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In vitro Growth Characteristics of *Fusarium langsethiae* Isolates Recovered from Oats and Wheat Grain in the UK

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Fusarium langsethiae is a fungus that has recently been implicated in the contamination of small-grain cereal crops such as oats, wheat and barley with high levels of HT-2 and T-2 toxins in many European countries. The epidemiology of this fungus is not well known and may therefore be a bigger problem than currently thought to be. A study was carried out investigating the *in vitro* growth characteristics of *F. langsethiae* isolates from contaminated oats and wheat at various temperatures; 15, 20, 25 and 30 °C. Results indicated similar growth trends of oats and wheat isolates of *F. langsethiae*. Wheat isolates grew significantly ($p<0.001$) faster than oat isolates although this difference may have been confounded by the age of cultures, with oat isolates collected one year earlier. The estimated optimum growth temperature for all isolates was 24 °C. Isolates were macro-morphologically categorized as having lobed or entire colony margins, and either possessing one of the following colony colours; white, orange or purple. Since the estimated optimum growth temperature of *F. langsethiae* is typical in temperate

summers when small-grain cereals are flowering, it is possible that this species can infect, colonise and possibly contaminate the developing grains with HT-2 and T-2 toxins which are of food safety concern.

Keywords: *Fusarium langsethiae*, HT-2 and T-2 toxins, mycotoxins, oats, wheat.

Fusarium langsethiae, whose status as a small-grain cereal crop pathogen has not yet been agreed upon by researchers, is a confirmed toxigenic fungal species known to contaminate oats, wheat and barley with high levels of HT-2 and T-2 trichothecene mycotoxins in Central and Northern Europe (Yli-Mattila 2010; Edwards et al., 2013; Imathiu et al., 2013). This fungus, which was formerly referred to as 'powdery *F. poae*' was first detailed in the 1990s as one whose spore morphology resembled that of *F. poae* (Torp and Langseth, 1999) and with a mycotoxin profile similar to that of *F. sporotrichioides* (Thrane et al., 2004). In this regard, it is possible that all T-2 toxin producing strains of *F. poae* reported earlier were actually *F. langsethiae* (Torp and Langseth 1999). According to Knutsen et al. (2004), due to the similarity of *F. langsethiae* and *F. poae* microscopically, the former may often have been identified as the latter. This fungus has been reported to differ from *F. poae* by its slower growth, production of fewer aerial mycelia and presence of powdery appearance on the surface of the colony, and lack of peach-like odour on synthetic media such as potato dextrose agar (Torp and Nirenberg 2004). On the other hand, *F. langsethiae* has been shown to differ from *F. sporotrichioides* in all aspects except the mycotoxin profile and conidial morphology, with the main macroscopic differences between the two species being the rate of colony growth and abundance of aerial mycelia, with *F. langsethiae* growing slower and producing less aerial mycelia in comparison to *F. sporotrichioides* (Thrane et al., 2004).

Due to its ability to colonize and contaminate small-grain cereals with high levels of HT-2 and T-2 toxins, *F. langsethiae* is considered of great public health concern as mycotoxin contamination of both foodstuff and feedstuff is an unwanted hazard in food and feed chain. These natural microbial toxins have emerged as a significant factor affecting the safety image of cereal grains as a raw material for the food and feed industry (Hietaniemi et al., 2004). T-2 and HT-2 (a deacetylated form of T-2) are type A trichothecenes which are usually found together in

contaminated cereal grains and their products. High levels of these mycotoxins have been reported in warm and dry summers in Europe (Langseth and Rundberget, 1999), conditions thought to be conducive for cereal infection and colonization by *F. langsethiae*. HT-2 and T-2 toxins are two of the most potent trichothecenes capable of inhibiting protein synthesis in eukaryotes (Ji et al., 1994; Smith et al., 1994).

As a result of the association of *F. langsethiae* with HT-2 and T-2 mycotoxin production, this fungal species has recently received great research attention. The natural occurrence of T-2 toxin has been of particular interest in Europe because of the association of T-2 toxin-producing *Fusarium* species, which caused an outbreak of alimentary toxic aleukia (ATA) that was responsible for the death of thousands of people in Russia during World War II in the 1940s (Desjardins, 2006). However, there is not sufficient information available with regard to the conditions under which *F. langsethiae* grows both *in vitro* and *in vivo* while producing these toxins in cereal grains during crop growth and/or storage. Although the European Commission (EC) has established a group tolerable daily intake of 100 ng kg^{-1} body weight for the sum of HT-2 and T-2 toxins, the Commission has recommended collection of more data on these toxins on cereals and cereal products including information about conditions contributing to their presence in these commodities, which is currently limited (EC, 2013).

