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Fusarium langsethiae (Torp and Nirenberg), investigation of alternative infection routes in oats

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Abstract *Fusarium langsethiae* is a recently characterized fungus within the genus *Fusarium*. It is found as a grain contaminant of small grain cereals such as oats and barley, and to a lesser extent wheat. *Fusarium langsethiae* is particularly widespread in the Nordic countries and the UK where it poses a serious problem as the main producer of T-2 and HT-2 mycotoxins. The biology of *F. langsethiae* and its interaction with the plant remains poorly understood, partly hampered by difficulties reproducing a natural level of infection under controlled conditions. The reported study was designed as a series of glasshouse experiments to advance our understanding of *F. langsethiae* biology by investigating alternative infection routes and its proliferation in oats, *Avena sativa*. Various methods of seed, soil, and seedling inoculation, boot injection and spray inoculation, were tested. The results clearly show a strong preference of *F. langsethiae* for the panicle, ruling out alternative infection routes. At relatively low temperatures spray infection, accompanied by prolonged humidity, ensured a thorough establishment of the fungus both at flowering and at early dough stage. Boot injection proved to be a reliable working tool for production of an even and predictable grain infection. Apart from in the panicle, considerable fungal proliferation was only detected in flag leaf nodes, and was a direct consequence of the boot injection method. Fungal presence in the node tissue also correlated with significant stunting of infected shoots. In light of the results the pathogenic and endophytic abilities of *F. langsethiae* are discussed.

Keywords Boot inoculation · Fusarium head blight · Real-time qPCR · Spray inoculation · T-2 toxin

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Introduction

Fusarium head blight (FHB) is a devastating disease of small grain cereal crops world-wide. It is caused by a large group of fungal species, the most important being *F. graminearum* Schwabe, *F. culmorum* (W. G. Sm.) Sacc., *F. avenaceum* (Fr.) Sacc., *F. poae* (Peck) Wollenw., and *Microdochium nivale* (Fr.) Samuels and Hallett (Parry et al. 1995). FHB caused by these pathogens is in general associated with a reduction in grain yield, however many of these fungi also produce mycotoxins, posing a serious health threat if contaminated grain is used for animal feed or human consumption.

Fusarium langsethiae is a relatively new species described within the *Fusarium* complex and is known as one of the main producers of the very potent T-2 and HT-2 mycotoxins, belonging to the type A group

of trichothecenes (Torp and Langseth 1999; Torp and Nirenberg 2004). Over the last decade it represents an increasing problem in the Nordic countries and the UK (Langseth and Rundberget 1999; Torp and Langseth 1999; Edwards 2007). Minor levels have also been found throughout Northern and Central Europe (Torp and Nirenberg 2004; Torp and Adler 2004). Phylogenetically *F. langsethiae* is most closely related to *F. sporotrichioides* Sherb. and *F. poae* (Schmidt et al. 2004), morphologically resembling *F. poae*, but exhibiting a mycotoxin profile related to *F. sporotrichioides*.

In contrast to most of its relatives, colonization by *F. langsethiae* is rarely associated with visual symptoms, and does not result in reduction of grain yield (Torp and Adler 2004). Hence, seemingly healthy grain may contain large amounts of T-2/HT-2 mycotoxins. T-2 toxin is considered the most toxic of the trichothecenes and is particularly known for its immunosuppressive effect (Rocha et al. 2005). Evidence for the role of T-2/HT-2 toxins as fungal virulence factors in cereals is lacking, however, studies from *Arabidopsis* Heynh. suggest a phytotoxic effect of these metabolites, distinct from that of deoxynivalenol (DON) (Nishiuchi et al. 2006; Masuda et al. 2007). In Europe no regulations are currently present for T-2/HT-2 toxin in commodities or any other products. In Norway, a maximum limit of 200 μg/kg has been defined for pig and horse feed.

Although the characteristics of the FHB disease are generally similar in small grain cereals, the majority of reports are on wheat, and symptoms regarding *F. poae* and *M. nivale* are somewhat disputed (Parry et al. 1995). General characteristics include initial infections appearing as small, water-soaked brownish spots at the point of infection, and water soaking and discolouration spreading in all directions. Infected grains may have a pink colour from fungal growth or appear shrivelled with a pale grey/brown color. Premature death or bleaching of cereal spikelets is also a common symptom. Compared to wheat and barley FHB in oats is associated with few and subtle symptoms (Tekauz et al. 2004). One reason for this might be the spacing of the spikelets on the panicle, making secondary infections rare. Despite the lack of severe symptoms high levels of DON and HT-2/T-2 have been found in oat grain (Torp and Langseth 1999; Tekauz et al. 2004; Edwards 2007; Hofgaard et al. 2009; Yan et al. 2010). Oats is one of the preferred crops for *F. langsethiae* (Torp and Langseth 1999; Edwards 2007).

The disease cycle of *Fusarium* spp. on small grain cereals has been extensively studied and is reviewed by Parry et al. (1995). Central to the infection process is the initial source of inoculum from *Fusarium* spp. surviving in the soil, either as saprophytic mycelium on crop debris or as thick-walled resting spores (chlamydospores), depending on the *Fusarium* species. Fusarium seedling blight (FSB) and foot rot may arise from contaminated debris and seed lots, and provide an air-borne inoculum in the form of conidia which may infect plant heads, resulting in FHB. Warm, relatively moist conditions are generally required for the production of conidia. In addition to dispersal either by rain-splashed conidia or air-borne ascospores, contaminated arthropod vectors and systemic fungal growth through plants have been suggested as ways of conidial dispersal (Parry et al. 1995). Little is known about the source of inoculum, and mode of dispersal by *F. langsethiae*. Recently, Imathiu et al. (2010) showed that *F. langsethiae* is unable to cause FSB in oats and wheat. Artificial panicle infection in glasshouse or field trials has not succeeded in producing levels of infection similar to what is found in natural fields (Imathiu 2008). The symptomless and as yet unknown colonization process of *F. langsethiae* has spurred a discussion of whether it should be considered a pathogen or whether it possesses endophytic abilities.

The aim of this study was to investigate the ability of *F. langsethiae* to use alternative infection routes for plant ingress and panicle colonization. Through this effort, we would also be able to address the ability of *F. langsethiae* to grow systemically and asymptomatically within vegetative tissues. Finally, our study aimed to develop a reliable and adequate method of artificial inoculation for *F. langsethiae*.

Materials and methods

Fungal strains and culture conditions

All plant inoculations conducted in this study were done using an equal mixture of three Norwegian isolates of *F. langsethiae*. Strain no. 9821-16-1 (IBT9951) and strain no. 9822-216-1F (IBT9956) originally isolated from oat and barley kernels,

respectively, have both been thoroughly characterized genetically and metabolically, and provide good representatives for the species *F. langsethiae* (Thrane et al. 2004; Torp and Adler 2004; Schmidt et al. 2004). One additional isolate from oats was included, 2010–58. Single conidial isolates were stored on Synthetischer Nährstoffarmer Agar (SNA) at 4°C, and were cultured on 9 cm petri dishes containing SNA for 1 week at room temperature (20–22°C) in transparent plastic bags on the lab bench. Microconidia were produced in 500 ml SNB shake cultures (SNA medium without agar) at 24°C, 150 rpm in the dark for 3 days, using one seven days-old SNA 9 cm petri dish with *F. langsethiae* as inoculum per 500 ml SNB culture. Microconidia were collected by centrifugation at 3,000 rpm for 20 min and washed once with sterile distilled water (SDW). Concentrations were determined using a Bürker haemacytometer and adjusted to working concentrations using SDW and 0.1% Tween20.

