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AGGRESSIVENESS OF *FUSARIUM LANGSETHIAE* ISOLATES TOWARDS WHEAT, BARLEY AND OATS IN AN *IN VITR*O LEAF ASSAY

ABSTRACT

Fusarium langsethiae has been identified as the primary producer of HT-2 and T-2 in European cereals. HT-2 and T-2 are considered as two of the most potent trichothecenes mycotoxins and a public health concern in Europe. There is currently no legislation on HT-2 and T-2, however, there is a discussion limit of 500 µg kg $^{-1}$ in unprocessed oats, 200 µg kg^{-1} for oat products and 50 µg kg^{-1} for infant food. There are limited data regarding F. *langsethiae's* pathogenicity and mycotoxin production, but it is evident that its behaviour deviates from traditional trichothecene-producing *Fusarium* species. This experiment was aimed at assessing the aggressiveness (measured by lesion length) of 20 different F. *langsethiae* isolates on wheat, barley and oats using an *in vitro* detached leaf assay. There was a significant (P<0.001) difference between lesion lengths formed by different F. *langsethiae* isolates used. Isolate Fl/2004/17(a) caused the shortest lesion on all cereals and this was significantly (P<0.001) different from that caused by isolate Fl/0/08/009/1 which caused the longest lesion on all cereals used. A highly significant difference (P<0.001) was also observed between lesions on the different cereals (wheat, barley and oats). Lesions on oats were the longest, followed by barley with wheat showing the shortest lesions.

Key words: aggressiveness, *Fusarium langsethiae*, HT-2, *in vitro,* leaf assay, T-2, trichothecenes.

INTRODUCTION

Fusarium langsethiae was first described in 1999 as a species that closely resembled F. *poae* morphologically and F. *sporotrichioides* in terms of metabolite profiling. It was initially called 'powdery' *F. poae* due to its powdery appearance on artificial growth media (Torp and Langseth 1999). It was recognized as a new species and named *F. langsethiae* in 2004 (Torp and Nirenberg 2004). Morphologically, both

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F. langsethiae and *F. poae* produce conidia that are globose to napiform in shape, however, F. *langsethiae* is differentiated from F. *poae* by its slower growth rate, producing less aerial mycelium with conidia borne on bent phialides as compared with straight monophialides of *F. poae* and the absence of falcate sporodochial conidia when cultured on synthetic low-nutrient agar (Torp and Nirenberg 2004). *Fusarium langsethiae* bares some morphological resemblance to F. *sporotrichioides* (similar conidia;-napiform in aerial mycelium) and the two species have a similar mycotoxin profile to *F. sporotrichioides*. Both species produce a number of mycotoxins; trichothecenes (T-2, HT-2, HT-2, DAS and NEO), culmorins, chrysogine and aurofusarin (Thrane *et al.* 2004).

The geographic distribution of F. *langsethiae* can not be described with certainty due to its recent identification and lack of experience in identifying this newly identified species (Edwards *et al.* 2009). Wilson *et al.* (2004) for example, indicated a situation where two isolates from Poland and Italy were initially identified as *F. sporotrichioides* but further analysis using PCR (ITS and *TRI5* sequences) confirmed that they were indeed F. *langsethiae*. This not withstanding, *F. langsethiae* has been reported mainly in Europe; Austria, the Czech Republic, Denmark, England, Germany and Norway (Torp and Adler 2004, Torp and Nirenberg 2004) and more 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 (Gautier 2007, Imathiu 2008). HT-2 and T-2 are considered as two of the most potent trichothecene mycotoxins and a public health concern in Europe (Edwards *et al.* 2009). Legislation for HT-2+T-2 was timetabled for July 2009. Although currently no legislation has been set, there is a discussion limit of 500 $\mu g\ kg^{-1}$ in unprocessed oats, 200 $\mu g \times kg^{-1}$ for oat products and 50 $\mu g \times kg^{-1}$ for infant food (Edwards *et al.* 2009). There is limited data regarding *F. langsethiae's* pathogenicity and mycotoxin production, but it is evident that its behaviour deviates from traditional trichothecenes producing *Fusarium* species (Imathiu 2008, Orlando *et al.* 2010).

The aggressiveness of a plant pathogen is the extent to which it can attack a susceptible host and it is measured by the amount of pathogen developing on or in a given host species (Bos and Parlevliet 1995). Aggressiveness of different strains or isolates of a given plant pathogenic species may vary, with the more aggressive ones attacking plants faster and/or more intensively. *Fusarium langsethiae* has been isolated from wheat, barley and oats from different parts of the UK and Europe as a whole. There are very limited data on the differences in pathogenicity and aggressiveness of the different isolates. There is therefore the need to study these different isolates to determine if there is any variability within the isolates and what factors influences the variability. For example, do isolates show any specialisation towards specific hosts? In *vitro* detached leaf assays as described by Diamond and Cook 1999 and Imathiu *et al.* 2009 have been used to study Fusarium head blight resistance in wheat and *F. langsethiae* pathogenicity and aggressiveness in wheat and oats respectively. The method, which makes use of components of partial disease resistance which include

incubation period, latent period and lesion length, has been shown to be a useful tool in predicting reaction of matured plants to FHB pathogens (Diamond and Cooke 1999). This work was aimed at determining the aggressiveness of 20 *F. langsethiae* isolates towards wheat, barley and oats using an *in vitro* detached leaf assay.