Fusarium species are normally field fungi whose extent of infection, colonization and mycotoxin production is heavily influenced by environmental factors such as temperature and humidity that cannot be controlled under natural conditions (Barbara et al., 2004). For this reason, complete elimination from agricultural commodities is unachievable. A key goal in the production of safe food and feed is, therefore, to develop and/or adopt practices that reduce the extent of contamination by these toxins to levels that are considered to have no significant impact on health (Desjardins, 2006). This cannot be achieved without an understanding of specific conditions that directly influence the growth of a particular fungus. It is important to understand the growth characteristics of an organism in order to devise ways of reducing mycotoxin contamination.

It is not known whether *F. langsethiae* is part of the fusarium head blight (FHB) complex of organisms whose growth characteristics and conditions in which they thrive optimally are well known and documented. Temperature is the single most important abiotic factor influencing all

aspects of behaviour of every living organism, small changes of which may greatly influence the incidence and severity of infection in small-grain cereals (Brennan et al., 2005). The aims of this study were; to isolate and characterize *F. langsethiae* isolates from commercial UK oats and wheat grains, and to investigate the effect of temperature on the *in vitro* growth rate of oats and wheat isolates of *F. langsethiae*.

Materials and Methods

Isolation of F. langsethiae *from oat and wheat grains*

Oats and wheat grain samples from years 2001, 2002 and 2004 UK harvests with known low and high levels of HT-2 and T-2 mycotoxins were identified from previous project material (Edwards 2007a; 2007b). *Fusarium langsethiae* was isolated from two oat samples with >500 µg kg^{-1}, four oat samples with <10 µg kg^{-1} and seven wheat samples with >50 µg kg^{-1} HT-2 and T-2 toxins. Grain samples were thoroughly mixed before representative subsamples were taken. Grains (approximately 20 g) were scooped into 50 ml sterile universal tubes. They were soaked in sodium hypochlorite (1.2% available chlorine) with added Tween 20 (0.05%) for 3 min to surface-sterilize. They were then rinsed three times with sterile distilled water before being allowed to dry in Petri dishes in a laminar air flow cabinet. Grains were plated (5 grains per plate) on potato dextrose agar (PDA, Merck, Germany) amended with streptomycin sulphate (130 µg ml^{-1}) to inhibit bacterial growth and incubated at room temperature (*ca.* 22 ºC) for 7 days. *Fusarium langsethiae* was identified by micro- and macro-morphology (Torp and Langseth 1999; Torp and Nirenberg 2004). The identity of the fungal isolates was confirmed by real-time PCR as described in Edwards et al. (2013) using the fungal isolates DNA extracted using a rapid DNA extraction method modified from Wash et al. (1991). Mycelia of the fungal isolates were removed from the surface of PDA plate cultures using a sterilized scalpel and placed in 2.0-ml Eppendorf tubes into which 250 µl chelex carbon buffer (1 g chelex 100 and 0.25 g charcoal (granular 20–60 mesh activated) made up to 20 ml using distilled water) was added. Mycelia

were crushed with a sterile micro-pestle and incubated at 56 °C for 20 min, vortexed and left to cool at room temperature for 30 min. The tube content was vortexed and centrifuged at 12,000×g for 15 min after which 50 µl supernatant was removed and added to 50 µl TE buffer (10 mM Tris–HCl, 1 Mm EDTA, pH 8.0) and vortexed. Extracted DNA was stored at 4 °C and 5 µl used directly for PCR reactions.