Seed material and propagation of plants

Two oat cultivars, cv. Gere and Belinda, were used. Gere was the only cultivar found to provide seed batches of both non-infected (batch Gere515) and naturally *F. langsethiae* infected grain (batch Gere2299), while no other *Fusarium* species were detected (data not shown). This was determined by screening several seed batches of various cultivars using real-time quantitative PCR (qPCR) species-specific assays for *F. langsethiae*, *F. culmorum*, *F. graminearum*, *F. poae*, and *F. avenaceum* (Table 1). Gere was used in treatments 1–6 and B1 (batch Gere515; all treatments except 2 and 3 which used batch Gere2299). For the remaining treatments cultivar Belinda was used. Belinda was found to accumulate large amounts of T-2/HT-2 mycotoxins in Norway in the years 2005–2007 (Hofgaard et al. 2009).

Plants were grown in a glasshouse using standard 1.5 l plant pots (LOG A/S, Norway) with perlite-added "P-jord", a mix of peat with 10% soil (LOG A/S, Norway). Five seeds were sown per pot, and plants were grown in white light (HPI) with 16 h photoperiod and relative humidity (RH) 75%. Plants were watered upon demand. Fertilized water was used after approximately 1 month of growth (ionic strength 1.1 mMho mSiemens^{-1}). Temperature during soil, seed, and seedling inoculation for repeat 1 was 14°C day, and 10°C night, but was otherwise kept at 18°C day and 14°C night.

Inoculation and sampling

All treatments and sampling stages are indicated according to plant developmental stage in Fig. 1. *F. langsethiae* microconidia were suspended in SDW containing 0.1% Tween20. The following treatments were included:

1) Non-inoculated control (C; batch Gere515 for boot inoculation control; Belinda for spray inoculation control).
2) Naturally *F. langsethiae* contaminated seeds.
3) Artificially vacuum-inoculated seeds with *F. langsethiae* conidial suspension. Seeds were submerged in microconidial suspension (10^6 conidia ml^{-1}), vacuum was applied twice for 30 s, upon which seeds were incubated in a sterile petri dish for 4 days under high humidity at room temperature in the dark. Seeds were surface sterilized prior to inoculation in order to prevent growth of saprophytes during the incubation period prior to sowing. Control seeds were vacuum treated with SDW in order to assess the effect of the vacuum on seed germination. Vacuum infiltration in itself did not greatly reduce germination as 25 out of 30 seeds germinated from vacuum control. Vacuum infiltration with *F. langsethiae* microconidia however, resulted in germination of only 16 out of 30 seeds.
4) Soil inoculation. Soil was watered once with 50 ml *F. langsethiae* microconidial suspension (0.7×10^6 conidia ml^{-1}) approximately 4 h after sowing.
5) Seedling stem base inoculation by application on the stem surface. Fourteen days old seedlings at growth stage (GS) 12 according to Zadoks scale (Z; Zadoks et al. 1974), were inoculated with 200–500 µl microconidial suspension (10^6 conidia ml^{-1}) by application into a cotton-wrap covering 2 cm of the surface at the base of the stem as described by (Mudge et al. 2006). The cotton, held in place by a sleeve of plastic wrap, was left on the plants throughout the growth period. In order to ensure sufficient humidity, RH was brought to 90% for the first week after inoculation.
6) Seedling inoculation by injection. Fourteen days old seedlings (GS Z12) were injected with micro-

Table 1 Overview of target genes, primers, and probes used for qPCR

Target organism	Target DNA	Primers/probe	Sequence (5′-3′)
F. avenaceum/F. arthrosporioides Sherb[a]	RAPD fragment	TMAV-f	AGATCGGACAATGGTGCATTATAA
		TMAV-r	GGCCCTACTATTTACTCTTGCTTTTG
		TMAV-p	TET-CTCCTGAGAGGTCCCAGAG ATGAACATAACTTC-TAMRA
F. culmorum[b]	RAPD fragment	culmMGB-f	TCACCCAAGACGGGAATGA
		culmMGB-r	GAACGCTGCCCTCAAGCTT
		culmMGB-p	FAM-CACTTGGATATATTTCC-MGB
F. graminearum[c]	β-tubulin gene	FGtub-f	GGTCTCGACAGCAATGGTGTT
		FGtub-r	GCTTGTGTTTTTCGTGGCAGT
		FGtub-p	TET-ACAACGGCACCTCTGAGCT CCAGC-TAMRA
F. langsethiae/F. sporotrichioides[a]	rDNA	TMLAN-f	GAGCGTCATTTCAACCCTCAA
		TMLAN -r	GACCGCCAAATTTGGG
		TMLAN -p	FAM-AGCTTGGTGTTGGGATCT GTCCTTACCG-TAMRA
F. poae[c]	RAPD fragment	poae-f	AAATCGGCGTATAGGGTTGAGATA
		poae-r	GCTCACACAGAGTAACCGAAACCT
		poae-p	FAM-CAAAATCACCCAACCGACC CTTTC-TAMRA
A. sativa Cherepanov	Cytochrome c oxidase gene	COX554-f	GGTTGTTGCCACCAAGTCTCTT
		COX554-r	TGCCGCTGCCAACTTC
		COX554-p	FAM-CTCCTATTAAGCTCAGCCTT-MGB

[a] (Halstensen et al. 2006)

[b] (Waalwijk et al. 2004)

[c] (Reischer et al. 2004)

conidial suspension (10^6 conidia ml^{-1}) by inserting a syringe into the stem. Ten to 25 μl were used, until suspension emerged from the 2nd leaf whorl.

B1–B5) Inoculation by boot injection. Oat plants at GS Z45 were inoculated using a syringe and carefully injecting microconidial suspension through the opening

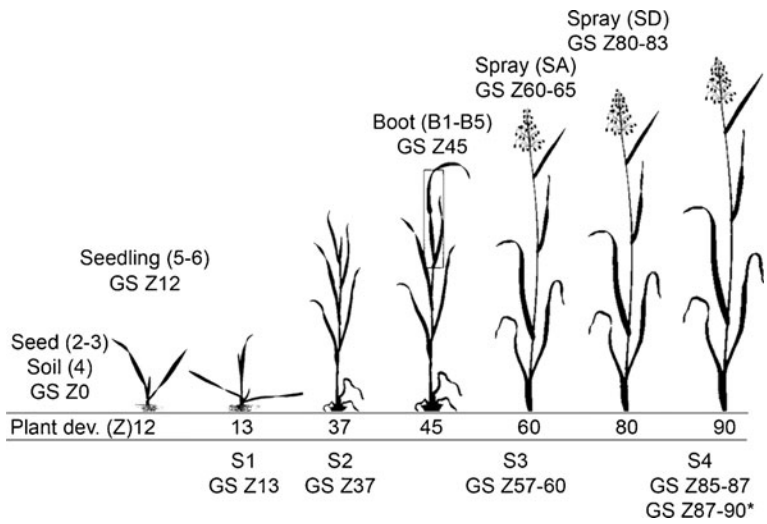

Fig. 1 Overview of treatments and sampling time-points relative to plant developmental stage.* Sampling stage S4 for treatments 1–6 and B1 was GS Z 85–87, whereas B2–B5, SA, and SD were at GS Z 87–90

in the flag leaf sheath into the swollen boot of main shoots. Care was taken to avoid wounding when inserting the syringe. Five different inocula, varying in conidial concentration as well as volume, were applied as boot injection; B1; 1 ml 10^6 conidia ml^{-1}, B2; 0.5 ml 10^6 conidia ml^{-1}, B3; 0.1 ml 10^6 conidia ml^{-1}, B4; 0.5 ml 10^5 conidia ml^{-1}, B5; 0.1 ml 10^5 conidia ml^{-1}. Boot control was treated with 0.5 ml SDW. Inoculated shoots were bagged with transparent plastic bags sprayed once inside with SDW. In order to avoid interference with shooting, bags were removed 3 days post inoculation.

