiae* DNA and HT-2+T-2 concentration among the cereals studied. Interestingly, there was a significantly higher quantity of HT-2+T-2 per unit *F. langsethiae* DNA for oats compared to wheat and barley.

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

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

under natural infections in commercial fields, suggesting that the infection route for *F. langsethiae* may not be that different from the other Fusarium head blight pathogens.

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

2. INTRODUCTION

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

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

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

Fusarium langsethiae has been identified as the primary producer of HT-2 and T-2 in European cereals (Langseth and Rundberget, 1999; Edwards *et al*., 2012). HT-2 and T-2 are considered as two of the most potent type A trichothecene mycotoxins and are a public health concern in Europe. The European Commission in January 2012 proposed discussion limits for HT-2+T-2 in cereals and cereal by-products. In unprocessed wheat, barley and oats for example, limits for discussion are 50, 200 and 1000 µg kg^{-1}, respectively. If legislation is set for HT-2+T-2, farmers will have to control *F. langsethiae* in their cereals if they are to be marketed for human consumption. However, the *F. langsethiae*-cereal interaction is poorly understood making the development of control strategies difficult. Attempts have been made to understand its pathogenicity and aggressiveness towards wheat, barley and oats using *in vitro* assays (Imathiu *et al*. 2009; Opoku *et al*. 2011) but

studies are still at the early stages and available information is not adequate to fully understand this interaction.

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

- To quantify *F. langsethiae* development and HT-2+T-2 production during crop development in commercial crops of wheat, barley, oats and triticale.
- To determine differences in disease development and severity in wheat, barley and oats caused by *F. langsethiae.*
- To develop artificial inoculation method(s) to infect wheat, barley and oats with *F. langsethiae*
- To quantify *F. langsethiae* development and HT-2+T-2 production during crop development in artificially inoculated experiments of wheat, barley and oats.

3. INVESTIGATIONS

3.1. Development of *Fusarium langsethiae* in commercial cereal production: a field survey

To understand the infection and development of *F. langsethiae* in cereals in the UK, a survey was carried out in commercial fields of wheat, barley, oats and triticale in Shropshire and Staffordshire during the 2009, 2010 and 2011 cropping seasons. All fields were within 30 km of Harper Adams University College, Newport, Shropshire UK. In all cropping seasons, sampling was done between April and August. Surveys were carried out in 25, 27 and 26 different fields in 2009, 2010 and 2011 cropping seasons, respectively. Sampling started from the tillering stage through to harvest. Sampled plants were visually assessed for any disease symptoms before processing. Processing involved washing of soil from roots, dividing into root, leaves, lower stem, upper stem and head sub-samples depending on the growth stage of the plant. Sub-samples were freeze-dried, milled and then DNA and mycotoxins analysis carried out.

Results from this study showed that: 1. *Fusarium langsethiae* biomass and HT-2+T-2 in harvested heads of the cereals differed, with oats having the highest concentrations in all three years of the study (Fig. 1). 2. There was a yearly difference in *F. langsethiae* biomass as well as HT-2+T-2 concentrations (Fig. 1) and, 3. The development of *F. langsethiae* in the cereals studied over the sampling periods followed a similar pattern with infection taking place at head emergence but before flowering (Fig. 2).

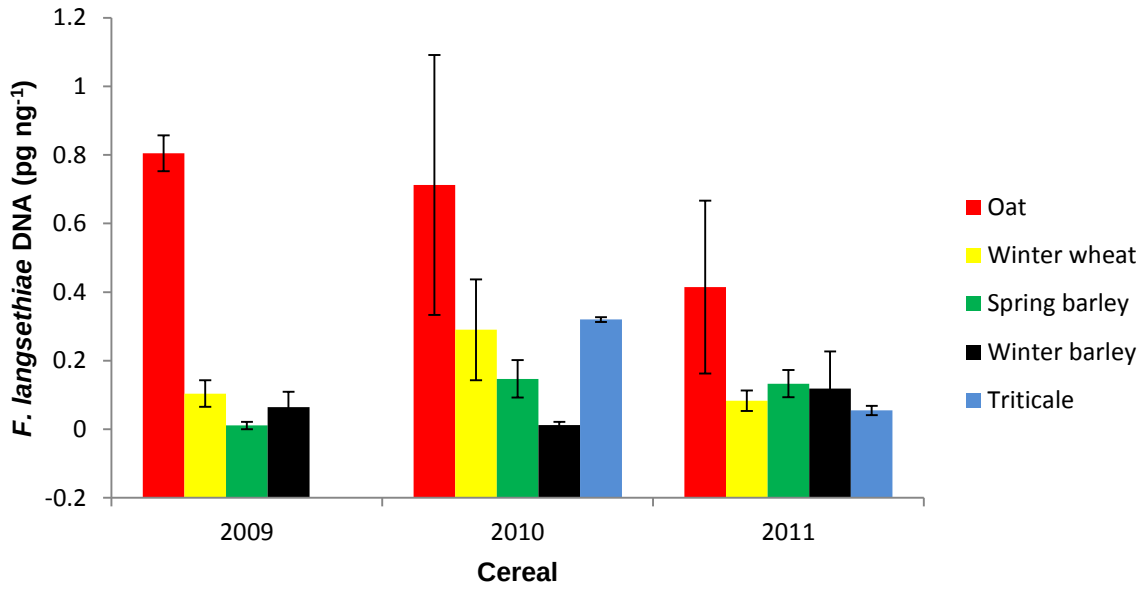

Figure 1. Mean *F. langsethiae* DNA in cereal head sub-samples of oat, winter wheat, spring barley, winter barley and triticale at harvest (G92) in 2009, 2010 and 2011 cropping seasons. Error bars represent standard error.

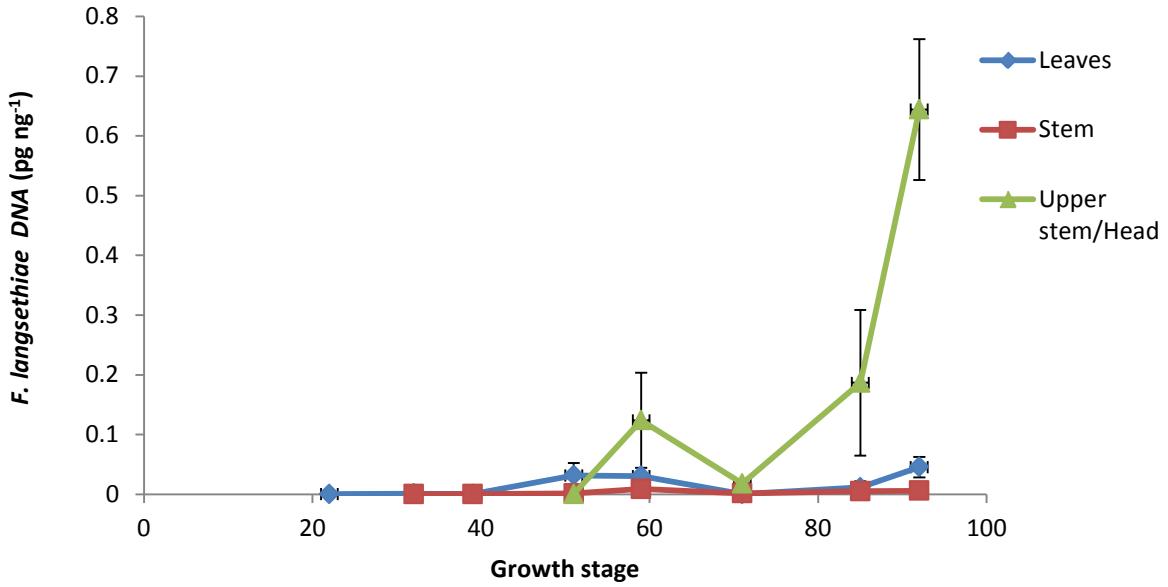

Figure 2. Mean *F. langsethiae* DNA in oat plants under commercial cultivation in 2009, 2010 and 2011 cropping seasons. (n = 24). Bars represent standard error.

A follow up study where three varieties each of winter and spring wheat, barley and oats were cultivated under identical field and cultural conditions at two different sites was conducted in the 2011 cropping season. This study again showed oat varieties to have the highest *F. langsethiae* biomass and HT-2+T-2 concentrations among the cereals studied (Tables 1 and 2). The results also showed winter oat varieties to have higher concentrations of *F. langsethiae* biomass and HT-2+T-2 than spring varieties (Tables 1 and 2).

Table 1. Log-transformed *F. langsethiae* DNA (pg ng^{-1}) for heads of wheat, barley and oats at harvest in 2011. Back transformed data in parentheses.

	Winter varieties	Spring varieties
Wheat	2.371 (0.0043)	2.420 (0.0037)
Barley	2.762 (0.0017)	2.597 (0.0025)
Oat	1.754 (0.0176)	2.239 (0.0058)
P-value	<0.001	0.01
%CV	7.1	8.4
s.e	0.48	0.22
LSD (5%)	0.176	0.234

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

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

The study also showed that after harvest, the concentrations of *F. langsethiae* biomass as well as HT-2+T-2 in grains and the rest of the cereal heads (comprising rachis, lemma and glume in wheat, the awns and the rachis in barley and rachis, rachis branches and the glumes in oats) differed, with higher concentrations occurring in the rest of the head (Figures 3 and 4).