To confirm the identity of the isolates previously identified by micro- and macro-morphology, DNA from the fungal isolate was subjected to *F. langsethiae* real-time PCR. An iCycler PCR machine (Bio-Rad, UK) was used for the amplification of fungal DNA extractions. *F. langsethiae* DNA standards (100–10−4 ng/µl) were included in duplicate, as well as negative and positive controls. The amplification mix consisted of 1 µM *F. langsethiae* primer pairs (FlangF3 5′-CAAAGTTCAGGGCGAAAACT and LanspoR1 5′-TACAAGAAGACGTGGCGATAT) (Wilson et al. (2004)) and SYBR Green Jumpstart Taq ReadyMix (Sigma, USA) reagent which was used according to manufacturer's instructions. The volume of DNA sample in the reactions was 5 µl in a total volume of 25 µl. In the negative control, 5 µl of PCR-grade water was used instead of DNA sample. The PCR programme had an initial denaturation for 2 min at 95 °C followed by 40 cycles with 15 s at 95 °C (denaturation), 10 s at 62 °C (annealing), 30 s at 72 °C (extension) and 10 s at 82 °C (fluorescence measurement).

In vitro *growth study*

A single isolate was maintained from each grain sample unless multiple isolates with distinct morphology colour were present. This reduced the chance of having multiples of the same isolate within the study. Ten *F. langsethiae* isolates (Table I) each from oats and wheat grains were randomly selected for the study. To obtain single spore colonies, each isolate was cultured on PDA as described above until profuse sporulation (powdery appearance) had occurred. Spores were carefully scrapped with a sterile wire loop and streaked on water agar (20 g of agar in 1 l of tap water) in Petri dishes and incubated at room temperature (*ca.* 22 ºC) for 3 days. A marker pen was used to highlight a single distinct colony from the reverse side of the plate which was then, by means of a sterile scalpel transferred onto a fresh PDA plate.

Five millimetre discs of single spore colonies from 12-day-old cultures were cut with a sterile cork borer from an advancing margin of colonies and plated at the centre of four fresh

PDA plates. The plates were incubated at 15, 20, 25 or 30 ºC in an incubator (Labheat Incubator, UK) in complete darkness for 12 days. Growth was measured by taking two diametric measurements of the colonies perpendicular to each other. The growth of cultures was measured after 3, 6 and 9 days of incubation and growth rate calculated as mm day^{-1}. Other secondary characteristics monitored were colony colour on day 6 and 12 after incubation, colony margin shape on day 6 and 9 and sporulation on day 3, 6, 9 and 12. Experiments were set up in randomized block designs with four replicates. The growth rate data was subjected to analysis of variance (ANOVA) using GenStat (Release 8.1, Rothamsted Experimental Station, UK).

Results

Isolation of F. langsethiae *from the grain*

From oat and wheat samples containing >500 and 50 µg kg^{-1} HT-2 and T-2 toxins respectively, *F. langsethiae* was isolated from 5-10% of the grain. No *F. langsethiae* was isolated from oat samples containing <10 µg kg^{-1} HT-2 and T-2 toxins. *Fusarium langsethiae* was identified micro-morphologically (globose to napiform conidia) and macro-morphologically (profuse 'powdery' appearance on short aerial mycelia on agar surface (Fig. 2)). The identity of the isolates was confirmed by real-time PCR methodology detailed by Edwards et al. (2013). The greatest challenge encountered in the isolation process was the fact that *F. langsethiae* was readily overgrown and outcompeted by faster growing fungi on the agar making it difficult to clearly see and effectively and efficiently isolate the fungus.

Effect of temperature on the in vitro *growth rate of oats and wheat isolates of* F. langsethiae

The growth trends of isolates of *F. langsethiae* recovered from oats and wheat were found to be similar at the four temperatures of study; 15, 20, 25 and 30 ºC (Fig. 1). All isolates grew least at the most extreme temperatures of 15 and 30 ºC with higher rates of growth

observed between 20 and 28 ºC. The optimum growth temperature for all isolates was estimated to be about 24 ºC. Although the wheat and oats isolates had similar growth trends at all temperatures studied, on average wheat isolates grew faster than the oat isolates at each temperature. Wheat isolates grew significantly faster ($p<0.05$) than oat isolates at 20, 25 and 30 ºC on day 3. On days 6 and 9, wheat isolates grew significantly faster ($p<0.05$) than oat isolates at 20 and 25 ºC with no significant growth differences at 15 and 30 ºC ($p>0.05$). The mean growth rates of oat and wheat isolates at 25 °C, a temperature which is close to the estimated optimum temperature for all isolates (24 °C) were as follows; 7.6, 7.7 and 7.7 mm day^{-1} for oat isolates on days 3, 6 and 9 respectively; 9.2, 9.3 and 9.1 mm day^{-1} for wheat isolates on days 3, 6 and 9 respectively. On day 6 which was considered the optimal day post-plating out for taking growth rate readings, oat isolate Fl 2004/59 had the slowest growth rate while wheat isolate W2001/69(b) had the fastest growth rate recorded over the four incubation temperatures.

