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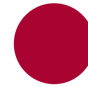

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Selection of fungal endophytes with biocontrol potential against Fusarium head blight in wheat

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ABSTRACT

Fusarium head blight (FHB) is a major disease in wheat and other cereal crops. Biological control is a promising alternative for control of this disease as current control options are not efficient. Fungal endophytes could represent a new source of biological control agents since they colonise plant tissues asymptomatically whilst sharing the same biological niche as pathogens. Here, we show that healthy wheat spikes and leaves collected in areas with high FHB incidence harbour fungal isolates that reduce disease severity and pathogen biomass inside the spikes. We employed an *in planta* screening method using detached spikelets to observe the performance of 15 fungal strains against *Fusarium* infection. The results were validated in greenhouse spike inoculation assays. Isolates *Sarocladium strictum* C113L, *Anthracocystis floculossa* P1P1, *A. floculossa* F63P and *Penicillium olsonii* ML37 were identified as potential biocontrol agents of FHB in wheat. Timing of application was important: the endophytes needed to be applied at least two days before the pathogen to have a significant effect. Reduced symptom expression correlated with reduced biomass of *Fusarium graminearum* in the infected spikelets. Interestingly, none of these isolates reduced *Fusarium* growth in confrontation assays on agar plates. Our results suggest that naturally occurring endophytes could be a new source of biological control agents to be used in integrated pest management strategies.

1. Introduction

Fusarium head blight (FHB) is a devastating disease in wheat crops worldwide (Savary et al., 2019). It reduces crop yield as well as grain quality as the pathogens infect the spike at flowering stage (Rojas et al., 2019a; Xu and Nicholson, 2009). Although many *Fusarium* spp. have been associated with FHB symptoms, *Fusarium graminearum* is often considered to be the most important species in Europe (Nielsen et al., 2011; Pasquali et al., 2016; Walder et al., 2017). FHB reduces grain quality by accumulation of mycotoxins such as trichothecenes (deoxynivalenol, DON) and the estrogenic toxin zearalenone (Hueza et al., 2014; Lee and Ryu, 2017). Likewise, infected seeds show a reduced germination rate and often exhibit Fusarium crown rot in the seedling stage (Jensen et al, 2000; Moya-Elizondo et al., 2011).

Considerable disease control can be achieved using agricultural management practices in relation to soil and crop debris as well as with crop rotation (Dweba et al., 2017). However, the adoption of reduced-tillage systems can increase the survival of *Fusarium* and augment inoculum pressure (Townsend et al., 2016). Fungicide treatments at anthesis can reduce disease levels moderately (Freije and Wise, 2015; Takemoto et al., 2018), but their use is being limited by their low efficacy and rising environmental concerns about their use among both

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farmers and consumers. New disease control alternatives are necessary in order complement or replace current approaches and sustain wheat productivity.

Biological control in relation to plant diseases is defined as the use of microbial antagonists or their properties to reduce pathogen activity (Cook and Baker, 1983; Eilenberg et al., 2001). It is a promising alternative that could enrich our set of tools to control FHB more effectively (Collinge et al., 2019, Rojas et al., 2019a) and its use is being increasingly promoted (Adams, 1990; Barzman et al., 2015; van Lenteren et al., 2018). Several organisms have been tested both *in vitro* and in plant assays for their efficacy to control FHB in wheat (Comby et al., 2017; Khan et al., 2001; Rabiey and Shaw, 2016; Sarrocco et al., 2019; Wang et al., 2015; Xue et al., 2014). However, very few of these discoveries have transformed into commercially available solutions for farmers, with inconsistent field performance being the major limiting factor in the development of biological control solutions (Parnell et al., 2016). Endophytic biological control agents may offer a route to more consistent effect in the field.

Fungal endophytes are naturally occurring plant colonisers and these microorganisms live inside plant tissues without causing visible damage (Collinge et al., 2019; Rodriguez et al., 2009). As fungal endophytes have evolved to exploit the same resources as plant pathogens, they can be considered as potential biological competitors (Silva et al., 2018). These can exhibit a diverse array of modes of action in disease suppression such as direct inhibition by competition, antibiosis or mycoparasitism and indirect inhibition by induced resistance (Latz et al., 2018). Moreover, their endophytic life-style could shield them from environmental factors, especially from fluctuating weather conditions and thus increase field performance (Card et al., 2016).

Several studies have investigated the fungal endophytic communities of wheat tissues, including heads, using cultivation dependent (Sieber et al., 1988; Vujanovic et al., 2012; Comby et al., 2016) and independent methods (Karlsson et al., 2017; Rojas et al., 2019b; Sapkota et al., 2015). These studies have shown that wheat tissues are colonised by a diverse but conserved array of fungal taxa that includes *Alternaria* spp., *Cladosporium* spp. *Epicoccum* spp., *Fusarium* spp., *Chaetomium* spp., *Zymoseptoria* spp., *Paratagonospora* spp. and *Sarocladium* spp. among others (Comby et al., 2016). Furthermore, these studies have explored how fungal endophytic communities are shaped by agronomic practices such as cropping system and fungicide treatments (Gdanetz and Trail, 2017; Hertz et al., 2016; Karlsson et al., 2014).

Recently, we reported that Fusarium infection modifies endophytic community dynamics. An *in silico* analysis showed that several fungal taxa negatively correlate with *Fusarium* pathogen abundance inside infected wheat spikes. Interestingly, some fungal taxa appear to be enriched in wheat spikes that remained healthy after pathogen exposure (Rojas et al., 2019b). This suggests that healthy wheat spikes in fields with high FHB incidence could harbour fungal endophytes with biocontrol capacity.