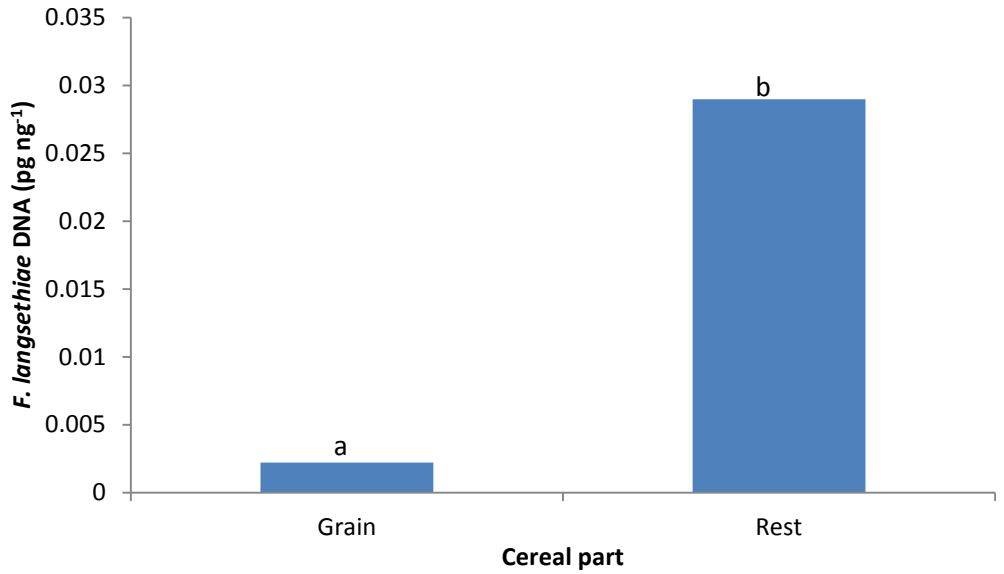

Figure 3. Back-transformed mean *F. langsethiae* DNA recovered from grains and the rest of the winter cereal heads. Bars with different letters are significantly different from each other (LSD = 0.01).

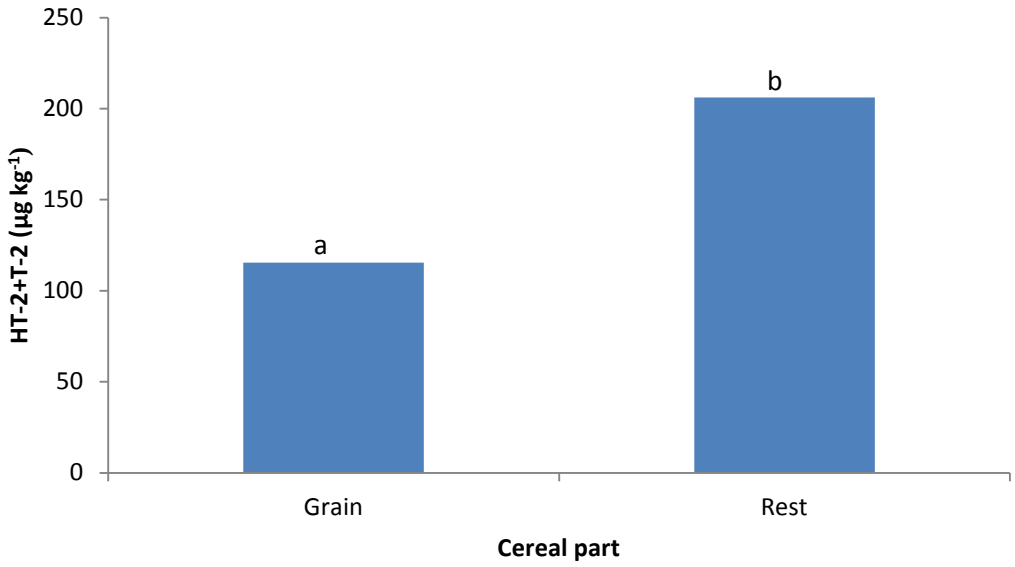

Figure 4. Back-transformed HT-2+T-2 in grains and the rest of the winter cereal heads. Bars with different letters are significantly different from each other (LSD = 1.8).

A simple linear regression between *F. langsethiae* DNA recovered from winter cereal heads and their corresponding HT-2+T-2 grouped by cereal species showed a highly significant ($P < 0.001$) regression best fitted by separate non-parallel lines. However, for wheat and barley alone, a single line accounted for 68% of the variance and parallel lines although significant accounted for only an additional 1.5% of the total variance observed. Thus, two lines were fitted, one for oat ($P < 0.001$, $r^2 = 0.79$) and another for wheat and barley ($P < 0.001$, $r^2 = 0.68$) (Fig. 5).

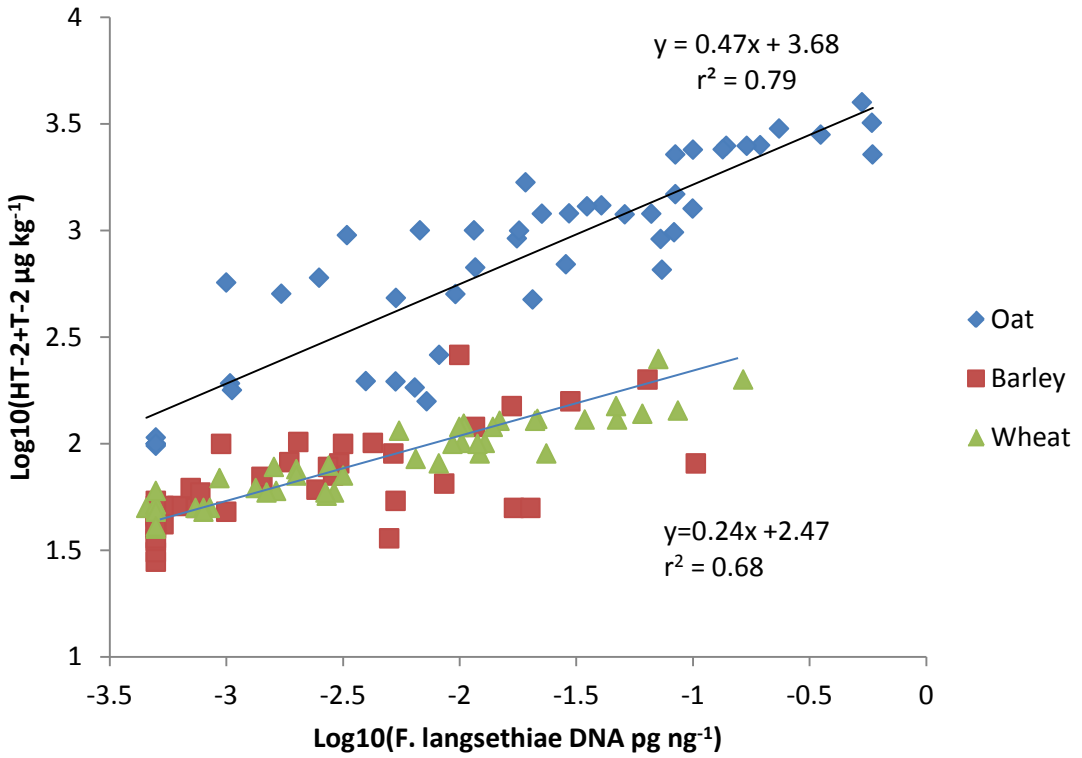

Figure 5. The relationship between *F. langsethiae* DNA and HT-2+T-2 concentration in winter cereal heads.