Fusarium langsethiae *isolates colony morphological characteristics*

In all isolates, colony margins were observed to be either entire or lobed (Fig. 2). The entire colony margin was characterised by smooth advancing colony edges while a lobed margin was characterised by uneven advancing colony edges. Colony mycelial colour of isolates was either white, orange or purple, which also applied to the reverse side of the plates. Only one isolate was purple in colour (isolate Fl 2004/59) (Fig. 2) which was isolated from oats and which had the slowest growth rate over the four incubation temperatures. Pigmentation tended to be more intense with increasing incubation temperature and was more pronounced at 25 ºC than at any other temperature. There was no relationship between colony colour and margin shape observed.

All isolate colonies exhibited a ‘powdery’ appearance with short sparse mycelia on the agar surface (Fig. 2). Onset and degree of sporulation (profuse ‘powdery’ appearance on agar surface) depended mainly on incubation temperature. It was observed that the higher the incubation temperature, the earlier was the onset of sporulation. Although very little growth occurred at 30 °C for all isolates, abundant sporulation was observed on the small colonies. The degree of sporulation was also found to increase with the increase in incubation period, with

greatest sporulation observed at 25 ºC on day 9. Of all the days that the readings were taken, it was evident that macromorphological characteristics for all the isolates were well defined on day 6.

Discussion

The finding that *F. Langsethiae* isolation was problematic as it tended to be overgrown by other types of fungi was not an unique observation. This finding agrees with that reported earlier by Torp and Nirenberg (2004) who reported that, due to its slow growth rate and sparse aerial mycelia, *F. langsethiae* is readily overtaken by other quick growing fungi such as *F. poae* and *F. culmorum*. This may also explain why low numbers (5-10%) of the fungus was recovered even from grain samples that were highly contaminated by HT-2 and T-2 toxins and assumed to be heavily infected.

Generally, most fungi particularly phytopathogenic ones are known to be mesophilic, growing well between 20-30 ºC. Most *Fusarium* species have also been found to grow well between these two temperatures (Smith et al., 1984). *Fusarium langsethiae* is therefore a mesophile and shares the same optimal growth temperature range with most *Fusarium* species. Most *Fusarium* species pathogenic to oats and wheat such as *F. poae*, *F. culmorum* and *F. graminearum* are known to have an optimum growth temperature of 25 ºC and an optimal growth rate in the range of 20-30 ºC (Brennan et al., 2003; Doohan et al., 2003; Hope et al., 2005). For instance, *in vitro* growth rates of *F. culmorum* and *F. graminearum* were found to increase between 10 and 25 °C and decrease between 25 and 30 °C; optimal growth occurred at 25 °C for *F. graminearum* and 20-25 °C for *F. culmorum*. However, a study carried out by Torp and Nirenberg (2004) indicated that the Norwegian isolates of *F. langsethiae* had an optimum growth rate at 27.5 ºC. Possible explanations for this difference may be attributed to regional differences including the environment from which the authors' isolates were recovered and the media conditions under which the isolates were grown. Brennan et al. (2003), in a study on *in*

vitro growth and pathogenicity of European *Fusarium* fungi showed different growth rates for different *Fusarium* species from different countries.

Although the oat and wheat isolates of *F. langsethiae* had similar growth trends at all temperatures studied, on average wheat isolates grew faster than the oat isolates at each temperature. It is possible that the significant differences in growth rate observed between oat and wheat isolates may be confounded by the original age of cultures used; oat isolate cultures were older than wheat isolates by one year, while wheat isolates were freshly used in the study after isolation. Hallsworth and Magan (1996) reported that increased age of the culture can contribute to the decline of fungal spore viability. The authors were investigating the effects of culture age, temperature and pH on the polyol and tetrahalose contents of fungal propagules. Although they did not comment on the growth rates of fungal colonies, age may also influence the rate of growth as observed in the study.

Both colony micro- and macromorphological characteristics which include colony diameter, colony colour and colony margin characteristics have been used in *Fusarium* taxonomy for a long time and have been known to provide useful secondary criteria for distinguishing isolates (Burgess et al., 1988; Summerell et al., 2003). For many *Fusarium* species, these characteristics are the only ones that are well described and widely available, and therefore they are very important in distinguishing isolates compared with biological species information and DNA sequence information which are recent advances and not readily available (Summerell et al., 2003.