The aim of the current study is to 1) isolate endophytic fungi from healthy wheat spikes and to 2) test their efficacy against FHB and their ability to inhibit *F. graminearum* infection and FHB symptoms. We isolated and identified fungal endophytes with consistent biocontrol capacity against FHB in detached wheat spikelets and greenhouse assays. Our results provide evidence that naturally occurring fungal endophytes can be a novel source for biocontrol agents.

2. Materials and methods

2.1. Endophyte isolation

Plots with winter wheat cultivars KW-Nils and SW14308 were grown at Arhus University Flakkebjerg, Denmark (55° 19′ 31.278″ N, 11° 23′ 28.619″ E) and at Lantmännen SW Seed AB, Svalöv, Sweden (55° 54′ 39.65″ N, 13° 6′ 48.283″ E), respectively, during 2016 and 2017. These plots were part of two independent FHB disease scoring trials. In such trails, multiple wheat lines are tested for their response to *Fusarium* infection. Plots were artificially infected with a mixture of *Fusarium* spp. (mainly *F. graminearum and F. culmorum* isolates from the area) during anthesis (BBCH 60–69, Lancashire et al., 1991). The inoculation methods are described previously (Rojas et al., 2019b). The aforementioned cultivars are reported to be moderately tolerant to FHB and showed the lowest levels of infection on a visual assessment among all the tested cultivars. Spikes that remained visually healthy at 14 days after anthesis, at the ripening stage (BBCH 85), were harvested. Eighteen spikes were harvested from each location and season.

Spikes were processed within 48 h of sampling. From each spike, three spikelets were removed from the middle section and dissected. Glumes, lemmas, paleas and kernels were separated and, rachises were discarded. Organs were then surfaced sterilised based on the protocol by Comby (et al., 2016) and the procedure optimized for floral tissues as follows: 96% ethanol for 1 min, 2% sodium hypochlorite for 3 min and 96% ethanol for 30 sec followed by rinsing twice with sterile Milli-Q water. A volume of 100 µl from the last water rinse was plated on Potato Dextrose Agar plates (PDA) (Difco™) to assess the efficiency of the surface sterilisation. The different organs were bisected longitudinally (Supplementary Fig. 1) and plated on three different media: malt extract agar (15 g malt extract l^{-1}), PDA (0.78 g PDA l^{-1}) and yeast extract peptone dextrose agar (10 g bacto-peptone, 5 g yeast extract, and 10 g dextrose l^{-1}). All three media were supplemented with streptomycin sulphate and chlorotetracyclin hydrochloride (30 µg·ml^{-1} of each). Plates were maintained at room temperature in the dark and were examined every day for 28 days. Emerging hyphae or colonies were picked and cultured in new PDA plates. Serial dilutions were performed to obtain single spore/cell cultures of all the isolates.

All isolates were grouped according to macroscopic similarities. Several isolates from each group were selected for molecular identification. Selected isolates, representing the different morphological types were cultured in 20 ml of complete medium (CM) (Pham et al., 2008). Mycelium was recovered and DNA extracted using the DNeasy Plant Mini ® DNA Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The Internal Transcribed Spacer (ITS) region from the rRNA gene was amplified using the primers ITS1f and ITS4 (Manter and Vivanco, 2007). For isolates with biocontrol potential, identity was verified further by amplifying the actin, β-tubulin and translation elongation factor 1-α genes using the primers reported by Roe et al., (2010), see Supplementary Table 1. PCR reactions were prepared in 20 µl volume, consisting of: 4 µl 5X Phusion® HF buffer (Thermo Scientific, Waltham, USA), 0.4 µl dNTPs (2.5 mM each), 1 µl of each primer (10 µM), 0.2 µl (5U/µl) Phusion® High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, USA), 1 µl DNA template and 12.4 µl ddH_2O. The PCR programme for the first reaction was 98 °C for 30 sec followed by 30 cycles (98 °C for 5 sec, 56 °C for 30 sec, 72 °C for 30 sec) and a final elongation step at 72 °C for 6 min. Amplicons were purified using Promega Wizard® DNA Clean-Up System. Samples were sequenced using Sanger sequencing at Eurofins MWG (Ebersber, Germany). Sequences were aligned against the UNITE database version 7.2 (Abarenkov et al., 2010) and the NCBI database to verify maximum identity and coverage.

Additionally, two fungal isolates, ML37 and ML38, originally recovered from wheat leaves at Lantmännen SW Seed AB were also included in the screening. These isolates were reported to significantly reduce the wheat foliar disease Septoria tritici blotch caused by *Zymoseptoria tritici* (Latz et al., 2020).

2.2. Detached-spikelet screening assay

A detached-spikelet assay was performed essentially as described by Comby et al. (2017), but using 6-well plates instead of 12-well plates and a different *Fusarium graminearum* strain. Yeast-like endophytes were grown in 20 ml complete medium for 4 days. Cultures were centrifuged and re-suspended in autoclaved MilliQ water twice to remove

medium traces. Filamentous endophytes were cultured on PDA plates for 15 days. Spores were collected by flooding the plate. The spore suspensions were filtered using Miracloth®, centrifuged and re-suspended in autoclaved Milli-Q water. Spores or cells were counted using a haemocytometer and their concentrations adjusted to 10^7 cells/spores ml^{-1}. All endophyte cell/spore inoculation suspensions and control treatments were prepared using autoclaved MilliQ water + Tween 20 (0.05%) as surfactant.