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

For spring cereal varieties, simple linear regression between *F. langsethiae* DNA recovered from heads and their corresponding HT-2+T-2 grouped by cereal species was highly significant ($p < 0.001$) and was best fitted by separate non-parallel lines. However, for wheat and barley alone, a single line accounted for 73% of the variance and parallel lines, although significant, accounted for only an additional 5.3% of the total variance observed. Thus, two lines were fitted, one for oat ($P < 0.001$, $r^2 = 0.78$) and another for wheat and barley ($P < 0.001$, $r^2 = 0.73$) (Fig.6)

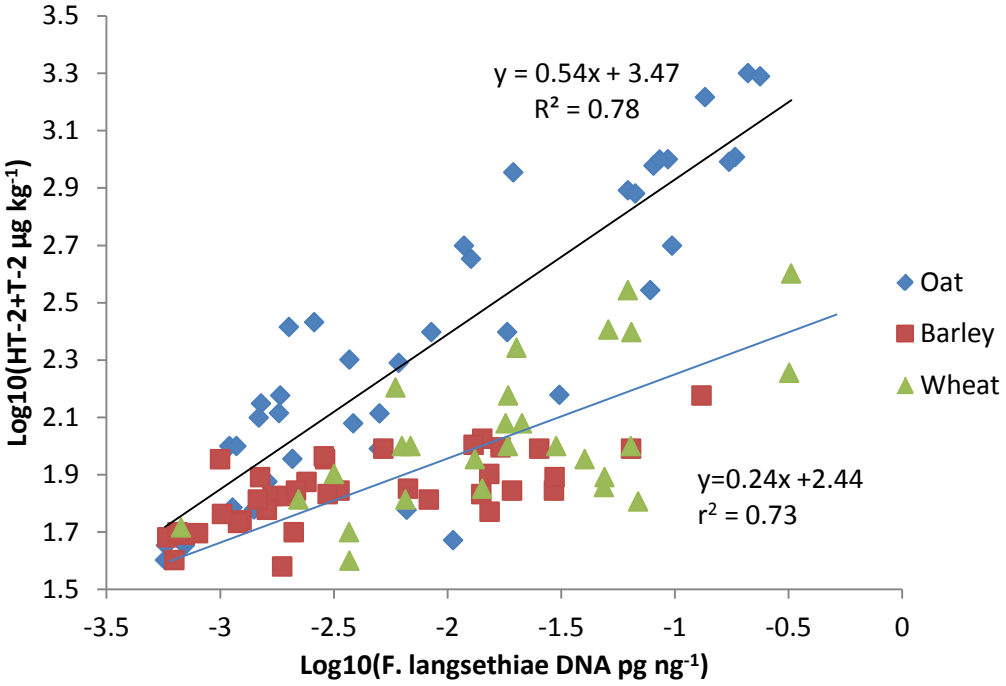

Figure 6. Relationship between *F. langsethiae* DNA and HT-2+T-2 concentration in spring cereal heads.

A visual comparison of Fig. 5 and 6 showed that the regressions for winter and spring oats were similar and the regression for winter and spring wheat and barley were nearly identical. Regression analysis was repeated for all oats grouped by sowing date (winter and spring). The data was best fitted by separate parallel lines (Fig. 7) which accounted for 84% of the observed variance.

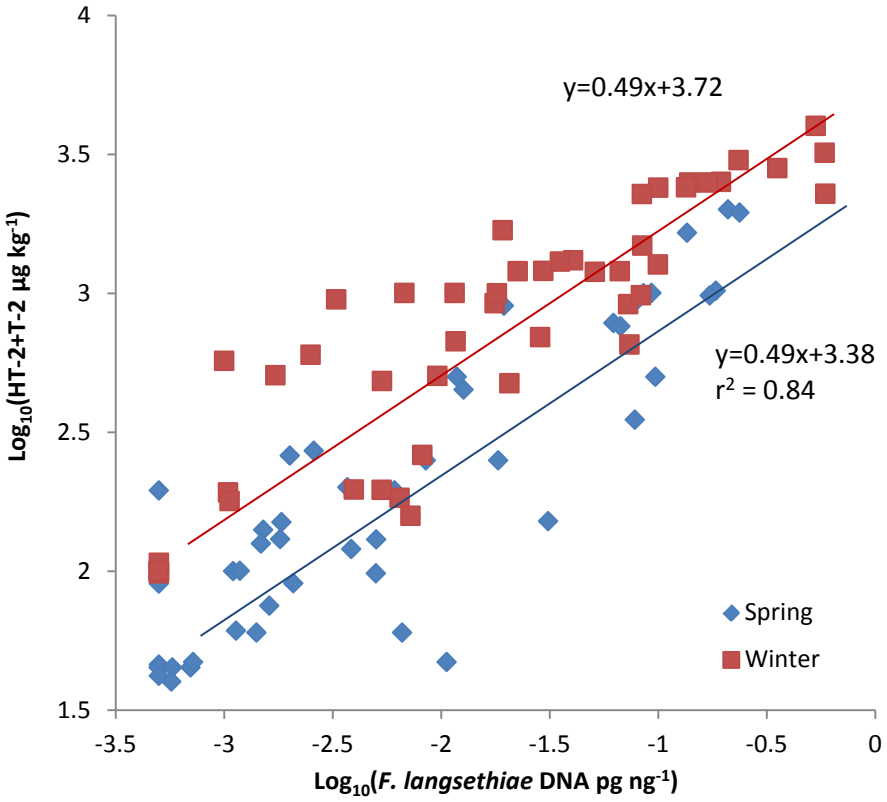

Figure 7. Relationship between *F. langsethiae* DNA and HT-2+T-2 concentration in winter and spring sown oats.

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

3.2. Resistance of UK wheat, barley and oat varieties to *Fusarium langsethiae*

An *in-vitro* detached leaf assay was used to screen UK varieties from the HGCA Recommended Lists in 2010 of wheat, barley and oats for resistance against *F. langsethiae* infection. Results from the experiment showed that none of the cereal varieties screened had total resistance to *F. langsethiae* infection, however, in oats, varieties with low HT-2+T-2 under field conditions (data from Edwards, 2012) also had shorter lesion lengths *in-vitro,* suggesting that the detached *in-vitro* leaf assay could be a good predictor of HT-2+T-2 concentration in harvested grain.

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

To study the mode of infection of *F. langsethiae* in cereals, an initial seed infection experiment was carried out to determine if *F. langsethiae* which had been shown to be seed borne (Torp and Adler, 2004; Torp and Nirenberg, 2004; Imathiu, 2008) could be systemically transmitted through the seed to the other parts of oats. This study involved the sowing of untreated seed samples of Gerald (five with high, one with low and four with undetectable HT-2+T-2 levels) from the 2008 harvest. Seeds were sown in bread baskets and the experiment carried out in a polytunnel.

The results from the experiment showed that although *F. langsethiae* was isolated from the oat seeds with high *F. langsethiae* DNA and high HT-2+T-2 concentration, at harvest, *F. langsethiae* DNA was not quantified in the resulting heads and HT-2+T-2 levels were mostly below the limit of quantification. This demonstrated that *F. langsethiae* was not likely to be a systemically seed transmitted pathogen. This does not however, indicate that seeds may not play any role in the infection process of *F. langsethiae* in cereals.

Three artificial inoculation trials (stem base inoculation, boot inoculation and spray inoculation) were carried out to determine if a suitable one could be identified and optimised for F. *langsethiae*-cereal studies. The stem base infection study showed that, whilst *F. graminearum* inoculated plants, used as a positive control, showed symptoms of stem infection, *F. langsethiae* inoculated plants showed no symptoms and throughout the study period there was no observable difference in control plants (uninoculated) and *F. langsethiae* inoculated plants (Figures 8–11). This indicated that *F. langsethiae* could not cause stem base infection even when in close contact with the stem base of the cereals studied.