MATERIALS AND METHODS

Fungal species and inoculum production

Twenty isolates of *F. langsethiae* from the Harper Adams Culture Collection were selected for this experiment. These were originally isolated from wheat or oats between the periods of 2001 and 2009 (Table I) and single spore isolates stored on potato dextrose agar (PDA, Merck, Germany) slopes at 4°C.

Table 1

***Fusarium langsethiae* isolates used in detached leaf assay**

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

Inoculum was produced by sub-culturing isolates from PDA slopes onto PDA plates (amended with streptomycin sulphate (130 µg ml^{-1})), incubated at room temperature (*ca*. 22°C) under natural light for 14 days. Spores were harvested by flooding individual cultures with SDW (*ca*. 5 ml) and gently agitating culture surfaces with a sterilised L-shaped glass rod. The spore

suspension was filtered through two layers of sterile muslin cloth to remove mycelia. The culture surface was rinsed with SDW (*ca*. 2.5 ml), filtered and added to the spore suspension. Spore concentration was then determined using a haemocytometer (Weber Scientific International, UK), aliquoted into 5 ml volumes and stored at -20°C. Viability of spores was assessed by culturing a 10-fold dilution series of spore suspension on Rose-Bengal Chloramphenicol Agar (Merck, Germany).

Production of leaf material

One variety each of oats (Gerald), wheat (Claire) and barley (Tipple) were used for the experiment. These varieties were selected for the experiment because they are currently among the most important winter varieties in the UK. Seeds were surface sterilized with sodium hypochlorite (1.2% available chlorine) amended with 0.05% Tween 20 for three minutes, rinsed three times with sterile distilled water (SDW) and allowed to dry in Petri dishes in a laminar air flow cabinet. Thirty seeds were sown in plastic trays (21.5 × 15 cm) containing sterilized John Innes Compost Number 2 at a depth of about 1 cm. Experimental trays were placed in a growth cabinet (Sanyo Versatile Environmental Test Chamber, Japan) at 20°C and a 12 h photoperiod. Leaves were harvested 14 days after sowing by cutting 4 cm length segments of the tip of the primary leaves.

Leaf inoculation and disease assessment

Harvested leaves were wounded at the middle of the upper surface with a sterile 10 µl micropipette tip. Wounded leaves were carefully placed in a Petri dish with 0.5% water agar amended with kinetin (10 mg × l^{-1}) with the adaxial surface facing up. Each Petri dish had a wounded leaf segment of wheat, barley and oat representing one replicate. A 5 µl conidial suspension was placed on each wound. The same volume of SDW was used as control inoculation. Each treatment was replicated thrice and arranged in a complete randomized design in a growth cabinet (Sanyo Versatile Environmental Test Chamber, Japan); 20°C, 10 h light 14 h darkness.

Seven days post inoculation, aggressiveness of the different F. *langsethiae* isolates were determined by measuring lesion lengths on a light box. Lesions were identified as water-soaked necrotic and/or chlorotic area.

Data were analyzed with GenStat (Twelfth Edition, VSN International Ltd, UK) using linear regression with groups and a split plot ANOVA and means separated by the Tukey's test at 5% significance level.

RESULTS

No lesions developed on control leaf segments (SDW inoculated). All *F. langsethiae* isolates used for the experiment caused visible lesions on wheat, barley and oats. Lesion characteristics were similar on all three cereals and identified as water-soaked and/ or chlorotic area (Fig. 1).

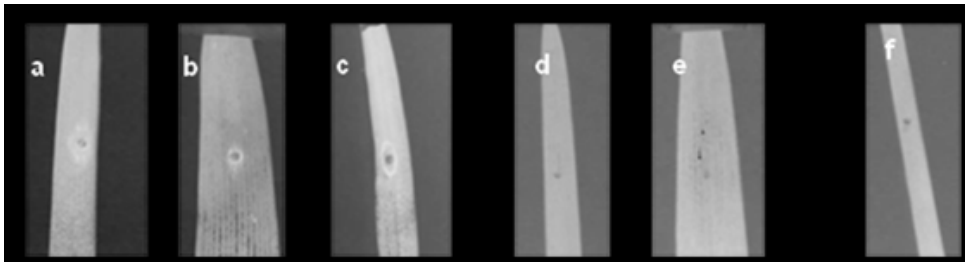

Fig. 1. Wounded detached leaves 7 days post-inoculation. a, b and c are oat, barley and wheat leaves inoculated with *F. langsethiae* and d, e and f are oat, barley and wheat leaves inoculated with SDW (control)