Spring wheat plants cv. Diskett (Lantmännen SW Seeds AB) were grown to heading stage in a greenhouse (18–20 °C, 60% relative humidity, 16 h light/8h darkness). This cultivar is moderately susceptible to FHB. Spikes were harvested at the pre-anthesis stage, BBCH 59–60. Eight to ten spikelets from the middle of spikes were detached, surface sterilised with 96% ethanol for 30 s and rinsed with autoclaved MilliQ water twice. Spikelets were dipped in 20 ml endophyte suspension with agitation at 200 rpm for 25 min. Three spikelets per treatment were placed vertically in 6-well plates containing 4 ml water agar (2 g l^{-1}) in each well (Supplementary Fig. 2). Six treatments, including two controls inoculated with water, were assigned in each plate. Plates were sealed and kept in a growth chamber (15 °C, 60% relative humidity and 16 h light/8 h darkness).

After 4 days, spikelets were removed from the plate and sprayed on both sides with a macroconidial suspension of *Fusarium graminearum* isolate WC-091-7 (kindly provided by Dr. Lise Nistrup Jørgensen, Aarhus University). The macroconidia suspension was prepared using *F. graminearum* WC-091-7 agar plugs cultured in 20 ml liquid sporulation medium (Imholte and Schramm, 1968) for 7 days. Spores were collected by filtering the culture through autoclaved Miracloth® and the concentration was adjusted to 10^5 spores ml^{-1} using autoclaved MilliQ water + Tween 20 for inoculation. Control spikelets were sprayed with autoclaved MilliQ water + Tween 20. After spraying, spikelets were reintroduced to their respective wells. The plates were sealed and kept in the growth chamber at 19 °C and 16 h of light. In total, five replications (plates) were used in each experiment. Experiments were repeated at least four times for each endophyte isolate.

Visual assessment of symptoms was performed at 7 days after *Fusarium* inoculation. Spikelets were assessed under a stereoscope and each spikelet was given a value from the following scale: 0 = No visible symptoms of FHB; 1 = Small bleached or necrotic areas; 2 = General bleaching and large necrotic areas, small *Fusarium* mycelia growing on top of the tissue; 3 = General necrosis of spikelets, abundant mycelium on top of the spikelets (Supplementary Fig. 3). Disease severity calculation was modified from the method of Comby et al. (2017). Thus, instead of calculating the mean values for the spikelets in each well, the sum of the three values was calculated. This variable is termed '*aggregated disease severity'*. This was done in order to obtain a larger range for data dispersion before the statistical analysis. The 0–3 scale was thus changed to a 0–9 scale.

An additional experiment was performed using this system on promising candidates. Four candidates were evaluated for their efficacy to reduce FHB symptoms on detached spikelets when *Fusarium* macroconidia were sprayed at different times after endophyte inoculation. Spikelets were treated as mentioned above, but *Fusarium* macroconidia suspensions were inoculated at 0, 1, 2 and 3 days after endophyte inoculation. This experiment was repeated three times.

2.3. *Fusarium head blight greenhouse assay*

Spring wheat cv. Diskett was grown under greenhouse conditions until the flowering stage. Endophyte suspensions were prepared as described previously and individual spikes were sprayed using 100 ml spraying bottles at heading stage (BBCH 59), with approximately 1 ml per spike. Three to five days later, during full anthesis (BBCH 65), spikes were sprayed with a *F. graminearum* WC-091-7 macroconidial suspension (10^5 spores ml^{-1}), approximately 0.5 ml per spike. All control treatments were sprayed using autoclaved MilliQ water + Tween 20. Spikes were covered with a previously misted transparent plastic bag and sealed using adhesive tape around the straw. Bags were removed after 48 h. Severity of infection was assessed at 5 days after *Fusarium* inoculation by counting the number of spikelets with FHB symptoms out of the total number of spikelets in each spike. Seven replications were used in each experiment. Experiments were repeated at least four times for each isolate.

2.4. *qPCR quantification of fungal DNA in endophyte-treated spikes*

Spikes from the FHB greenhouse tests were collected five days after *Fusarium* inoculation. Six middle spikelets were removed and freeze dried for 48 h. DNA extraction was performed using the E.Z.N.A.® Plant DNA Kit Plant (Omega Bio-tek, Norcross, GA, USA). The specific primers Tri6-10F 5′-TCTTTGTGAGCGGACGGGACTTTA-3′ and Tri6-4R 5′-ATCTCGCATGTTATCCACCCTGCT-3′ were used to detect the *tri6* gene in *F. graminearum* (Horevaj et al., 2011). Specific primers to detect elongation factor gene *TEF1* in wheat: EF1α-F 5′-TCTCTGGGTTTGAGGGTGAC-3′ and 5′-GGCCCTTGTACCAGTCAAGGT-3′ (Nicolaisen et al., 2009), were used as plant DNA reference. Reactions of 20 µl were prepared using Brilliant III Ultra-Fast SYBR® Green qPCR Master Mix with Low ROX, 10 µl per reaction of master mix, 1 µl of each primer, 1 µl of template DNA and 7 µl of water. All reactions were prepared in triplicates. The thermocycler programme was 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles of 15 sec at 95 °C + 60 sec at 60 °C. A dissociation curve was elaborated from 60 to 95 °C for each primer to verify the specificity of the PCR reaction.