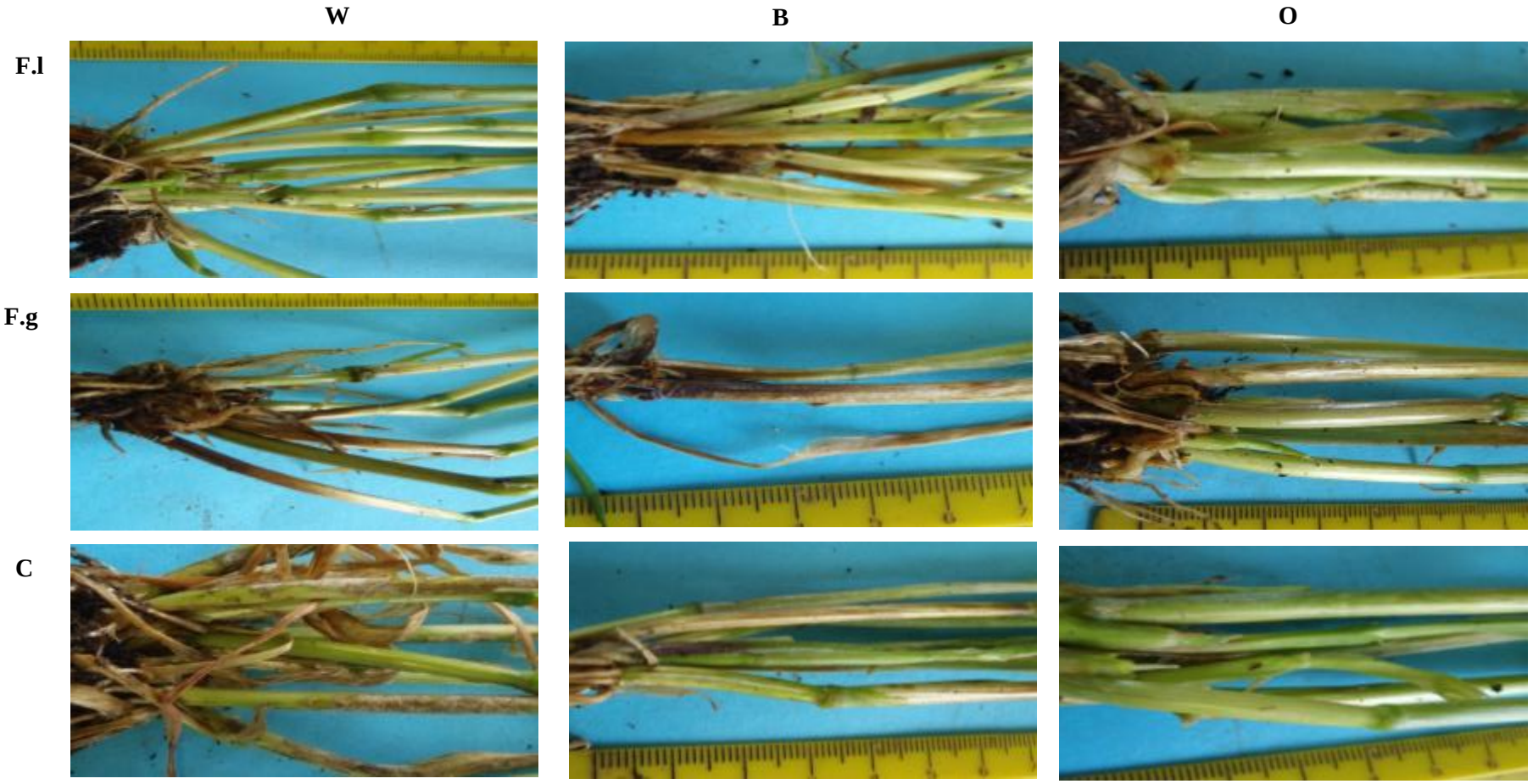

Figure 8. Mycelium-plug inoculated wheat (W), barley (B) and oat (O) (28 DPI). F.l = *F. langsethiae*, F.g = *F. graminearum* and C = PDA plug inoculated seedling.

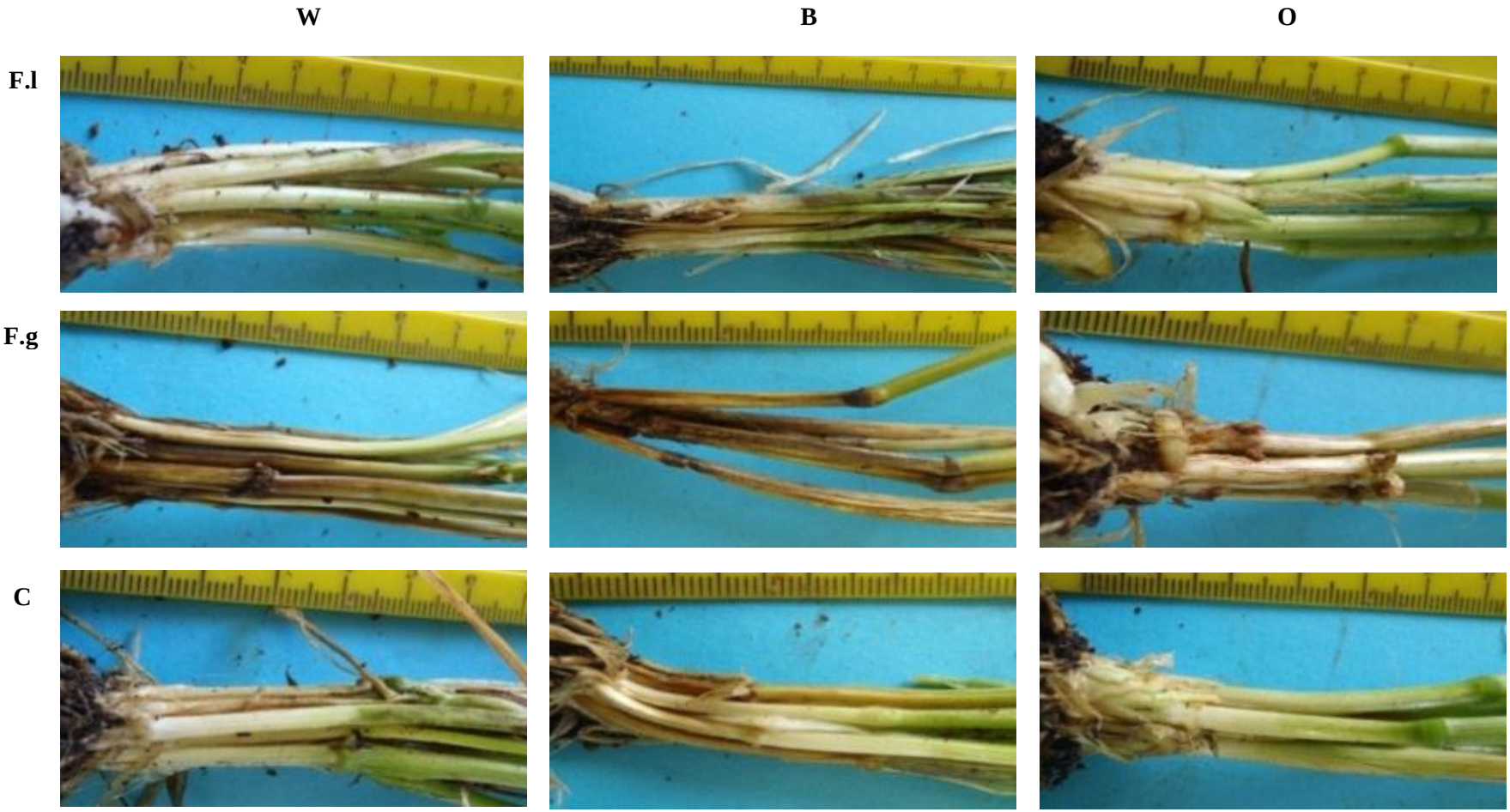

Figure 9. Spore-agar inoculated wheat (W), barley (B) and oat (O) (28 DPI). F.l = *F. langsethiae*, F.g = *F. graminearum* and C = water-agar inoculated seedlings.

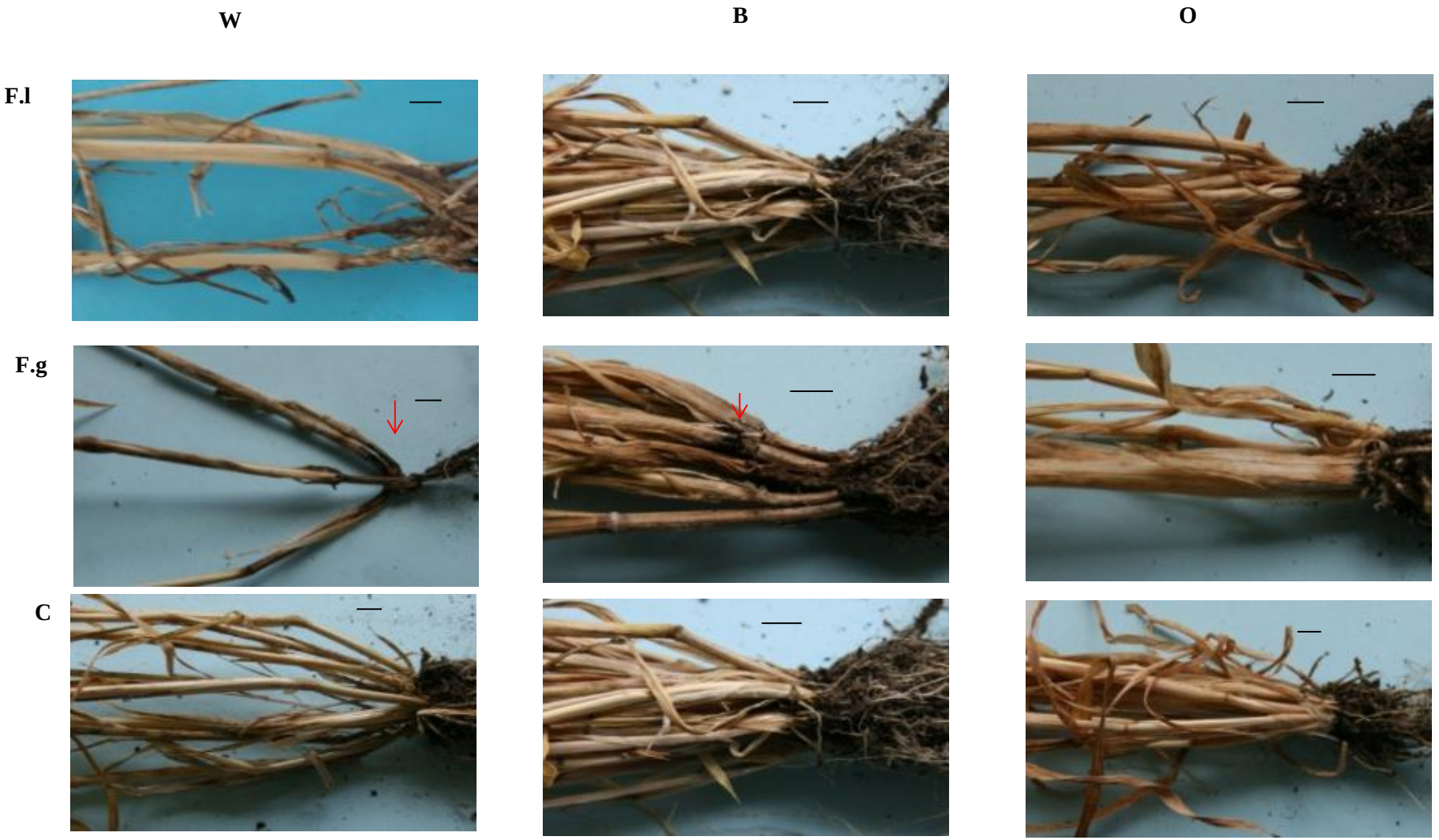

Figure 10. Mycelium-plug inoculated wheat (W), barley (B) and oat (O). A, B and C = *F. langsethiae, F. graminearum* and PDA plug inoculated plants as seen at harvest. Bar = 1 cm. Red arrow indicates position of perithecia.