SA) Spray inoculation of panicles at anthesis (GS Z60-65). Five sprays, approximately 4.7 ml micro-conidial suspension (10^6 conidia ml^{-1}) were applied per panicle. Controls were sprayed with the same volume of SDW. Inoculated shoots were bagged for 6 days post inoculation with transparent plastic bags sprayed once inside with SDW prior to bagging.

SD) Spray inoculation of panicles at early dough stage (GS Z80-83) was performed as for SA.

All treatments were done in two biologically independent repeats (i.e. inocula and plants were prepared independently for each repeat), and each sampling consisted of a pool of five plants. Sampling was done at four developmental stages (Fig. 1). Ten plants were harvested from S4, of which five plants were used for re-isolation of the fungus, and five plants were stored in −20°C for DNA extraction.

Re-isolation of *F. langsethiae*

To confirm viability of *F. langsethiae* during the trial, plant parts were surface sterilized using 70% ethanol, washed twice with SDW and blot-dried before incubation on moist filter paper for 10 days at 24°C in dark. Monitoring of fungal viability in soil in treatment 4 was done by taking 1 g samples of soil from a layer 1–2 cm under the soil surface. Samples were diluted in 50 ml SDW, filtered through sterile cheese-cloth, and 500 µl were spread on Czapek–Dox iprodione dichloran (CZID) agar (Abildgren et al. 1987). Plates were incubated for 1 week in dark at room temperature, upon which the number of white fungal colonies was counted. Plates were then moved to 24°C with 12 h photoperiod (white + black light). This made white fungal colonies turn orange. Fungal colonies emerging upon incubation were determined using a Leitz Aristoplan light microscope (Leica, Montréal, Quebec, Canada).

DNA extraction and real-time quantitative PCR

Plant and fungal DNA was extracted from 100 mg ground tissue. Grain and roots were extracted using the FastDNA SPIN for Soil Kit (MPBiomedicals, France). Prior to application of the FastPrep instrument grain was ground with an A11 Basic Analytical mill device (IKA, Germany) and roots were ground in liquid nitrogen with a mortar and pestle. Other plant tissues and fungal control were ground in liquid nitrogen with a mortar and pestle, and DNA was extracted using the DNeasy Plant Mini Kit (Qiagen Inc., Germany) according to the manufacturer's descriptions. DNA was eluted in 100 µl elution buffer, and DNA integrity was verified by agarose gel electrophoresis.

Quantification of plant and fungal DNA was investigated by TaqMan-qPCR using the Applied Biosystems 7900HT instrument with a standard 96-well block (Applied Biosystems, UK). Each TaqMan reaction was carried out in a 25 µl volume, containing 1×PCR Master Mix for Probe Assays (Eurogentec, UK), 300 nM primers (Invitrogen Ltd, UK), 100 nM probe (final concentrations), and 2 µl 5× diluted template DNA. The target DNA and the sequences of primers and probes for each assay are listed in Table 1. The probes (Applied Biosystems) were labelled at 5′ end with reporter dye FAM (6-carboxyfluorescein) or TET (6-carboxy-4,7,2′,7′-tetrachlorofluorescenin), and at the 3′ end with either a quencher dye TAMRA (6-carboxy-N,N,N′,N′-tetrachlorofluorescein) or a minor groove binder (MGB). All reactions were run with the following parameters: 2 min at 50°C and 10 min at 95°C, followed by 15 s at 95°C and 1 min at 60°C for 40 cycles. The data were analyzed using Sequence Detection Software (SDS) version 2.2.1 (Applied Biosystems).

Amount of DNA in the samples was quantified by a standard curve algorithm using five 10-fold dilutions of known amounts of DNA, starting with 1 ng for fungal and 50 ng for oat DNA. Samples were tested in two technical PCR replicates, differing by a $C_q \leq 1$. Amount of fungal DNA in inoculated plants was calculated relative to plant DNA by dividing mean total quantity of two fungal replicates with mean total quantity of respective two plant replicates.

Enzyme immunoassay for detection of T-2 toxin

Quantification of T-2 toxin was performed on grains and flag leaf nodes (FLN) from two biological repeats

using the ELISA Ridascreen® FAST T-2 Toxin kit (R-Biopharm, Germany) according to the manufacturer's description. Absorbance was measured at 450 nm with an Expert Plus Microplate Reader (Biochrom, UK), and amount of toxin was calculated as a mean of two technical replicates according to standards provided in the kit.

Statistical analysis

Variation in culm length of plants from treatments C, B1–B5, SA, and SD gave a reason to assume unequal variance among these groups. Non-parametric statistical tests were therefore used for comparisons according to median. Data were analyzed statistically in Minitab version 15 using the Mann–Whitney pairways comparison test, the Kruskal-Wallis multiple-comparison test, and the Spearman's rho test for linear correlations.

Results

Investigation of alternative infection routes of *F. langsethiae* in oats

Artificial infection with *F. langsethiae* has proven difficult to reproduce both in oats and wheat (Imathiu 2008). A glasshouse trial was designed to investigate the ability of *F. langsethiae* to infect oat grain through different routes and tissues. In addition to a non-inoculated control, various treatments were set up to cover possible infection routes through contamination in the seed, soil, seedling, boot and panicle (Fig. 1). The amount of colonization was determined by real-time qPCR.

Initially, mature grain was sampled at the last sampling stage, S4 (GS Z85-90), from treatments 1–6, B1, SA and SD. Presence and quantity of *F. langsethiae* DNA was determined. Overall, none of the alternative infection ways using seed, soil or seedling inoculations (treatments 1–6) yielded any detectable level of *F. langsethiae* DNA in the grain. This was confirmed by culturing grain from additional five plants from S4 on moist filter paper (data not shown). All plants developed normally and showed no signs of disease. Viability of *F. langsethiae* in the soil in treatment 4 was confirmed at sampling stages S1, 2 and 4 (Fig. 1), ranging from 15 to 40 colonies per plate (data not shown). For plants receiving treatment 5 survival of fungus on the cotton was confirmed at S1, 2 and 4. Slight fungal growth was also observed on the stem surface in contact with the cotton, however, no sign of wounding was apparent.

Boot injected plants (B1) showed clear signs of infection already at sampling stage S3, 2 weeks post inoculation, however the symptoms were not similar to FHB. Whereas inflorescence emergence of control plants was completed at this stage, inoculated panicles remained largely inside the boot, failing to emerge from the flag leaf sheath. Several plants showed browning on the stem surface, protruding through the flag leaf sheath from inside of the boot. Infected panicles showed nearly 100% floral abortion with chlorotic undeveloped flowers at first rachis node, and about 50% at 2nd rachis node (Fig. 2a). Extensive browning, lesions and fungal growth were noted on the rachis internodes in this area (Fig. 2b). Together, this reflects the considerable volume of conidial suspension filling up the lower half of the boot during the injection. Only a few floral abortions were noted on the third rachis node. Upper half of the panicle seemed healthy with no signs of browning or fungal growth on the plant surface. At the last sampling (S4; 6 weeks post boot inoculation), spikelets appeared slightly more yellow than control, and yellowing protruded into the stem down to the flag leaf node (FLN). Also, flag leaves of infected shoots were completely yellow and senesced. Most apparent, however, was a considerable stunting of infected shoots compared to the control (Fig. 2c). Measuring the length from FLN to the first rachis node, the stunting of injected shoots was statistically significant as compared to the control using the Mann–Whitney test ($P<0.001$; Fig. 2d).