Standard curves for DNA quantification were built using six 10-fold dilutions starting from 50 ng of pure target DNA (wheat or *F. graminearum*). Reactions were run using AriaMx Real-Time PCR System G8830A (Agilent Technologies, Santa Clara, CA, United States). The ratio between the total amount of *F. graminearum* DNA and the total amount of *T. aestivum* DNA was calculated as the rate of infection for each treatment. Experiments were performed three times.

2.5. *In vitro dual-culture assay*

Endophyte isolates that showed consistent reduction in FHB disease levels were evaluated in direct confrontation assays against *Fusarium graminearum* isolate WC-091-7. Agar plugs from 7-day-old cultures of the pathogen were placed in the centre of a PDA plate. Endophyte isolates were inoculated on a transverse line on one side of the plate as it is shown in Supplementary Fig. 4. Plates were incubated at room temperature with normal day light conditions. *F. graminearum* radial growth towards the antagonist (Ra) and towards the edge of the plate (Rc) against each endophyte isolate was measured three times per day until pathogen and endophyte growth made contact. Five replications of each combination were cultured and the experiment was performed three times.

2.6. *Statistical analysis*

All statistical analyses were carried out in R environment version 3.5.2 (R Team 2018). Detached spikelet data were analysed using a generalized linear model specifying a Poisson distribution. FHB greenhouse assay data were analysed using a generalized linear model specifying a binomial distribution. All statistical comparisons were performed against FHB controls and were considered significant when $P \leq 0.05$. The *in vitro* dual culture data were analysed using the function 'compareGrowthCurves' from the *statmod* package in R (Giner and Smyth, 2016) to compare *F. graminearum* radial growth towards the antagonist (Ra) and towards the edge of the plate (Rc) for each endophyte isolate. The analysis uses a permutation test of the difference between two groups over time with 10,000 permutations and computes a *P*-value for each pair of groups. Such *P*-values were adjusted using Bonferroni corrections. *Fusarium* DNA quantification using qPCR was

Table 1
Fungal endophytes isolated from wheat spikes used for biocontrol screening.

Code	Isolation				Taxonomy[a]			Source
	Location	Medium[b]	Year	Tissue	Phylum	Genus	Closest BLAST hit	
ML38	Sweden	Malt Extract Agar	2016	Leaf	Ascomycota	*Acremonium*	*Acremonium alternatum* [c]	Latz et al 2020
F93G	Sweden	Malt Extract Agar	2016	Glume	Ascomycota	*Alternaria*	*Alternaria alternata*	This study
D103Lb	Denmark	Malt Extract Agar	2016	Lemma	Ascomycota	*Aureobasidium*	*Aureobasidium pullulans*	This study
A24L	Denmark	Malt Extract Agar	2016	Lemma	Ascomycota	*Cladosporium*	*Cladosporium herbarum*	This study
F33G	Sweden	Malt Extract Agar	2016	Glume	Ascomycota	*Cladosporium*	*Cladosporium macrocarpum*	This study
ML37	Sweden	Malt Extract Agar	2016	Leaf	Ascomycota	*Penicillium*	*Penicillium olsonii* [c]	Latz et al 2020
C113L	Denmark	Malt Extract Agar	2016	Lemma	Ascomycota	*Sarocladium*	*Sarocladium strictum* [c]	This study
F63P	Sweden	Malt Extract Agar	2016	Palea	Basidiomycota	*Anthracocystis*	*Anthracocystis flocculosa* [c]	This study
P1P1-S	Sweden	Potato Dextrose Agar	2017	Palea	Basidiomycota	*Anthracocystis*	*Anthracocystis flocculosa* [c]	This study
E103L	Sweden	Malt Extract Agar	2016	Lemma	Basidiomycota	*Anthracocystis*	*Anthracocystis grodzinskae*	This study
P12P1-S	Sweden	Potato Dextrose Agar	2017	Palea	Basidiomycota	*Cryptococcus*	*Cryptococcus stepposus*	This study
A103LB	Denmark	Malt Extract Agar	2016	Lemma	Basidiomycota	*Sporobolomyces*	*Sporobolomyces roseus*	This study
D63P	Denmark	Malt Extract Agar	2016	Palea	Basidiomycota	*Sporobolomyces*	*Sporobolomyces roseus*	This study
Y12K1-S	Sweden	Yeast Extract Agar	2017	Kernel	Basidiomycota	*Vishniacozyma*	*Vishniacozyma victoriae*	This study
A103La	Denmark	Malt Extract Agar	2016	Lemma	Basidiomycota	*Vishniacozyma*	*Vishniacozyma victoriae*	This study

[a] Identification based on ITS alignment against UNITE database version 7.2 and NCBI database.
[b] Medium concentration was modified from standard preparation see Materials and methods section.
[c] Identity was verified by alignment of multiple loci using actin, β-tubulin, and translation elongation factor 1-α genes, see Supplementary Table 1.

analysed using multiple regression analysis of the ratio between ng *F. graminearum* DNA and ng wheat DNA for each condition. All statistical comparisons were performed against FHB controls and were considered significant when $P \leq 0.05$.

3. Results

Endophyte isolation from healthy wheat spikes resulted in 163 fungal isolates, belonging to 20 different species. Isolated fungal communities were largely dominated by two genera, namely *Cladosporium* and *Alternaria.* These fungi represented around 90% of the total fungal abundance recovered. Other commonly found genera were *Fusarium*, *Epicoccum* and *Parastagonospora*. Among the less frequently isolated organisms were several yeast-like fungi from genera such as *Vishniacozyma*, *Anthracocystis* and *Sporobolomyces* (basidiomycetes). After taxonomic classification, reported wheat pathogens and reported human pathogens, as well as known toxic or allergenic species, were discarded. Thus, 13 of these isolates as well as two isolates with demonstrated potential to control Septoria tritici blotch were chosen for further screening. A full list of the tested isolates (location, isolation media and wheat tissue) is shown in Table 1.