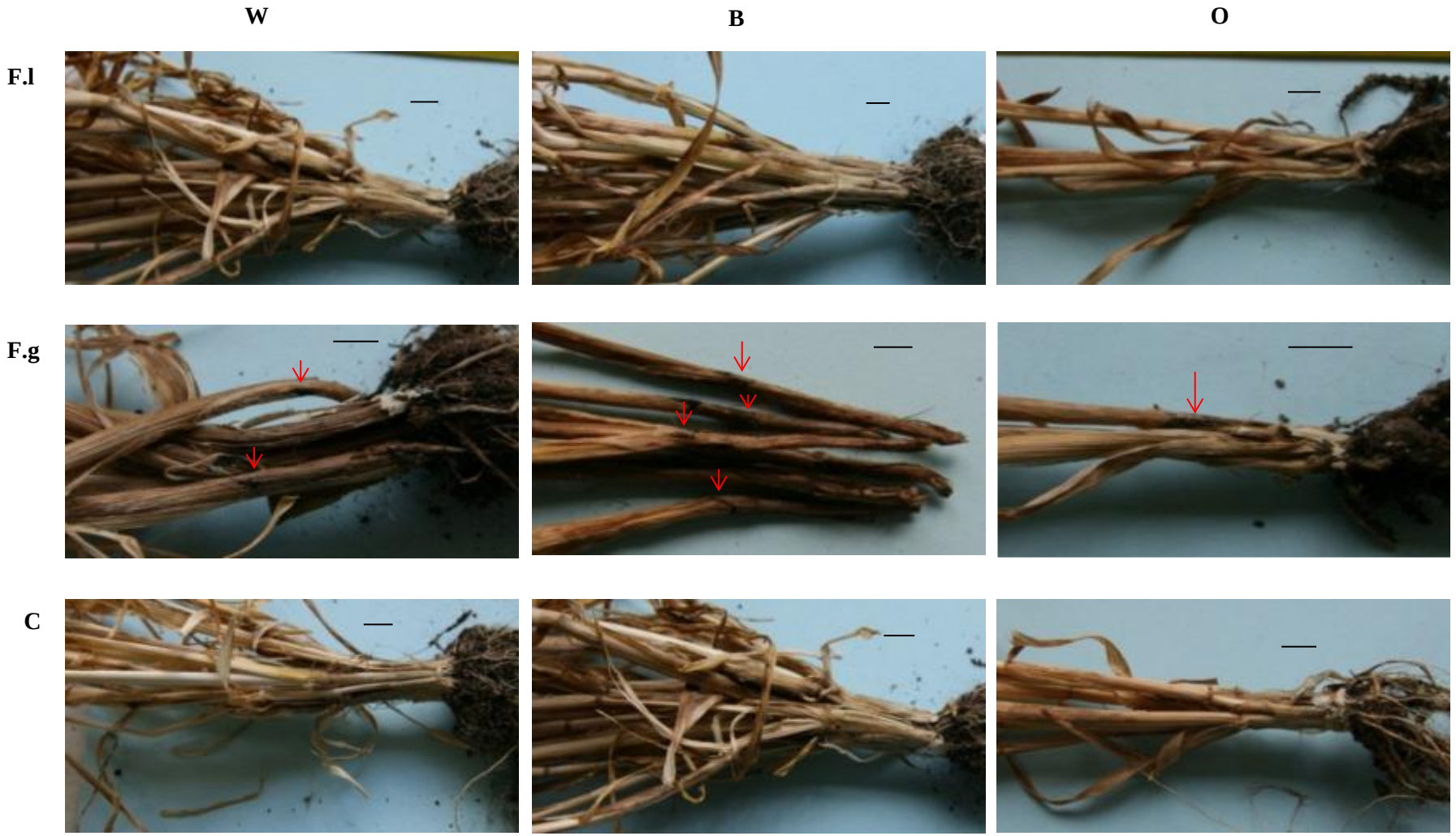

Figure 11. Spore-agar inoculated wheat (W), barley (B) and oat (O). A, B and C = *F. langsethiae, F. graminearum* and water agar inoculated plants as seen at harvest. Bar = 1 cm. Red arrow indicates position of perithecia.

The boot inoculation method resulted in disease symptoms in the form of lesions on the boots 14 days after inoculation (Fig. 12).

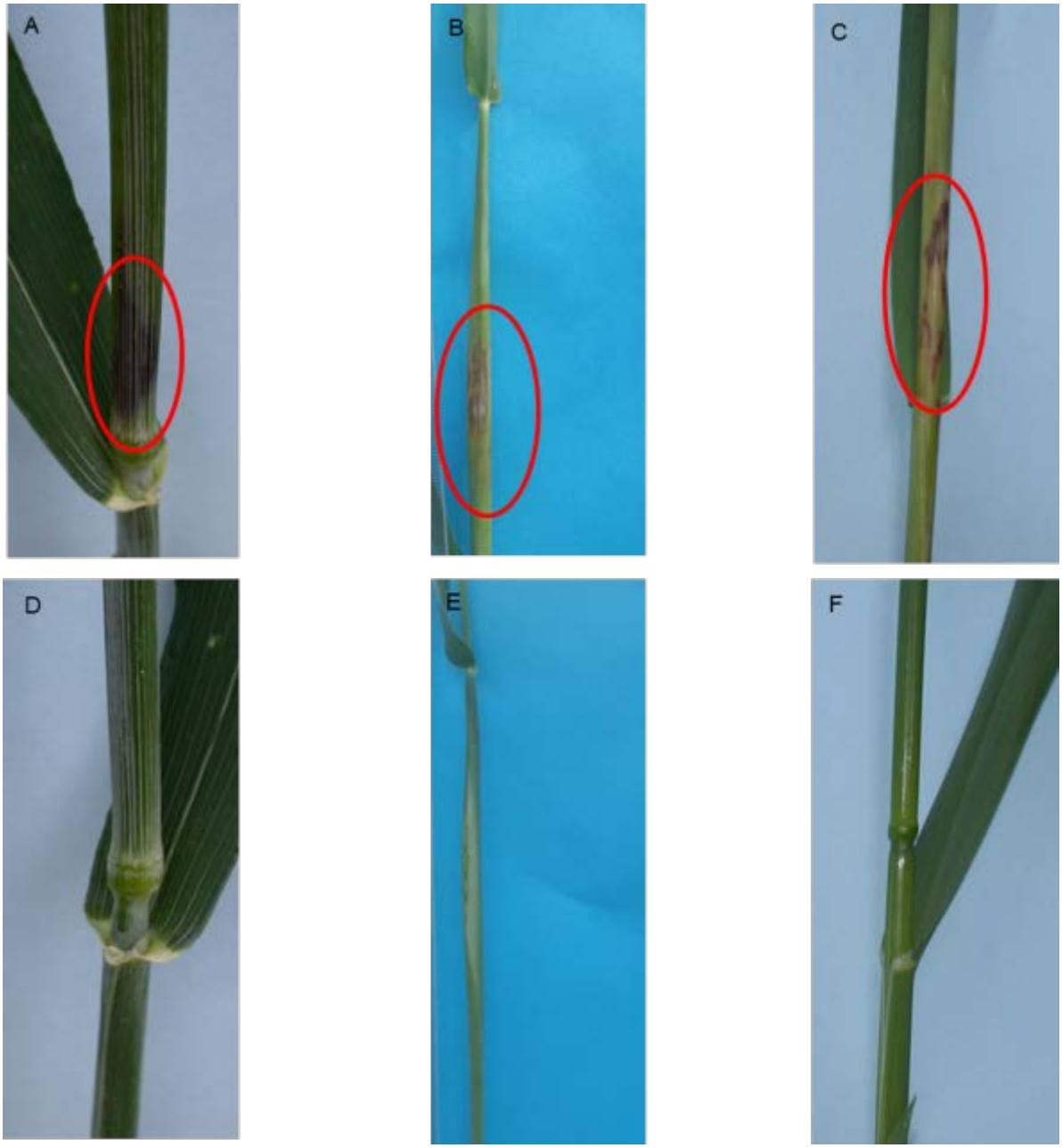

Figure 12. *F. langsethiae* boot inoculated wheat, barley and oat (A, B and C, respectively) (inoculation at GS47 with 10^5 spores ml^{-1}) and the corresponding water inoculated plants (D, E and F, respectively) in the glasshouse. Pictures were taken 14 DPI. Bar = 1.8 cm on A, 1.6 cm on B and 2 cm on D.