A number of colony macroscopic attributes; mycelial colour, colony margin shape and sporulation were assessed in order to characterise the isolates of *F. langsethiae*. Colony pigmentation is known to be uniform within some *Fusarium* species while in others, it is a variable character (Burgess et al., 1988). In this study, *F. langsethiae* appeared to be one of those species that may naturally produce different colour shades irrespective of whether isolates are recovered from oats or wheat grains. The different shades of colour in *F. langsethiae* cultures can be explained by the fact that some of the strains of this fungus have been reported to produce a pigment called aurofusarin (Thrane et al., 2004), which is produced by nearly all strains of *F. poae* and *F. sporotrichioides* and influences colony colour development. *Fusarium poae* and *F. sporotrichioides* have been reported to be closely related to *F. langsethiae* micro-

morphologically and this might be another extra character they have in common. Similar results on *F. langsethiae* pigmentation were observed by Torp and Nirenberg (2004) who used words like white to pinkish white, pale red or pastel red and orange tint to describe colony pigmentation of this fungus. There is also a possibility that different shades of colour may result because the isolates in the three groups (white, orange and purple) are different subgroups within the same species, which warrants further studies. Colony margins for all the isolates were observed to be either entire or lobed. This was an inconsistent attribute as it could differ in the four replicates of the same isolate at the same incubation temperature indicating that although this secondary attribute can be used to aid isolate identity in some fungi, it is of no use in the characterisation of *F. langsethiae* isolates.

'Powdery' appearance on the agar surface which is as a result of profuse production of spores is an attribute which is obvious with the unaided eye and taxonomically one of the characters used to differentiate *F. langsethiae* from all other *Fusarium* species (Torp and Langseth 1999; Torp and Nirenberg 2004). UK *Fusarium langsethiae* isolates used in this study seem to share all of the growth characteristics reported for European isolates used in a study by Torp and Nirenberg (2004).

Day 6 post-plating out would be considered the most appropriate time for taking all readings on growth rate, pigmentation and other macroscopic characters as at this day, all isolates had grown substantially with all attributes well developed. Day 3 would not be appropriate because all the characters were not well defined. By day 9, a few isolate colonies had grown to reach the edge of the agar plates (20 and 25 °C only), which would make assessment of colony margins difficult. The fact that most readings on *Fusarium* species are taken on day 6 and 7 when cultured on synthetic media (Smith et al., 1984) supports the above statement.

In conclusion, this study has found out that oats and wheat isolates of *F. langsethiae* can grow and sporulate at a wide range of temperatures from 15-30 ºC (and probably lower or higher temperatures at a slower rate). With the optimum growth temperature of *F. langsethiae* being about 24 ºC, it is possible therefore that this species of fungus can infect and colonise small-grain cereals at these temperatures that are common in temperate summers when crops are flowering. The differences in colony colour observed in *F. langsethiae* isolates may indicate underlying differences in genetic makeup. It would, therefore, be useful to carry out DNA profiling to

establish whether isolates differing in colony colour, as well as oat and wheat isolates are distinct subgroups of *F. langsethiae*.