In agreement with the presence of disease symptoms, approximately 100 pg fungal DNA per 100 ng plant DNA were detected in S4 grains of boot inoculated plants (treatment B1; Fig. 3). Between 98 and 100% of the harvested grains confirmed the presence of *F. langsethiae* in culture. Boot injected plants were also tested for fungal DNA in the rachis. Due to extensive damage to floral tissue on the lower part of these panicles the rachis was divided into upper (URa) and lower (LRa) rachis between second and third rachis node. Whereas URa contained *F. langsethiae* DNA levels comparable to the grain, we found a 10-fold increase in LRa (data not shown),

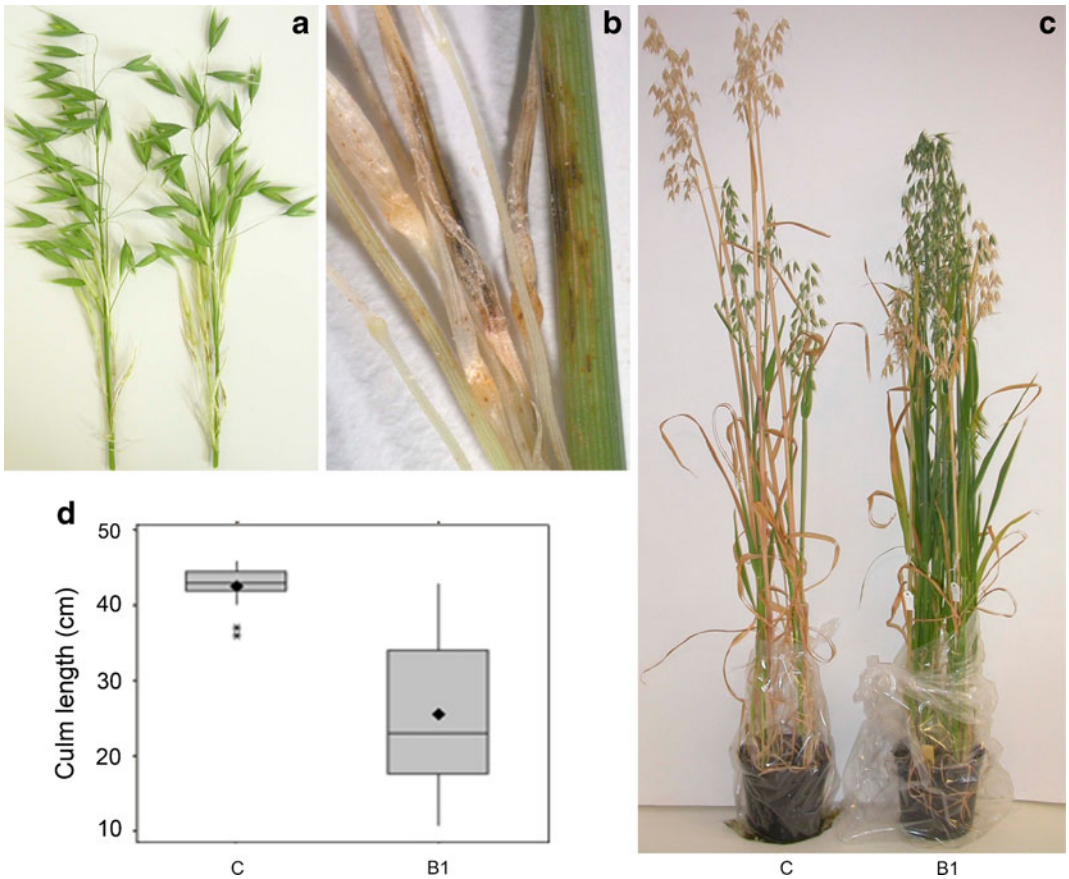

Fig. 2 Typical *F. langsethiae* symptoms upon boot inoculation. **a** and **b** Sampling stage S3, 2 weeks post B1 boot inoculation. Colonized panicles showed extensive floral abortion with chlorotic undeveloped flowers at the first and second rachis nodes (**a**), as well as browning, lesions and fungal growth on peduncle and rachis internodes in the same area (**b**). **c** Stunting of boot injected shoots as compared to control, at sampling stage S4, 6 weeks post boot inoculation. **d** Whiskers plot showing the distribution of culm lengths of control and inoculated shoots. Length was measured as cm from the FLN to the first rachis node. Using the Mann–Whitney test for comparison of group C and B1, the groups were deemed significantly different ($P<0.0001$). $N_C=15$, $N_{B1}=19$

reflecting the extensive fungal growth on the plant surface in this area.

Spray inoculated plants also showed symptoms of disease. However, these resembled FHB symptoms, and were quite distinct from that of boot injected plants. At 6 days post inoculation, upon debagging, fungal growth was evident on the apical part of individual spikelets. The visible mycelia gradually disappeared, and apical tips of palea and lemma developed a dark brown colour towards maturation (Fig. 4a). Occasionally, diffuse grey discolouring appeared as spots on the glumes (Fig. 4c) or extended into the glumes from the apical tips of the grain (Fig. 4b). The symptoms were clearly distinguishable, albeit not conspicuously, from control and boot injected (B1) plants. Spots or areas of browning were also occasionally noted on the pedicel, peduncle and rachis (Fig. 4a, c). Symptoms were most easily noted on plants inoculated at early dough stage.

Consistent with the symptoms, fungal DNA confirmed in all tests a successful infection, both from spray inoculated grain at anthesis (SA; 305.6 and 1101.7 pg fungal DNA per 100 ng^{-1} plant DNA) and early dough stage (SD; 529.1 and 792.2 pg fungal DNA per 100 ng^{-1} plant DNA; Fig. 3). Fungal DNA seemed more abundant in SA and SD as compared to B1, however the difference was not found statistically significant using the Kruskal-Wallis multiple comparison test ($P=0.180$).