F. graminearum WC-091-7 inoculation caused classical infection symptoms on detached spikelets in plates after 7 days. A four-level visual scale of increasing symptoms was used to assess the effect of fungal endophyte inoculation on *Fusarium* infection (Supplementary Fig. 3). Four endophyte treatments reduced the appearance of bleached and necrotic lesions on spikelets and diminished the growth of *Fusarium* mycelium over the spikelet (Fig. 1B) and thus reduced significantly *Fusarium* severity infection values (Fig. 1A). The identity of these isolates was further verified using alignment actin, β-tubulin, and translation elongation factor 1-α genes (Supplementary Table 1). There four isolates were identified as *Sarocladium strictum* C113L, *Anthracocystis flocculosa* P1P1, *Anthracocystis flocculosa* F63P and *Penicillium olsonii* ML37.

We selected these four isolates to test the effect of time between endophyte treatment and pathogen arrival on the biocontrol efficacy. Interestingly, none of the isolates reduced FHB symptoms when co-inoculated with the pathogen (0 dai). However, isolates *Sarocladium strictum* C113L and *Anthracocystis flocculosa* F63P significantly reduced FHB symptoms when applied at 2 dai and 3 dai before the pathogen, whereas isolates *A. flocculosa* P1P1 and *Penicillium olsonii* ML37 did so only with a 3 day interval (Fig. 2). A clear progression in the reduction of symptoms on detached spikelets was observed for all four candidate isolates.

The four isolates were tested further in whole plant experiments under greenhouse conditions. The FHB severity at 5 dai was significantly reduced when endophytes were inoculated onto the spikes at 3–5 days before anthesis (Fig. 3A). FHB severity reduction was similar for all isolates and ranged between 70% (*A. floculossa* P1P1 and F63P) and 80% (*S. strictum* C113L and *P. olsonii* ML37) (Fig. 3B). Additionally, three isolates that did not show any significant reduction in the detached spikelet assay (*Acremonium alternatum* ML38, *Sarocladium* sp. P12P1 and *Vishniacozyma victoriae* Y12K1) were tested to corroborate the accuracy of the detached spikelet assay selection. These isolates showed no significant reduction in FHB severity in wheat spikes and in some cases, increased FHB severity (Fig. 3A)

The amount of pathogen DNA in endophyte-treated spikes was estimated using qPCR. The ratio between *F. graminearum* DNA and wheat DNA was significantly reduced when spikes were treated with fungal isolates (Fig. 4). This reduction was around 70% for all isolates.

Finally, the four endophyte candidates were tested for their effect on *F. graminearum* growth *in vitro* in dual cultures. No significant effects were observed in *Fusarium* growth on the plates for any of the isolates after 7 days of co-cultivation (Table 2).

4. Discussion

Biological control solutions are in high demand for many pathosystems. They will supplement the set of tools available to control plant diseases and, in most cases, these innovative solution will be environmentally friendlier than traditional pesticides. However, care must be taken to avoid selecting unsuitable organisms, *e.g.* those capable of causing disease in other plants (cultivated or wild), humans and livestock. Fungal endophytes are wide-spread and closely associated with plants. They could represent a source of new potential biological control agents. Here, we show that naturally occurring fungal endophytes isolated from healthy wheat spikes in areas with high levels of FHB can reduce disease severity and pathogen growth inside wheat spikes. Similarly, we found that a detached spikelets assay (Comby et al., 2017) is a fast and reliable screening method for fungal biocontrol candidates.

In the past, several fungi isolated from cereal tissues have been tested as biocontrol agents against FHB. Isolates such as *Cryptococcus flavenses* OH182.9 (Khan et al., 2001) and *Clonostachys rosea* (Jensen et al., 2000) have shown positive results in reducing *Fusarium* symptoms in spikes and seedlings. Similarly, *Phoma glomerata*, *Aureobasidium proteae* and *Sarocladium kiliense* were observed reducing FHB symptoms