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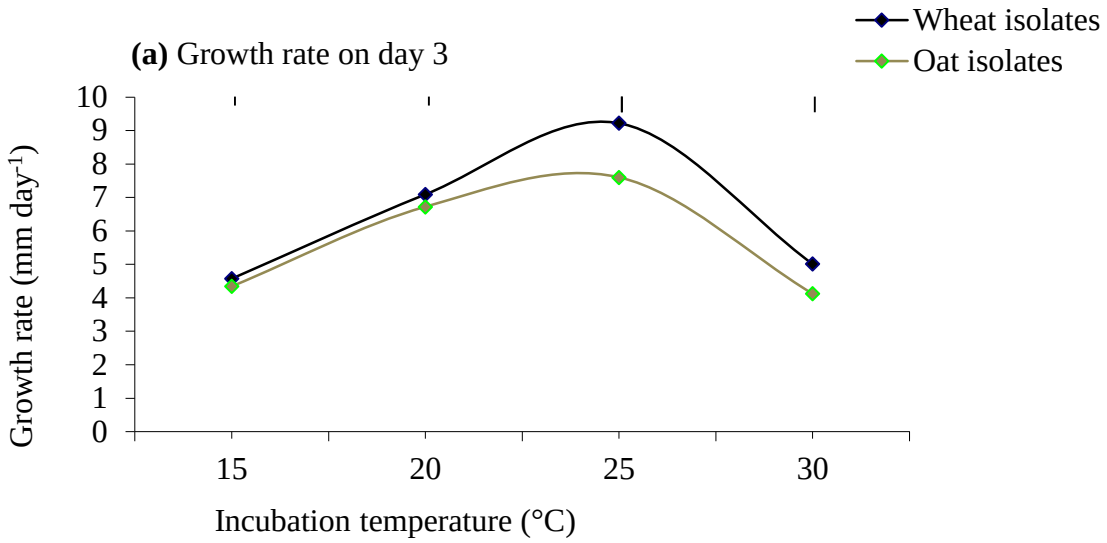
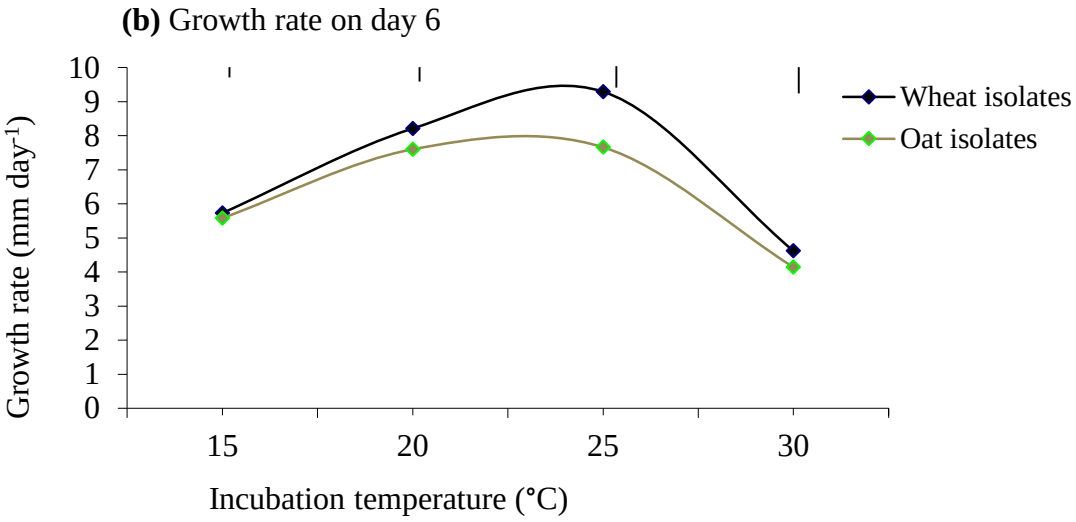
Table 1

Oat and wheat *Fusarium langsethiae* isolates used in the *in vitro* growth rate study

Isolate	**Host cereal**
Fl 2004/59	Oat
Fl 026/1	Oat
Fl 04/O2	Oat
Fl 04/O3	Oat
Fl 2004/37/1	Oat
Fl 2004/37/2	Oat
Fl 077/3	Oat
Fl 2004/31	Oat
Fl 062/1	Oat
Fl 062/2	Oat
W2004/171a	Wheat
W2004/140a	Wheat
W2001/69a	Wheat
W2004/171b	Wheat
W2001/69b	Wheat
W2001/17	Wheat
W2004/140	Wheat
W2001/1	Wheat
W2004/170	Wheat
W2004/171c	Wheat


(a) Growth rate on day 3
Wheat isolates
Oat isolates
Growth rate (mm day-1)
10
9
8
7
6
5
4
3
2
1
0
15
20
25
30
Incubation temperature (°C)


(b) Growth rate on day 6
Wheat isolates
Oat isolates
Growth rate (mm day-1)
10
9
8
7
6
5
4
3
2
1
0
15
20
25
30
Incubation temperature (°C)