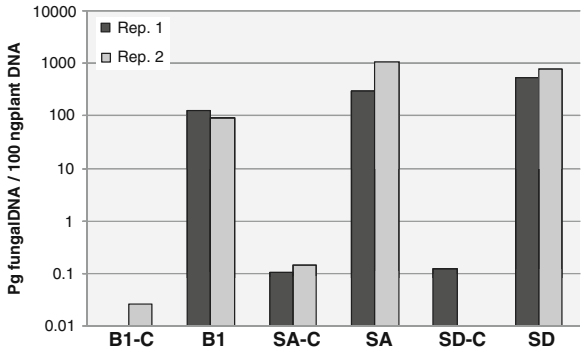

Fig. 3 Monitoring of fungal DNA in grain of boot injected and spray inoculated plants using real-time qPCR. Infection level at S4 is shown as pg fungal DNA per 100 ng plant DNA, visualized on a logarithmic scale. The experiment was carried out in two biological repeats (*dark and light bars*) where each repeat is a pool of five plants. *B1* boot injection, *B1-C* B1 control, *SA* spray at anthesis, *SA-C* SA control, *SD* spray at dough stage, *SD-C* SD control

In addition to testing of the grains, the uppermost three culm nodes (FLN, N-1, N-2), and roots (R) from all treatments were tested at the last sampling stage at maturity (S4). Treatments 1–6 (testing seed, soil, and seedling inoculation methods) yielded only arbitrary values of 5–50 pg fungal DNA per 100 ng plant DNA in any of the nodes (data not shown), mostly not consistent between biological repeats and/or technical replicates, and all failed to confirm in culture. In B1 boot inoculated FLNs, however, as much as 2,100–6,600 pg fungal per 100 ng plant DNA were found, a 10–15 fold increase to that of B1 grains (Fig. 5b). This was confirmed by culturing nodes of an additional five plants from S4 on moist filter paper. Fungal DNA was also detected in N-1 nodes of B1 boot injected plants although at much lower concentrations (16.9 pg fungal DNA per 100 ng plant DNA; data not shown). Being in the range of background levels, importantly, this was confirmed in culture as live fungus emerged from 2 out of 5 N-1 nodes in one of the biologically independent repeats. The control always remained negative. Hence, 16.9 pg designates the lowest quantity of fungal DNA confirmed in culture in this study. N-2 of B1 boot injected plants, as well as grain and nodes of their tillers, were found free of *F. langsethiae* DNA (data not shown). For spray inoculated plants only FLNs were tested for presence of fungal DNA. Interestingly, no consistent levels of fungal DNA were detected in these nodes (data not shown).

The only treatments that yielded any consistent detection of fungal DNA in the S4 sampling stage of roots were seedling infections both by application (treatment 5) and injection (treatment 6) of the stem. The amount of detected fungal DNA per 100 ng plant

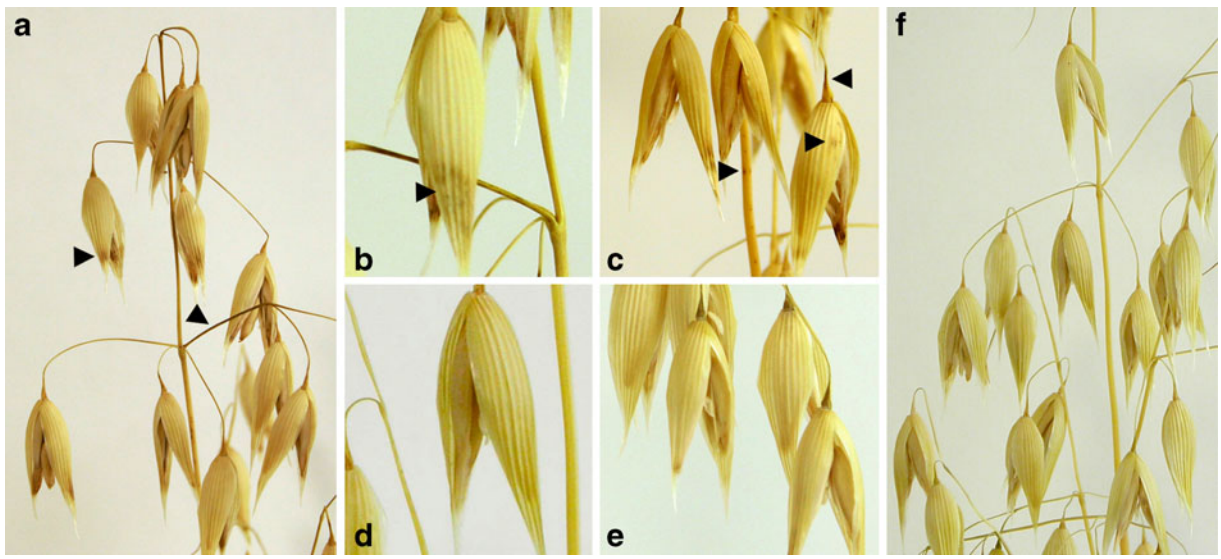

Fig. 4 Typical *F. langsethiae* symptoms upon spray inoculation at early dough stage. **a**–**c** Colonized panicles and single grains showing browning at grain tips (**a**), areas and spots with browning on rachis, peduncle and pedicel (**a**, **c**), and grey discoloration in glumes (**b**, **c**). **d**–**f** Non-inoculated control

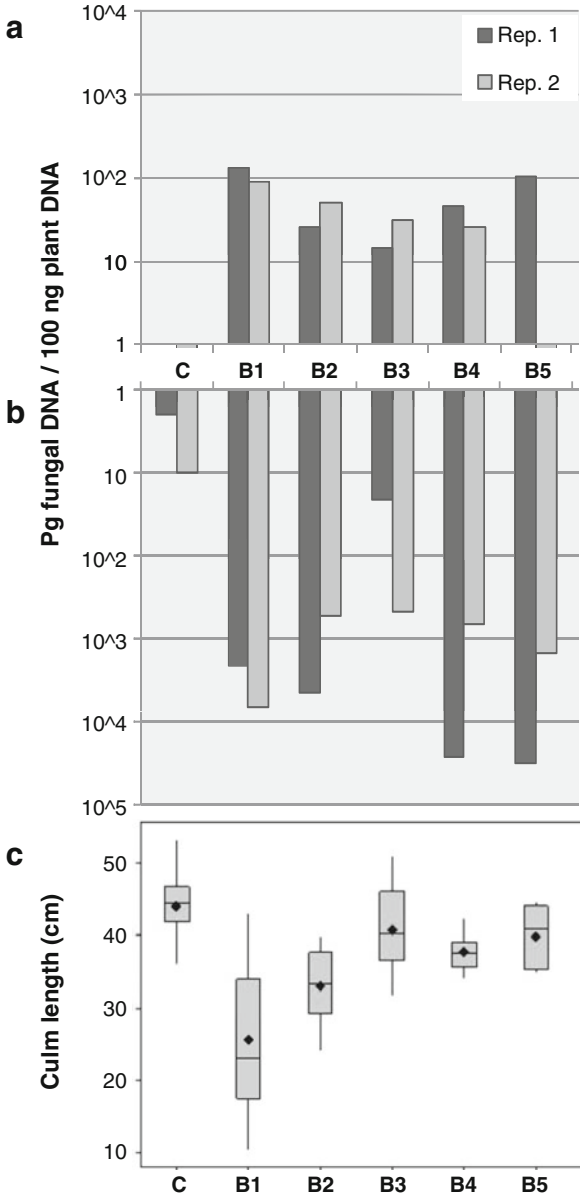

Fig. 5 Assessment of the boot inoculation assay. **a** and **b** Level of colonization at sampling stage S4 in grains (**a**) and FLNs (**b**) shown as pg fungal DNA per 100 ng plant DNA, visualized on a logarithmic scale. The treatments were carried out in two biological repeats (*dark and light bars*) where each repeat is a pool of five plants except for C-1 (3 plants). Spearman's rho and Kendall's tau tests for linear correlation between conidial load and amount of DNA in FLN were significant over B1–B3 (P=0.014). **c** Whiskers plot showing the distribution of culm lengths resulting over the five boot inoculation treatments. Length was measured as cm from the FLN to the first rachis node. In order to get a robust measure of the control, culm length of control plants from all boot and spray inoculation treatments were pooled. Culm length of B1, B2 and B4 was significantly different from control at $P<0.000$. $N_C=42$, $N_{B1}=19$, $N_{B2\text{-}B5}=10$

DNA varied greatly however, between biological repeats, showing a particularly low level in second biological repeat (7.4 pg vs. 1 pg for treatment 5, and 61.3 pg vs. 1.3 pg for treatment 6). For these treatments additional S1 and S2 samplings of stem and root tissue were tested, but consistent levels of fungal DNA were not detected, nor was any of this confirmed in culture (data not shown).