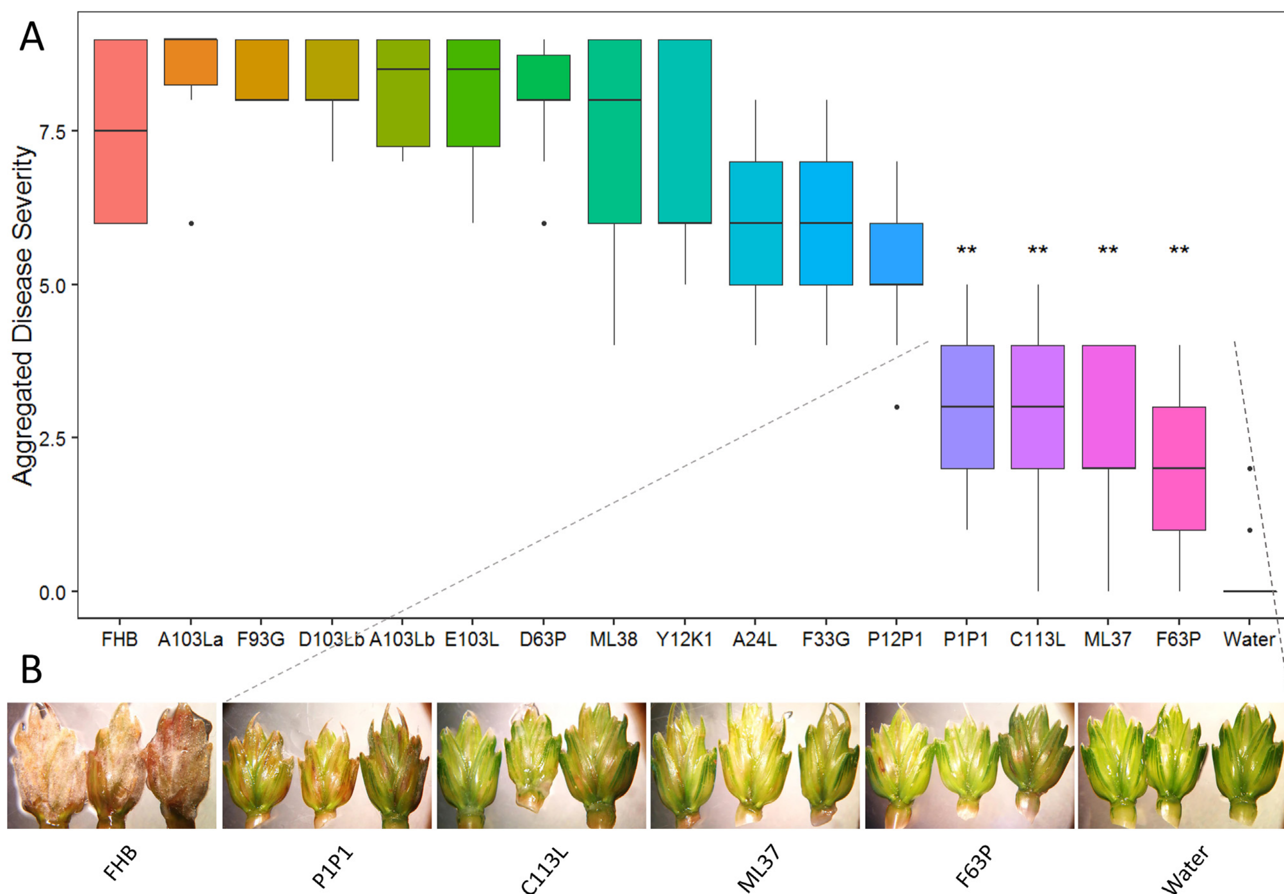

Fig. 1. Effect of endophyte isolates on development of symptoms caused by *Fusarium graminearum* on wheat spikelets. A). Aggregated disease severity (sum of severity of individual spikelets) of *Fusarium* infection on detached spikelets at 7 days after pathogen inoculation. Boxplot shows range and median severity for each treatment. Points below and up boxes are outlier data points. Significant differences ($P < 0.01$) compared to FHB control are denoted as '**'. B. Fusarium symptoms developed on detached spikelets sprayed with fungal isolates that significantly reduced FHB symptoms: *Sarocladium strictum* C113L, *Anthracocystis flocculosa* F63P and *A. flocculosa* P1P1 and *Penicillium olsonii* ML37.

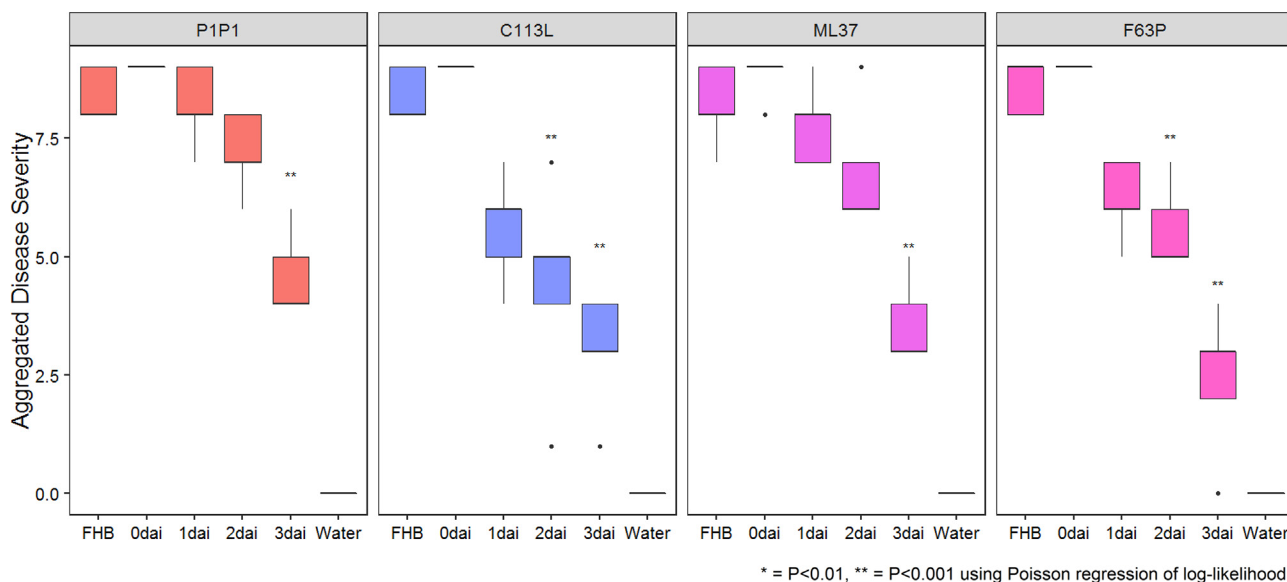

Fig. 2. Effect of endophyte isolates on *Fusarium* disease severity on detached spikelets at different intervals before pathogen inoculation (0, 1, 2 and 3 days after endophyte inoculation). Boxplot shows range and median of aggregated disease severity (sum of severity of individual spikelets) for each treatment. Points below and up boxes are outlier data points. Significant differences ($P < 0.01$) compared to FHB control are denoted as '**'.