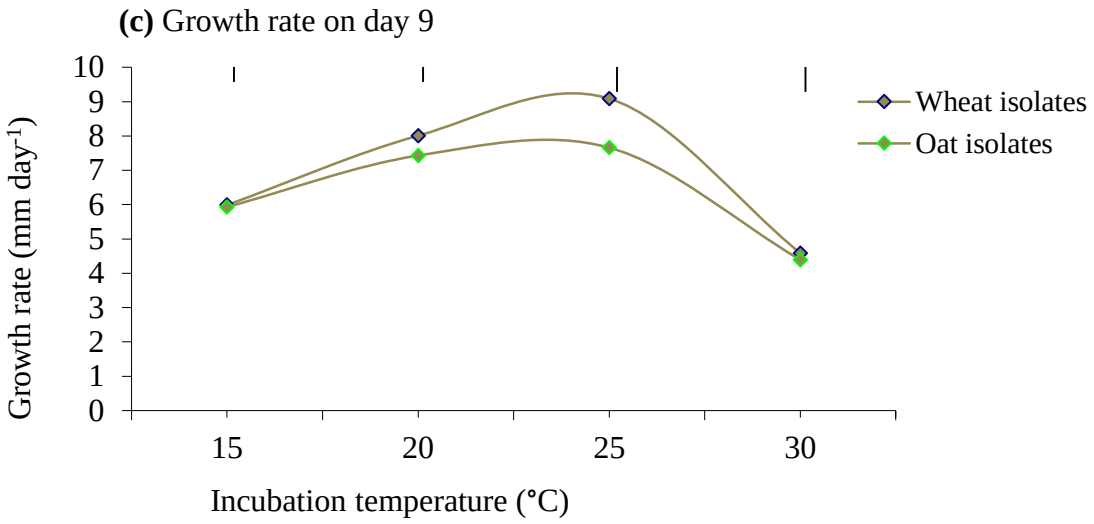

(c) Growth rate on day 9
Growth rate (mm day-1)
10
9
8
7
6
5
4
3
2
1
0
15
20
25
30
Incubation temperature (°C)
Wheat isolates
Oat isolates

Fig. 1.

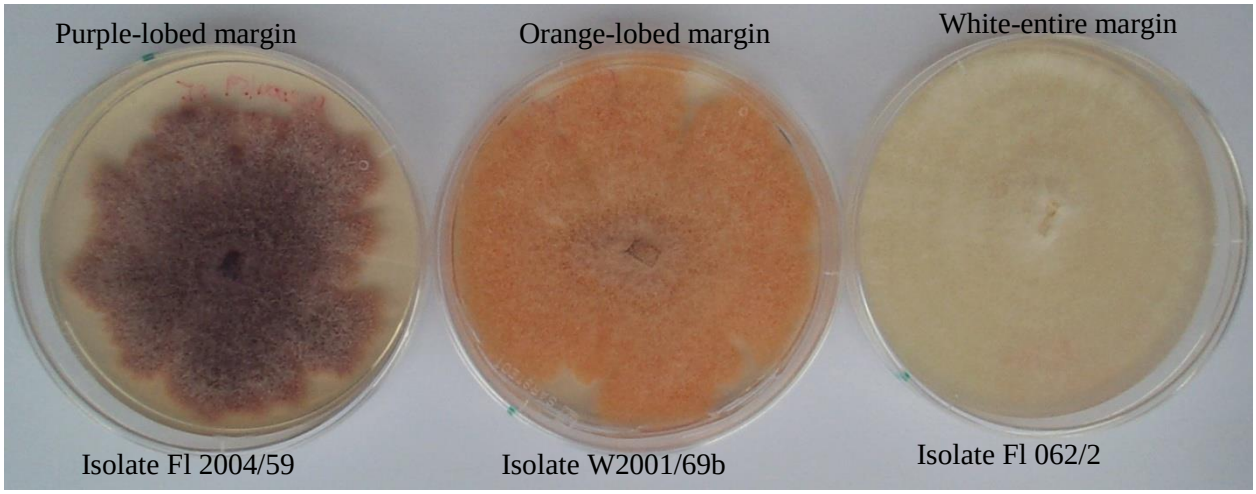

Purple-lobed margin
Orange-lobed margin
White-entire margin
Isolate Fl 2004/59
Isolate W2001/69b
Isolate Fl 062/2

Fig. 2.

Legend of Figures

Fig. 1. Effect of temperature on the growth rate of *F. langsethiae* isolates obtained from oat and wheat grains contaminated with HT-2 and T-2 toxins. Values are the means of four replicates. Bars represent least significant difference (LSD (5%))

Fig. 2. Three categories of *F. langsethiae* isolates based on colony colour