Sensitivity assessment of the boot inoculation assay

Analysis of boot injected plants from treatment B1 indicated that this method could be used as a reliable assay to achieve even distribution of *F. langsethiae* infection throughout the entire oat panicle. In order to test the limits of the boot injection assay, four additional boot injection treatments were made, using various volumes and conidial concentrations (Fig. 5). Plants were only sampled once at maturity (S4; GS Z85-90), and the amount of *F. langsethiae* DNA in grain and FLNs was assessed using real-time qPCR. Consistent with injection of a decreasing total number of conidia, the degree of colonization in boot injected grain decreased gradually over the three highest inocula from an average of 110 pg in B1 to 22 pg in B3 per 100 ng plant DNA (Fig. 5a). Over these three treatments both total number of conidia and inoculation volume decreased gradually while conidial concentration was kept constant at 10^6 conidia ml^{-1} (table in Fig. 5). In treatment B4, receiving a medium volume combined with a lower conidial concentration (10^5 conidia ml^{-1}), the colonization level increased slightly despite a further reduction in total number of conidia. In B5, where both total number of conidia and volume were lowest, the level of infection varied greatly, giving poor reproducibility between the two biological repeats (Fig. 5a).

Compared to the grain, overall more fungal DNA was found in the FLNs of boot injected shoots (Fig. 5b). Good correlation between total conidial inoculum and amount of fungal DNA in node was seen for the three largest inocula (B1–B3; Spearman's rho = 0.837). Culm elongation was compared between all the different boot inoculation treatments (Fig. 5c), and was significantly shorter than control (P<0.0001)

for treatments B1, B2, and B4. Consistent with the absence of fungal DNA in nodes of spray infected plants (SA and SD), these were found to be significantly different from B1 at α=0.05 using Kruskal-Wallis test with Bonferroni adjustment (Fig. 6). Roughly, over the five boot inoculation treatments, a negative correlation of culm length was seen, partly to total number of conidia inoculated (B1–B5; Spearman's rho = −0.729), and partly to the amount of fungal DNA measured in the FLN (B1–B3; Spearman's rho = −0.607).

In order to quantify the level of mycotoxin in *F. langsethiae*-colonized plants, amount of T-2 toxin was measured in S4 grain samples from treatments B2–B5, SA and SD using ELISA immunoassay. T-2 toxin was found in the range of 86–2,230 μg/kg (ppb; data not shown). Significant correlation between T-2 and *F. langsethiae* DNA in the grain was found across the treatments (Spearman's rho = 0.888) and also within the boot injection treatments alone (B2–B5; Spearman's rho = 0.683). Using ELISA, the presence of T-2 toxin was confirmed for FLNs as well (up to 2,350 ppb), however, the material was insufficient to make correlation to DNA quantity informative.

Growth of *F. langsethiae* in oat node tissue

The presence of *F. langsethiae* in FLNs was used to gain additional insight into its biology in vegetative tissues. Upon culturing *F. langsethiae*-colonized nodes on moist filter paper, fungal colonies emerged from the node and adjacent areas (Fig. 7a). The appearance of fungal colonies, especially on the basal side adjacent to FLN, was restricted to dark, parallel, vertical rays of tissue between the vascular bundles of the stem (Fig. 7b). Comparison to non-inoculated control nodes strongly indicated that the protruding fungal colonies coincided with the presence of stomata (Fig. 7c and d).

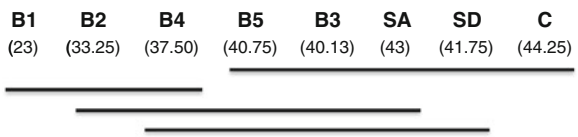

Fig. 6 Multiple comparison of culm length between treatments. Treatments combined with a line are not significantly different according to the Kruskal-Wallis test at α=0.05 with Bonferroni correction. Treatments (*bold*) are ordered according to average ranking, and numbers in brackets indicate median. N_C=42, N_{B1}=19, $N_{B2\text{-}B5,\ SD}$=10, N_{SA}=9

The emergence of fungus from stem tissue adjacent to FLN basal side might indicate that *F. langsethiae* to some extent had actively traversed the node tissue. Consistent with this we were occasionally able to confirm the presence of live fungus also at the second culm node from the top (N-1) in treatment B1. In addition, when comparing real-time qPCR data from FLN of B1 boot injected plants at 2 and 6 weeks post inoculation (S3 and S4, respectively), an increase in fungal DNA was noted (Fig. 8). The injected conidial suspension was absorbed already by the time of S3, hence we infer that fungal proliferation might occur within the FLN tissue.

Discussion

In the present study the ability of *F. langsethiae* to colonize oat panicles through various infection routes and tissues was investigated. Due to difficulties in scoring disease according to FHB symptoms in oats (Tekauz et al. 2004) and the general lack of symptoms upon *F. langsethiae* colonization (Torp and Adler 2004; Edwards 2007), real-time qPCR detection of fungal DNA was employed in order to quantify the level of colonization. This method provides a sensitive and specific measure of fungal quantity, independent of disease symptoms.

Based on the data investigated, we found no strong evidence to suggest a different infection route for *F. langsethiae* compared to well-studied *Fusarium* species such as *F. graminearum* and *F. culmorum*, causing FHB. Only infections directly to the panicle, i.e. boot injection and traditional spray inoculations, resulted in detectable accumulation of fungal DNA in the grain. Spray inoculations gave roughly 6-fold more fungal DNA in the grain compared to the most successful boot injection experiment (B1). The difference between B1 and SA/SD was significant at only 82% confidence. However, providing strength to the results is the fact that each observation (two biologically independent repeats for each treatment) was a pool of five plants. One plausible explanation for the difference in colonization level might be that flowers at anthesis and developing grain provide a more suitable growth medium for *F. langsethiae* than developing florets and other vegetative tissues. This would be in agreement with the general biology as we know it for FHB-causing fungi (Parry et al. 1995; Lacey et al. 1999; Doohan et al. 2003).