Head emergence in *F. langsethiae* inoculated plants was delayed and on emergence heads were stunted with lesions on spikelets (wheat and barley) and panicle (oats) (Figure 13).

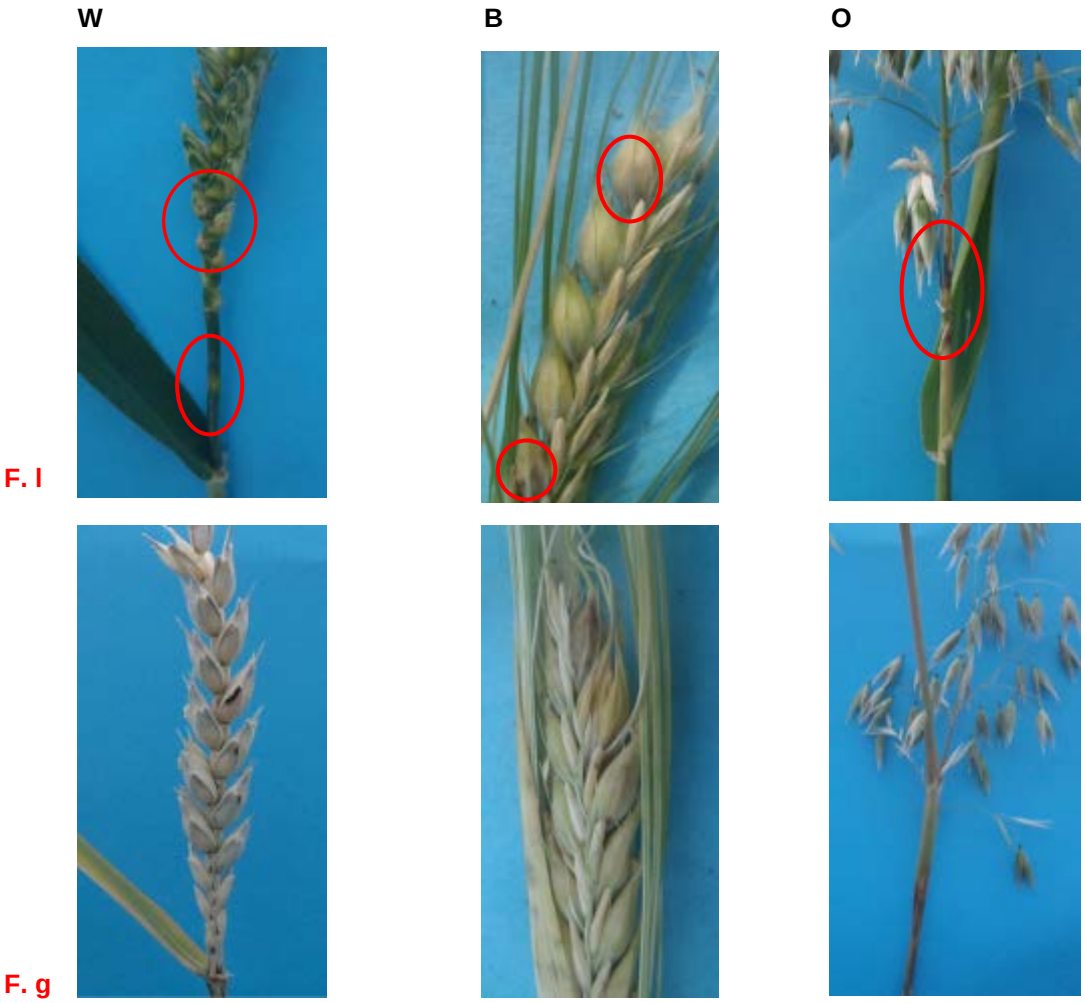

Figure 13. Boot inoculated wheat (W), barley (B) and oat (O) (inoculation at GS47 with 10^5 spores ml^{-1}) in the glasshouse. Pictures were taken 21 DPI. F. l = *F. langsethiae* and F. g = *F. graminearum* inoculated plants. Red circles show lesions on panicles and grain.

Although the method resulted in infection and subsequent disease symptoms, the method is not likely to mimic natural infection due to the mode of introduction of the inoculum into the boot (direct injection of inoculum with a hypodermic needle into the boot).

The spray inoculation method, where inoculum in the form of spore suspension was sprayed onto cereal heads at full head emergence but before flowering (GS57–GS59), resulted in *F. langsethiae* biomass and HT-2+T-2 concentrations comparable with that observed in the field under natural infection conditions, suggesting that it was a better method to mimic natural infection and thus a good artificial method for infecting cereals with *F. langsethiae.* The method also resulted in disease symptoms in the form of bleaching of cereal heads (Figures 14 – 16).

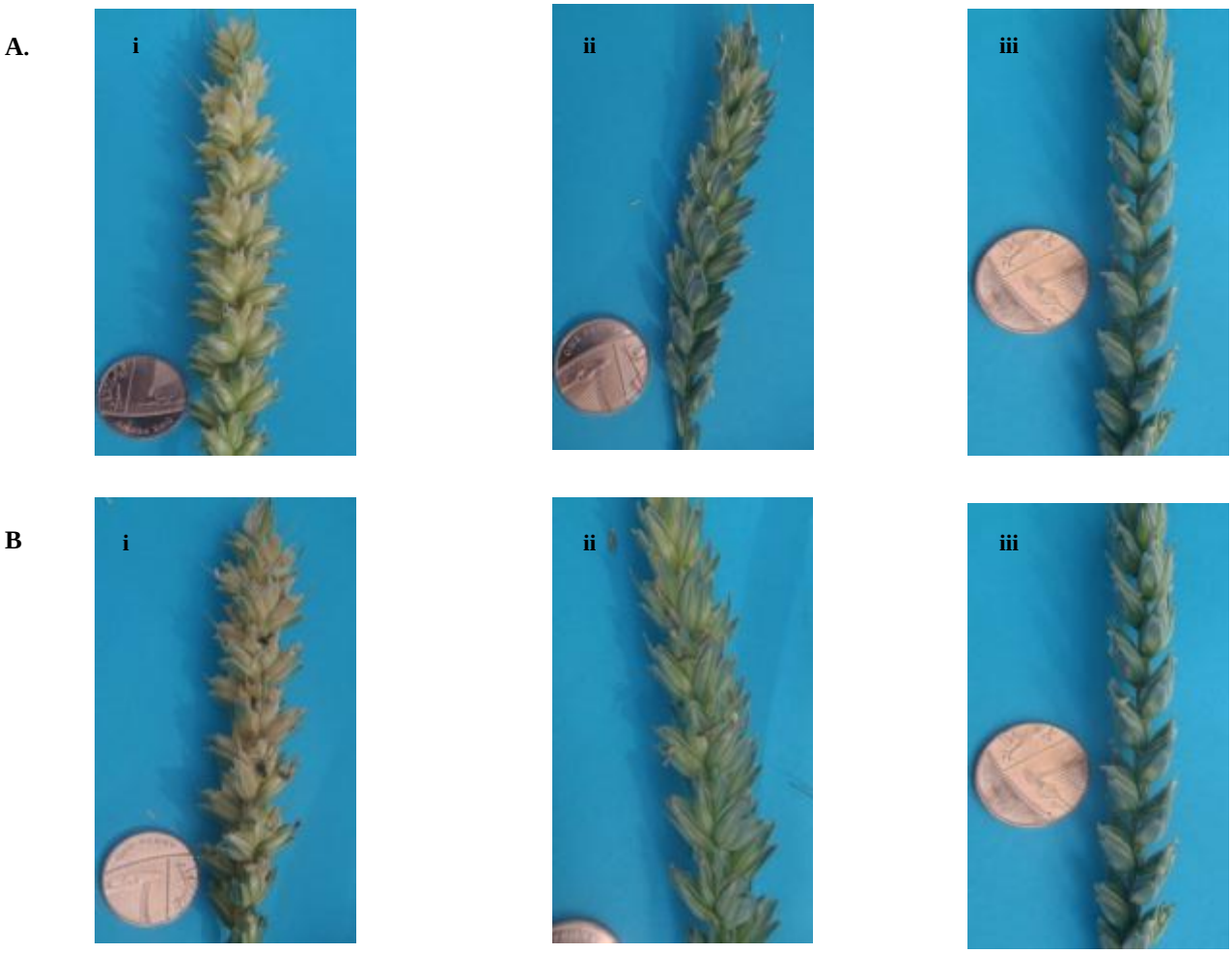

Figure 14. *Fusarium langsethiae* spray inoculated wheat heads; A and B are heads bagged with perforated and normal bags respectively (14 DPI). (i), (ii) and (iii) are inoculated and bagged, bagged but uninoculated and inoculated without bagging of heads, respectively.