A regression analysis of isolate age (length of time that each isolate had been stored) against isolate lesion length grouped by isolate host (cereal from which the isolate was originally isolated) and cereal (cereal species leaf inoculated) showed that both the age of isolate and the isolate host did not have a significant effect on lesion length ($P=0.38$ and $P=0.07$ respectively). There was a highly significant effect ($P<0.001$) of the type of cereal (wheat, barley or oats) on the length of lesion developed. There was no significant interaction between any of these factors ($P>0.05$). Based on these results a split plot ANOVA (where each Petri dish was considered as a plot with each cereal leaf as a split within the plot) was carried out and this showed that cereal/isolate interaction was not significant ($P=0.37$). There was a highly significant ($P<0.001$) difference between lesion lengths formed by different F. *langsethiae* isolates used (Table 2). Isolate Fl/2004/17(a) caused the shortest lesion (3.8 mm) on all cereals and isolate Fl/0/08/009/1 caused the longest (9.4 mm) lesion on all cereals used. Results from individual isolates showed a continuum of lesion lengths with no obvious segregation of isolates into distinct groups.

A highly significant difference ($P<0.001$) was found between the length of lesions on the different cereals (wheat, barley and oats) (Table 2). Lesions on oats were the longest, followed by barley with wheat showing the shortest lesions.

Table 2

Lesion length (mm) caused by 20 *F. langsethiae* isolates on wounded detached leaf assay of wheat, barley and oats. Values are means of three replicates. Values with the same superscript letter are not statistically different based on Tukey's test (P = 0.05).

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

DISCUSSION

Artificial wounds were created on leaf surfaces for this experiment because in a previous work by Imathiu *et al.* (2009), F. *langsethiae* isolates were found to be pathogenic to wounded oat and wheat detached leaves but only on oat

leaves in an unwounded leaf assay. The leaf surface serves as a physical barrier to fungal infection and therefore tougher cuticles may be harder for pathogenic fungi to penetrate into the underlying tissues. Creating artificial wounds on the leaf surface therefore eliminates this barrier and creates a uniform lesion for the initiation of infection.

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

The ability of *F. langsethiae* to cause chlorosis and necrosis on wheat, barley and oat leaves is not surprising. This is because *F. langsethiae* is a prolific producer of T-2 and HT-2, two of the most potent trichothecenes known (Edwards *et al.* 2009). T-2 and HT-2 are phytotoxic and have been reported to cause chlorosis and necrosis in plant tissues (Zonno and Vurro 2002). They are also known to inhibit RNA and DNA synthesis as well as triggering apoptosis; a process of programmed cell death in multicellular organisms (Uno *et al.* 1995, Yang *et al.* 2000). Although programmed cell death is essential for plants, necrotrophic pathogens are able to trigger programmed cell death in healthy plant tissues to cause disease (Coffeen and Wolpert 2004).

Variability in the agressiveness of different *F. langsethiae* isolates has been reported by Imathiu *et al.* (2009). Five of the seven most aggressive isolates in the current experiment were isolated from oat grains out of which three were in 2009 from grains harvested in 2008. This suggests three possibilities; their aggressiveness may be related to cereal species from which they were isolated; all the isolates may be from one population or their high agressiveness was due to the fact that they were isolated recently and had been stored only for a short period. *Fusarium* species, if not stored properly for a long period of time, like other fungal species are susceptible to cultural degeneration which can lead to decrease in virulence (Leslie and Summerell 2006). Results from the regression analysis showed that the length of storage (age) or the host isolates were cultured from did not significantly affect the length of lesions formed on the leaves of the different cereals. This is in agreement with the work of Imathiu *et al.* (2009) who reported that, the aggressiveness of six (three isolates from wheat and three from oat) *F. langsethiae* isolates on detached leaf assay was not de-

pendent on the source of isolation. This suggests that there is no preferential host specialization for the different *F. langsethiae* isolates used indicating that the differences observed in the lesion lengths on wheat, barley and oats leaves were mainly due to the cereal effect. *Fusarium langsethiae* was more aggressive on oats than barley and wheat in this assay, suggesting that oats may be the most suscptible among the three cereals used for the experiment. There are very limited data on the susceptibility of different cereals towards *F. langsethiae*, but the relative ease of isolation of *F. langsethiae* from oats coupled with high levels of T-2 and HT-2 in oats in the UK and the rest of Europe (Edwards *et al.* 2009), support this assertion.

CONCLUSIONS

This study has indicated that oats are the most susceptible species to *F. langsethiae*. Variability in virulence exists within the different isolates of *F. langsethiae* used for the experiment but this was not correlated to the period that isolates had been in storage or the host that they were originally isolated.

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