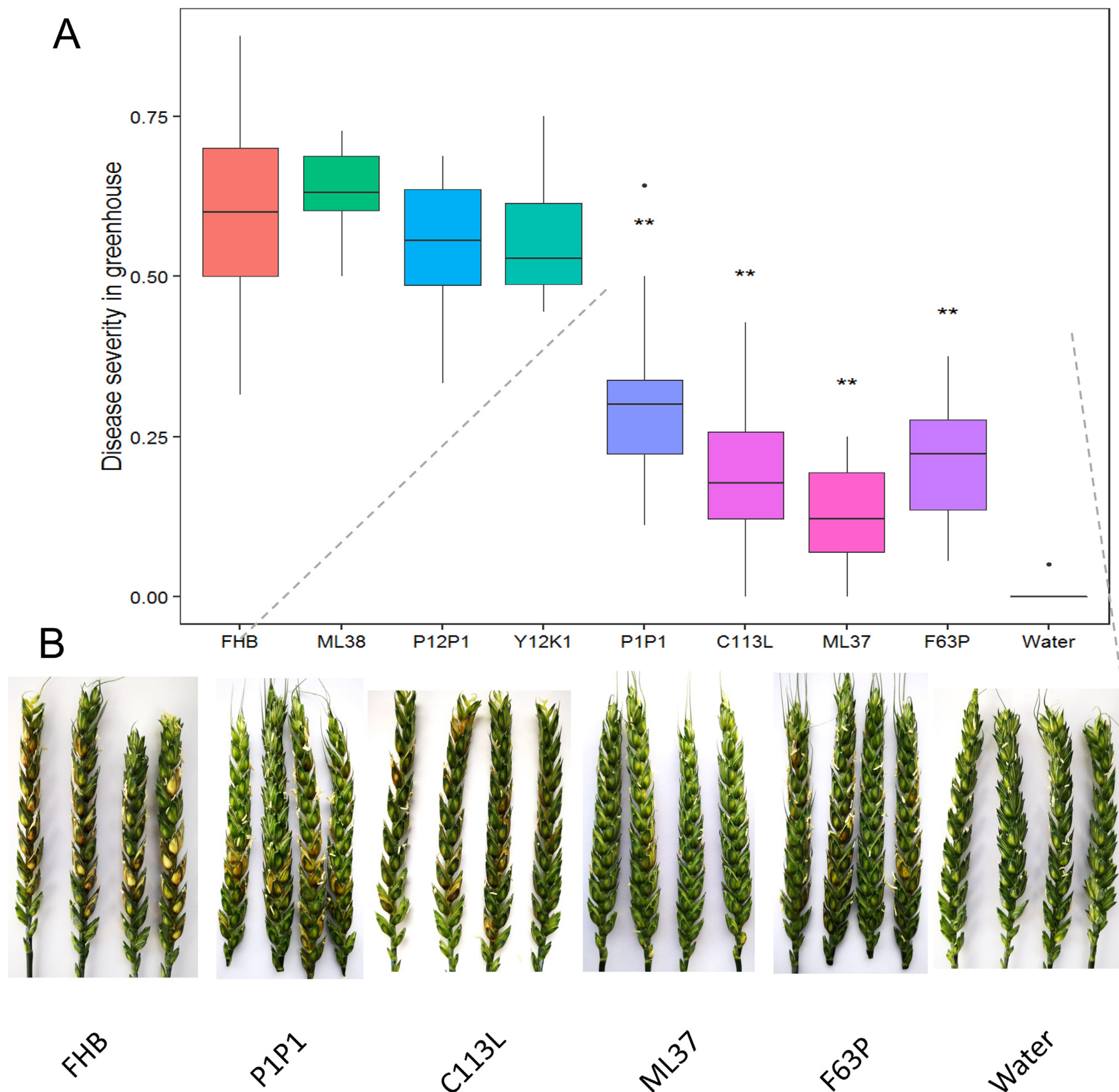

Fig. 3. Effect of endophyte isolates on FHB severity 5 days after pathogen inoculation during greenhouse assays. Spikes were sprayed with endophytes 3–4 days before pathogen was inoculated at anthesis. A. Boxplot shows range and median severity for each treatment. Points below and up boxes are outlier data points. Significant differences at $P < 0.01$ compared to FHB control are denoted as **. Seven isolates were tested and four candidates significantly reduced FHB severity (P-value < 0.01) on wheat spikes. B. FHB symptom reduction after treatment with endophytes. *Acremonium alternatum*. ML38, *Sarocladium* sp. P12P1, *Vishniacozyma victoriae* Y12K1, *Sarocladium strictum* C113L, *Anthracocystis flocculosa* F63P, *A. flocculosa* P1P1 and *Penicillium olsonii* ML37.

in detached spikelets (Comby et al., 2017). We identified four isolates: *Sarocladium strictum* C113L, *Anthracocystis flocculosa* F63P and *A. flocculosa* P1P1 and *Penicillium olsonii* ML37 with biocontrol effect against FHB. To our knowledge, this is the first report of these species as potential biocontrol agents in cereals against FHB

Sarocladium strictum (syn.: *Acremonium strictum*) is a well-known endophyte in Poaceae and has been isolated from wheat tissues in several studies (Sieber et al., 1988; Crous et al., 1995; Vujanovic et al., 2012; Comby et al., 2016). This species has been observed to reduce *in vitro* growth of *Helminthosporium solani* and suppressing the nematode *Meloidogyne incognita* in tomato (Goswami et al., 2008). Similarly, it has been reported to be a potential biocontrol agent against *Botrytis* in tomato (Gyung et al., 2009). Recently, Comby et al. (2017) identified *Sarocladium kiliense* as a potential biocontrol agent of FHB using detached spikelets.