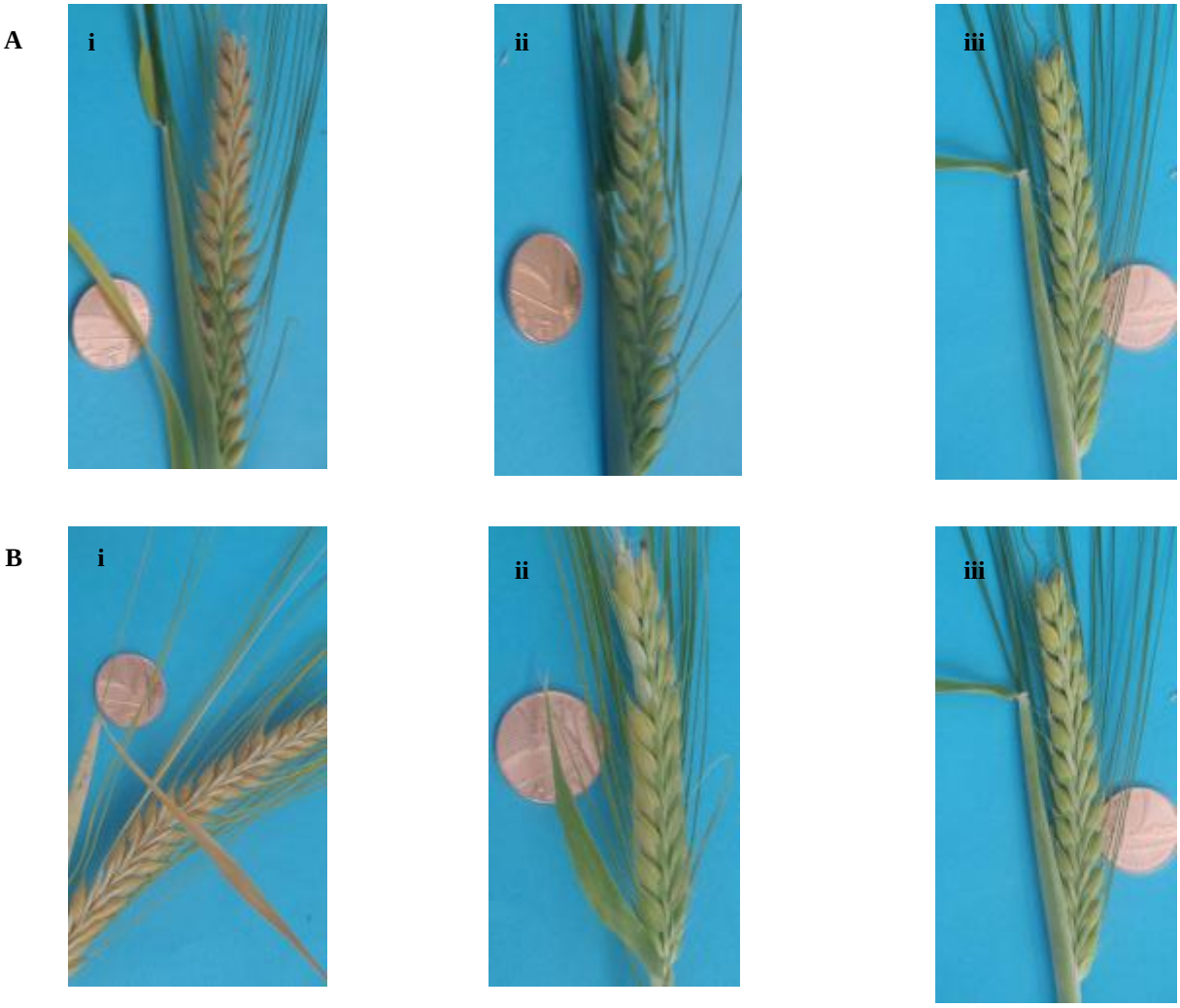

Figure 15. *Fusarium langsethiae* spray inoculated barley heads; A and B are heads bagged with perforated and normal bags respectively (14 DPI). i, ii and iii are inoculated and bagged, bagged but uninoculated and inoculated without bagging of heads, respectively.

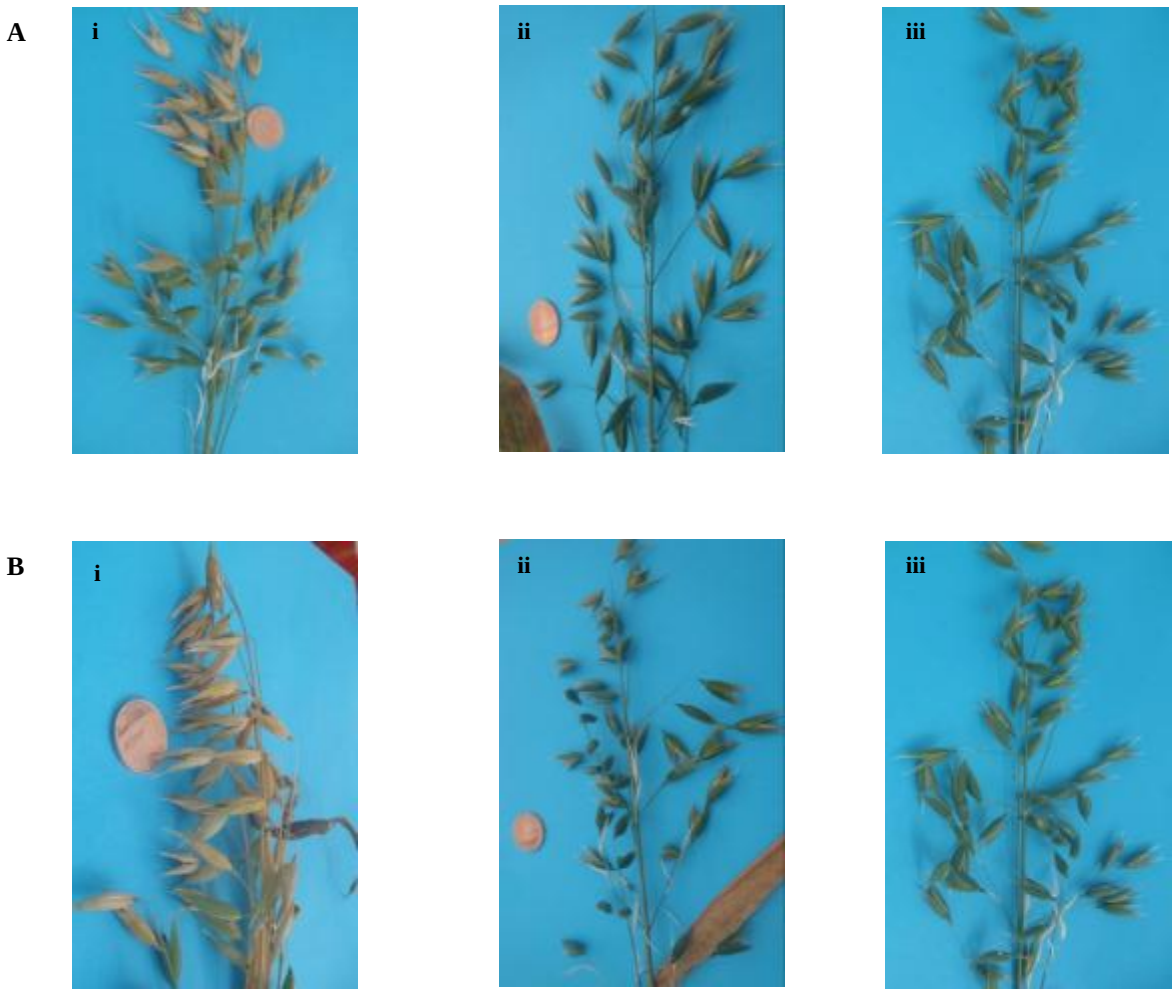

Figure 16. *Fusarium langsethiae* spray inoculated oat heads; A and B are heads bagged with perforated and normal bags, respectively (14 DPI). i, ii and iii are inoculated and bagged, bagged but uninoculated and inoculated without bagging of heads, respectively.

4. CONCLUSIONS

This study has provided novel information on the life cycle of *F. langsethiae* in wheat, barley, oats and triticale. In all three years of the field survey, no stem base infection was observed on any of the samples with high *F. langsethiae* DNA and HT-2+T-2 concentrations. This suggests that *F. langsethiae* may not cause stem base infections. When two stem base infections experiments were carried out, it was observed that, whilst *F. graminearum* inoculated plants showed symptoms of foot rot from 14 days after inoculation, *F. langsethiae* inoculated plants showed no such symptoms and at harvest there was no observable difference between the stems of control and *F. langsethiae* inoculated plants confirming the inability of *F. langsethiae* to cause stem base infection even when inoculum is in close contact with the host plant. The field survey data showed that if *F. langsethiae* head infection in wheat, barley, oats and triticale occurs, it is at full head emergence but before flowering. This information is very important in the development of a control programme for *F. langsethiae* in cereals in the UK. This is because the current chemical spraying regime against *Fusarium* infection in cereals does not seem to be effective against *F. langsethiae* in the

field (Edwards and Anderson, 2011) although under laboratory conditions some active fungicides such as prochloraz, tebuconazole and fenpropimorph seem to have antifungal activity on *F. langsethiae* (Mateo *et al*. 2011). Chemical spraying against FHB in wheat, barley and oat is done close to the infection period, that is early anthesis (GS61–65). However, data generated from this study has shown that at anthesis *F. langsethiae,* if present, would have already infected the cereal head. This is evident by the presence of *F. langsethiae* biomass in cereal heads at head emergence at relatively high levels. This may explain why these known fungicides, although effective against FHB species are not effective against *F. langsethiae*. This suggests that if the spraying time is optimised through experimentation, a chemical control could be effective against *F. langsethiae*.