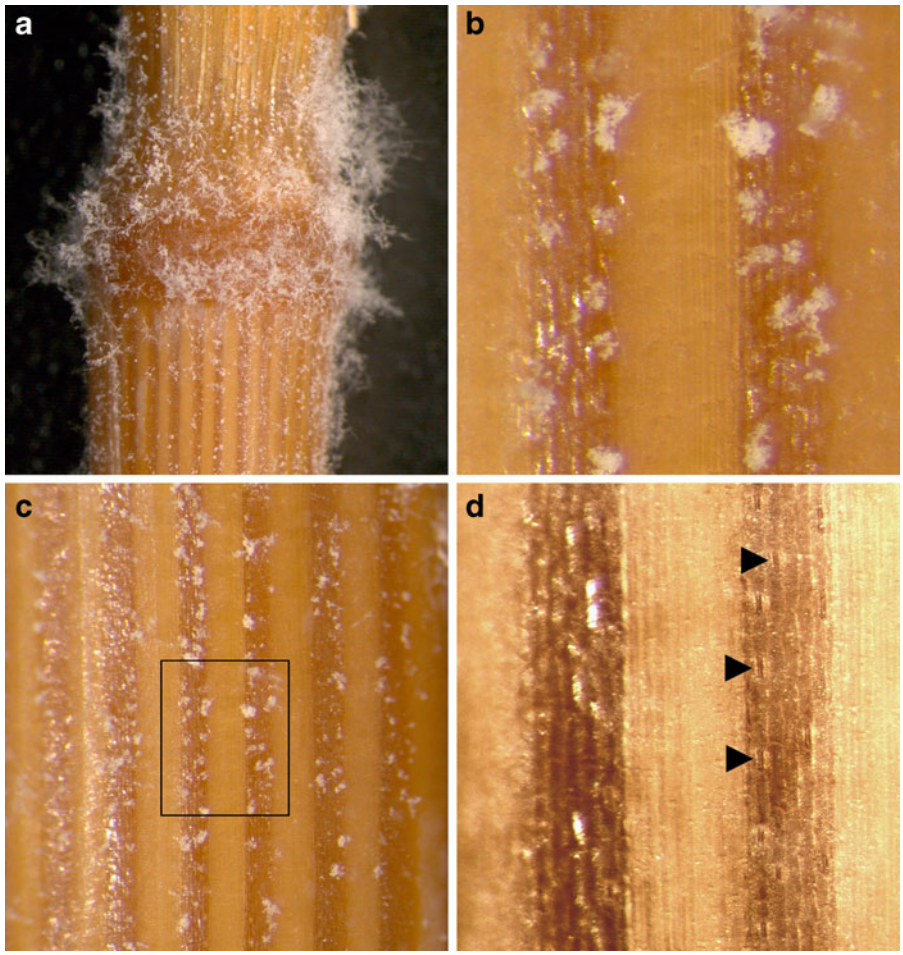

Fig. 7 Culture of *F. langsethiae*-colonized nodes. **a** Emerging *F. langsethiae* colonies from FLN and adjacent areas (0.8× magnification). **b** The appearance of fungal colonies was restricted to parallel, vertical rays of tissue between the vascular bundles of the stem on the lower side of the FLN (5× magnification). **c** Enlarged section of **b** showing *F. langsethiae* colonies emerging from stomata (8× magnification). **d** Control stem with stomata indicated by arrowheads (8× magnification)

The amounts of fungal DNA found in boot and spray inoculated grain in this study are comparable to what is found in commercial oat fields both in Norway and the UK (Imathiu 2008; Klemsdal, unpublished results). To our knowledge, this is the first report where successful artificial inoculation with *F. langsethiae* has been documented and quantified at the molecular level. The level of colonization achieved in this study was roughly 10–15 fold that of Imathiu (2008).

Sufficient moisture has been shown to be one of the most important factors influencing FHB disease in cereals. Irrigation for a period up to 3 days post inoculation (Lacey et al. 1999), and bagging time up to 24 h (Lemmens et al. 2004) increased infection by *F. culmorum* on wheat. Similarly, albeit very low levels of infection were achieved, bagging for 48 h resulted in significantly more colonization with *F. langsethiae* on oat panicles (Imathiu 2008). Taking into account the slower growth rate of *F. langsethiae* on nutrient media (Torp and Nirenberg 2004), the bagging time in our study was increased to 72 and 144 h, for boot and spray inoculation, respectively. This would ensure a favourable microclimate for the time required for conidial germination and early growth to occur. Differences in bagging time between spray and boot inoculations may also explain the more successful colonization upon spray inoculation. Bagging time was, however, not included as a test parameter in our experiments.

Temperature is another important factor influencing fungal growth. Whereas the optimal temperature for *F. graminearum* in in vitro culture is 24–28°C, *F. culmorum*, *F. avenaceum* and *F. poae* share an optimum temperature of 20–25°C (Doohan et al. 2003). This is also the case for *F. langsethiae*, characterized with an optimum temperature at 24–25°C (Torp and Nirenberg 2004; Imathiu 2008). In the

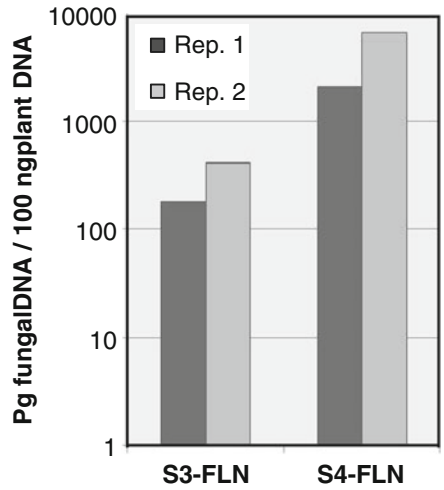

Fig. 8 Indication of *F. langsethiae* proliferation within the FLN tissue. *Fusarium langsethiae* DNA in FLNs of B1 boot inoculated plants was measured at 2 and 6 weeks post inoculation (sampling stages S3 and S4, respectively) using real-time qPCR. Values are shown as pg fungal DNA per 100 ng plant DNA, visualized on a logarithmic scale. The treatments were carried out in two biological repeats (dark and light bars) where each repeat is a pool of five plants

present study the temperature was set to simulate Norwegian conditions in the field. At the time of panicle infections (spray and boot), temperatures were oscillating between 12°C night and 21.5°C day, hence disregarding the optimum temperature for *F. langsethiae*. Higher temperatures and larger oscillations, well above the optimum for *F. langsethiae* (15°C to 25–45°C), did not yield successful infections (Imathiu 2008). It is possible that the favourable temperature for *F. langsethiae* during plant colonization is lower than that registered on potato dextrose agar (PDA) medium. Giorni et al. (2009) showed that the carbon nutritional patterns of *Aspergillus flavus* Link and *F. verticillioides* (Sacc.) Nirenberg are modified by environmental factors such as water activity and temperature. Interestingly, although *F. langsethiae* is considered a relatively slow-growing fungus, at temperatures of 18.5°C it will perform equally well or even better than species like *F. poae*, *F. sporotrichioides*, *F. avenaceum* and *F. graminearum* in growth assays on minimal medium (SNA with 0.8 g l^{-1} NH_4NO_3 as nitrogen source) with 2% amylopectin as carbon source (Divon, unpublished results). This indicates that *F. langsethiae* might have nutritionally favourable niches at temperatures outside its optimum.

Possible differences in infection efficiency may be due to variation in aggressiveness of the fungal isolates and/or resistance in oat cultivars used. In the present study a mix of three isolates originating from barley and oats was used. There is no reason to believe that the inoculum used in the present study was more aggressive than the inoculum used by Imathiu (2008) consisting of a mixture of 11 *F. langsethiae* isolates all originating from oats. The level of susceptibility in oats to *F. graminearum* has been shown to vary between cultivars (Tekauz et al. 2004). Spring oat variety Belinda, used for the spray inoculations in the present study, is known to accumulate high levels of T-2/HT-2 toxins in Norwegian oat grain (Hofgaard et al. 2009). In general winter oat varieties have been shown to be more susceptible than spring oat varieties in the UK (Edwards 2007). It is thus unlikely that Belinda is more susceptible than the winter oat variety Gerald, used by Imathiu (2008).