Based on all the information gathered from this research it can be postulated that the generalised life cycle of *F. langsethiae* deviates from that of the known FHB pathogens. These deviations include its inability to cause stem base infection and its earlier head infection in cereals (Figure 17). Such information is critical to the development of effective control measures to reduce the infection *F. langsethiae* in cereals and the subsequent contamination of cereals with HT-2+T-2.

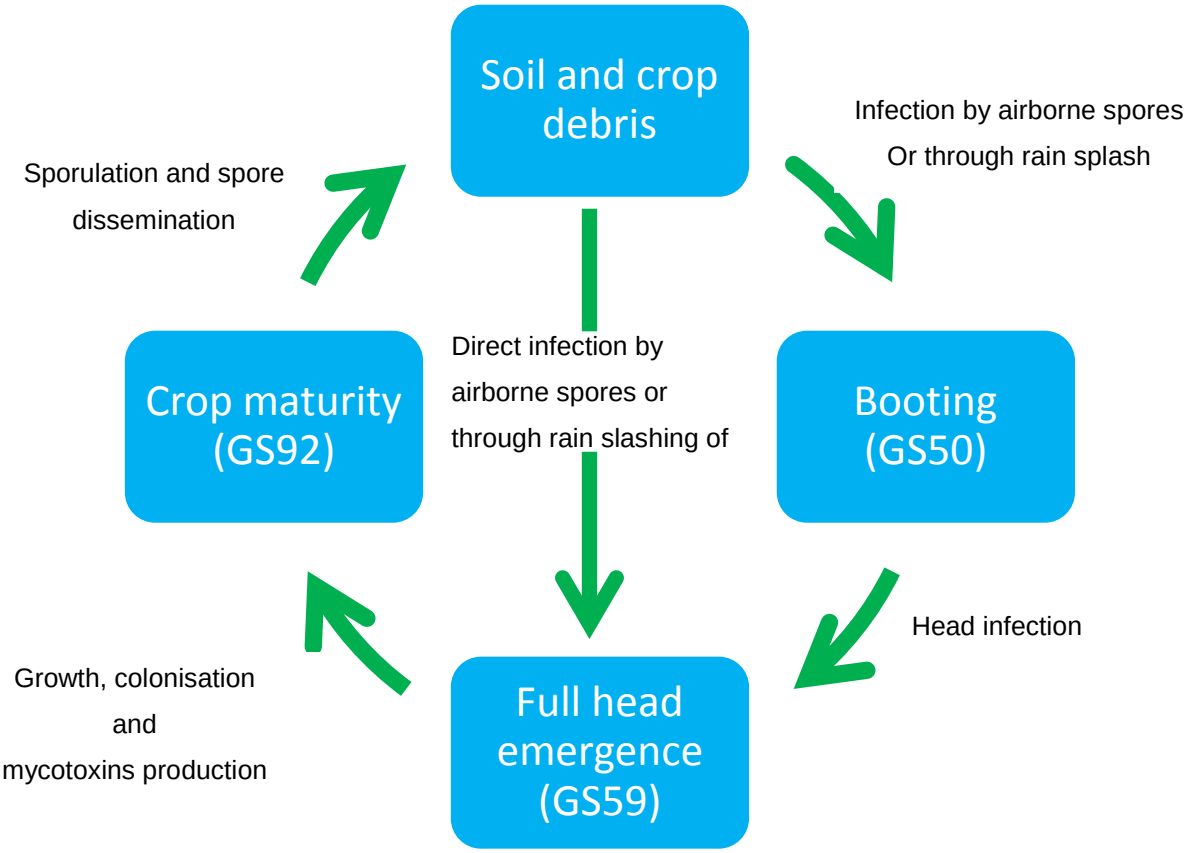

Figure 17. Postulated life-cycle of *F. langsethiae* in cereals based on a three-year field survey, artificial inoculations and the generalised life-cycle of *Fusarium* species.

4.1. Recommendations for future work

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

- This study could not identify how *F. langsethiae* overwinters. It was also not able to identify resting structures for *F. langsethiae.* It should be mentioned here that some sporodochia-like structures were identified on one-year old PDA plates (Fig. 18).

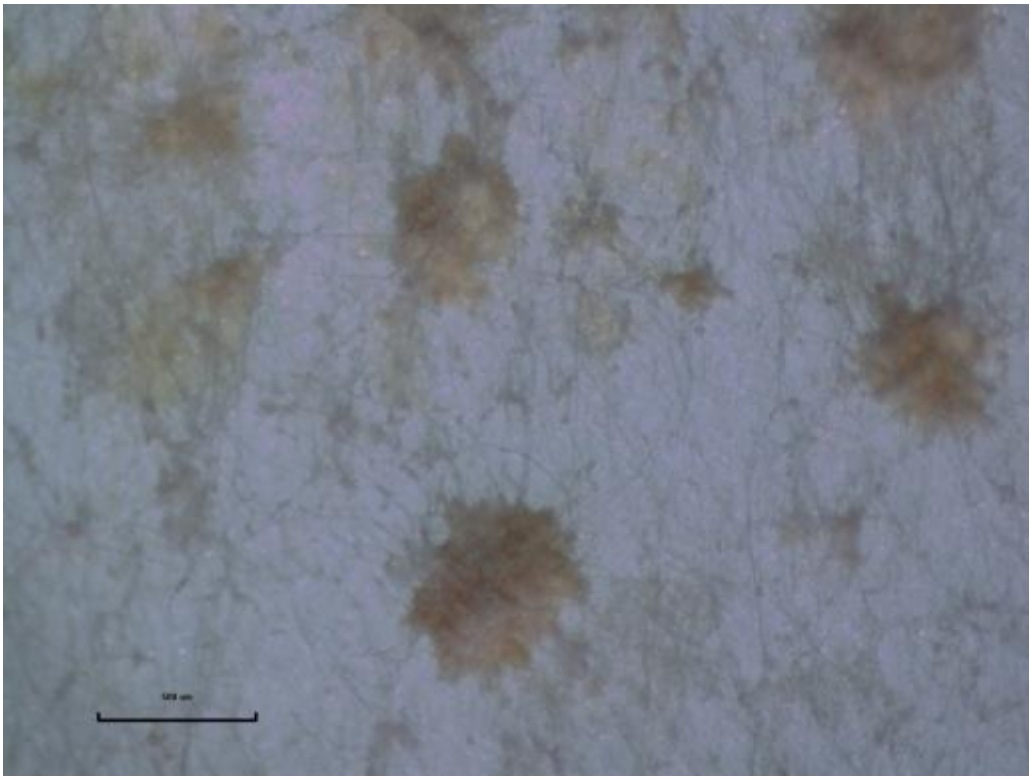

Figure 18. Sporodochia-like *F. langsethiae* structure on PDA plate left standing for one year Scale = 500 µm.

These structures, upon crushing in SDW, were found to contain a large number of microconidia. Morphological studies and PCR proved that spores were that of *F. langsethiae*. It will therefore be of interest if further studies are carried out to confirm or otherwise what these structures are and to properly describe them. These experiments could include culturing *F, langsethiae* on different low nutrient agars and other culturing methods under natural conditions over time.

- The field survey showed that *F. langsethiae* infection occurred at full head emergence. It was, however, not ascertained how the spores were transmitted onto the heads (although some assumptions have been made) or the conditions that stimulate the spores to be released. A spore trap experiment could be performed to monitor *F. langsethiae* dynamics

in air and to determine if it could be related to specific weather conditions and crop growth stages.

- It was observed that, under identical field and agronomic conditions, *F. langsethiae* infection was still highest in oats compared to wheat and barley. This relationship is poorly understood. There is, therefore, the need to carry out further studies to understand the factors leading to this observed preference of *F. langsethiae* for oats and, most importantly, the resistance mechanism and the genes that influence this relationship.

- Although this study has provided some information on the infection process of *F. langsethiae* it is still not clear how the actual head or grain infection takes place and the type of infection structures that are employed by this fungal species during infection. There is, therefore, the need to carry out more artificial inoculation studies coupled with microscopy studies to determine the mechanism of infection and the fungal and plant structures involved in this process. The use of GFP (green fluorescent protein) labelled genetically modified isolates of *F. langsethiae* would greatly facilitate such studies.

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