In the present study symptoms were recorded towards grain maturation and were of two main kinds, depending on the inoculation method used. Subtle symptoms were seen on grain upon spray inoculation, especially after spraying at the early dough stage. Although the symptoms were indisputable, they were most easily noted when comparing infected and control panicles side by side. The same material did not show any DNA contamination of other FHB-causing fungi, hence ruling out the possibility that the symptoms were caused by other pathogens. Few symptoms are associated with *F. langsethiae* infection of small grain cereals, and our results are largely in agreement with this. The subtle symptoms noted in this study were different from the lesions previously reported as “glume spots” on *F. langsethiae* infected wheat ears in Austria (Torp and Adler 2004). The symptoms on spray infected panicles were apparent only towards maturation when the grains turned yellow, hence any bleaching effect would not be detected. The symptoms did however, remind of FHB. It has e.g. been noted that severely affected wheat ears may show a dark browning of the peduncle (Parry et al. 1995).

Boot injection did result in relatively high levels of fungal DNA in the grain, however, compared to spray infection, not enough to indicate that this might be the natural infection route for *F. langsethiae*. The boot injection assay may provide a useful tool e.g. for molecular studies or when screening host cultivars for

physiological susceptibility to *F. langsethiae*. We found that the assay produced a reliable quantity of fungal DNA in the grain steadily increasing with total number of conidia over the three highest inocula (B1–B3). In addition to total conidial load, inoculum volume might also influence the rate of infection, as indicated in treatment B4. At B5 we did not achieve reproducible results between the two biological repeats, probably due to a combination of low number of conidia and small volume, reducing the effect of the inoculum to the outer range of sensitivity of the assay. A larger volume might be required for the assay to perform reproducibly, and it is possible that yet lower conidial concentrations will yield good colonization if larger volumes are used. Similar to the grain, fungal DNA in the uppermost culm node was well correlated with conidial load over B1–B3. At B4 a higher level of fungal DNA was detected in the node, possibly as a result of the increased inoculation volume. For reasons unclear, B5 showed relatively high levels of fungal DNA in the node.

FHB of boot injected grain was not observed, however boot injected shoots showed a significant stunting, and panicles failed in many cases to emerge from the flag leaf sheath. Additional symptoms were yellowing of the stem down to the FLN, and extensive wilting of the flag leaf. Similar non-specific symptoms are usually caused by vascular wilt and root and stem rot diseases, due to disruption of the nutrient uptake. Stunting is however not a symptom associated with FHB. Degree of stunting was correlated with inoculum concentration over all five boot injection treatments, and with amount of fungal DNA in the node tissue over the three largest inocula (B1–B3). Coinciding with the absence of fungal DNA in node tissue of spray inoculated shoots, culm length of SA and SD was significantly different from B1. We therefore conclude that the effect on culm elongation is connected to the inoculation method and presence of fungus in the node. Boot injection is a commonly used and accepted method for susceptibility screening of wheat germplasm to karnal bunt, caused by the fungus *Tilletia indica* Mitra (Warham et al. 1986; Riccioni et al. 2008), but to our knowledge stunting is not a common symptom of these infections. It is therefore possible that stunting is caused by some *F. langsethiae*-produced component interfering with plant development. Intercalary meristems adjacent to the node tissue are responsible for most of the cell production required during shooting, and fungal presence in the node area could interfere with plant cell cycle and thus result in stunted plants. High levels of T-2 toxin were in some samples detected in node tissue. This is consistent with *Tri5* activity in *F. graminearum* infected wheat nodes (Ilgen et al. 2009). It is plausible that the presence of T-2 toxin in the node is responsible for the reduced culm elongation in boot injected shoots. Indeed, the phytotoxic effect of T-2 toxin has been demonstrated in *Arabidopsis* resulting in dwarfism of toxin treated seedlings (Masuda et al. 2007). The dwarfism was due to inhibition of cell extension in petioles and leaves. It is tempting to speculate that this is the case also in monocots, however more studies are needed to confirm the phytotoxic mechanism of T-2. The presence of other phytotoxic factors with similar effect can also not be ruled out.

Our investigations revealed no, or very limited ability, of *F. langsethiae* to actively enter, grow or spread within vegetative tissues other than the node. Injection of microconidial suspension into seedlings had no notable effect on growth and development of the plant. Similarly, application of microconidial suspension on the stem surface, or *F. langsethiae* contamination of the soil did not cause any visible lesions on stem or roots, nor did it impair plant development. This is in accordance with other reports ranking *F. langsethiae* as a weak pathogen relative to other FHB-causing fungi. Although *F. langsethiae* has been shown to cause lesions on oat and wheat leaves in a detached leaf assay (Imathiu et al. 2009), it was not able to cause seedling blight on oat or wheat cultivars as compared to other FSB pathogens (Imathiu et al. 2010).

The poor aggressiveness of *F. langsethiae* has spurred a discussion of whether *F. langsethiae* is to be considered an endophyte (Imathiu 2008). Some endophytes have developed a close relationship with the host, and depend on host dissemination for their spread (Saikkonen et al. 2004). Horizontally transmitted fungal endophytes on the other hand, may be closely related to pathogenic fungi and disseminate via similar routes. It has been suggested by Saikkonen et al. (2004) that due to conflicting selective forces the benefits from any such interaction are rarely symmetric and under constant evolution. Hence, plant endophytism should be understood as a continuous spectrum ranging from mutualistic symbiosis to

antagonistic parasitic relationships between the host and microorganism (Saikkonen et al. 2004). In some cases genetic variation in one single locus is enough to shift the endophyte-pathogen equilibrium to either direction. This is exemplified by the non-pathogenic *path-1* mutant of *Colletotrichum magna* Jenkins and Winstead (Freeman and Rodriguez 1993) and the fungal endophyte, *Epichloë festucae* Leuchtm., Schardl and Siegel, rendered pathogenic to ryegrass upon mutation in the *noxA* gene (Tanaka et al. 2006). Also pathogenic species within the genus *Fusarium* have been found to contain endophytic isolates which engage in symbiotic associations with the host, e.g. an endophytic isolate of *F. culmorum* conferring salt tolerance to the perennial grass species *Leymus mollis* (Trin.) Hara (Rodriguez et al. 2008). In addition, asymptomatic colonization of wheat occurs with FHB causing fungi such as *F. graminearum*, *F. pseudograminearum* O'Donnell and Aoki, *F. culmorum*, and *F. avenaceum* (Clement and Parry 1998; Mudge et al. 2006). In fact, upon stem base inoculation similar to treatment 5 in this study, Mudge et al. (2006) recovered *F. graminearum* and *F. pseudograminearum* isolates growing asymptomatically through the plant to the flag leaf and head. In the present study we failed to detect any directional spread of *F. langsethiae* against gravity, whether from seed or seedling infections, hence excluding the possibility of a general endophytic behavior for *F. langsethiae*. Our impression is that the fungus reveals a strong preference for the panicle, and, given the opportunity to produce a sufficient biomass, *F. langsethiae* might be able to overcome/surpass the plant resistance response, interfere with plant development, and eventually will also produce disease symptoms both in grains and vegetative tissues.

The present study demonstrated that panicle infection, similar to that of FHB-causing fungi, seems to be the prime route of infection also for *F. langsethiae*. Testing of alternative infection routes revealed no seed-to-head systemic transfer of the fungus. We favour a view of *F. langsethiae* as a weak pathogen, at least in part attributed to its slow growth, possibly strongly specialized for the cereal grain. Further studies are needed to answer diverse topics such as source(s) of inoculum, molecular mechanisms influencing aggressiveness, and modes of colonization. Histological studies to further investigate the colonization process of *F. langsethiae* are under way.

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