Anthracocystis flocculosa (syn.: *Pseudozyma flocculosa*) has been reported as a plant epiphyte or saprophyte (Avis and Bélanger, 2002). It is known for its capacity to reduce disease levels of powdery mildews, including *Blumeria graminis* f.sp. *hordei* in barley, using a combination of antimicrobial substances and mycoparasitism (Laur et al., 2017). Interestingly, two of the potential biocontrol isolates in our study were identified as *Anthracocystis flocculosa* (isolates F63P and P1P1) and they showed similar levels of biocontrol capacity in both screening tests. These microorganisms were isolated from fields in Sweden, but in different years. This suggests that *Anthracocystis flocculosa* strains with biocontrol properties are established in the fungal communities within the agroecosystem.

On the other hand, *P. olsonii* ML37 was observed as a potential

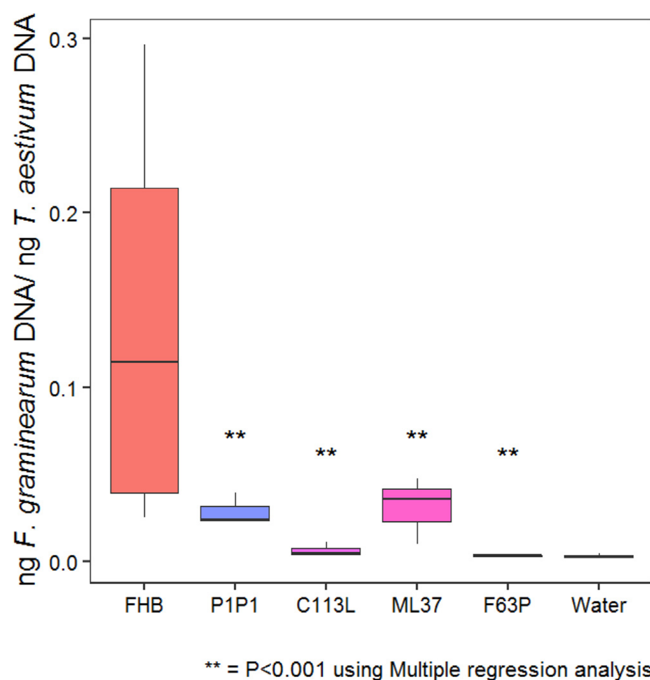

Fig. 4. Abundance of *Fusarium* DNA in wheat spikes pre-treated with endophyte isolates *Anthracocystis flocculosa* P1P1, *Sarocladium strictum* C113L, *A. flocculosa* F63P and *Penicillium olsonii* ML37 before *F. graminearum* inoculation. Samples harvested at 5 days after pathogen inoculation. Boxplot shows range and median of the ratio between *Fusarium* and wheat DNA for each treatment. Significant differences ($P < 0.01$) compared to FHB control are denoted as '**'.

Table 2
Comparison of *F. graminearum* radial growth towards the antagonist (Ra) and towards the edge of the plate (Rc) for each endophyte isolate.

Dual culture test	Difference in radial growth (cm)	P-value
F. graminearum vs C113L	−0.393	0.660
F. graminearum vs F63P	0.582	0.561
F. graminearum vs P1P1	−0.18	0.103
F. graminearum vs ML37	−0.331	0.561

biocontrol agent of *Zymoseptoria tritici* on wheat under controlled conditions and in the field (Latz et al., 2020). *Penicillium olsonii* has been described as a woody plant endophyte (Nicoletti et al., 2014) and recently, as causing post-harvest fruit rot in tomato (Anjum et al., 2018). It has been studied for its ability to degrade pectin and the production of polygalacturonases (Wagner et al., 2000). Other *Penicillium* species have been reported to be common colonisers of wheat tissues (Larran et al., 2007). Some non-pathogenic plant-associated species, such as *P. adametzioides, P. chrysogenum* and *P. citrinum* have shown the ability to reduce pathogen infection of root diseases and post-harvest losses and have been used as biocontrol agents (Ahmed et al., 2015; Waqas et al., 2015; Zheng et al., 2017). Here, we observed no direct inhibitory effect against *F. graminearum* growth.

Interestingly, the time between biocontrol inoculation and pathogen arrival had an effect on the observed biocontrol efficacy for all the isolates. Whereas isolates *Sarocladium strictum* C113L and *Anthracocystis flocculosa* F63P reduced FHB symptoms significantly after 2 days, isolates *A. flocculosa* P1P1 and *Penicillium olsonii* ML37 required 3 days (Fig. 3). This 'establishment' period and the natural variation between isolates could provide an advantage when delivering the inoculum in the field. It is well known that fungicide efficiency against FHB is limited due to application timing and limitations in coverage of the spikes during anthesis. Delivering the endophytes at the heading stage, and perhaps using mixtures of several isolates with different time responses, could provide a wider interval of protection to the spikes.

Despite promising results in identifying biocontrol agents, only a few discoveries have led to commercial products against FHB in wheat (Legrand et al., 2017). Indeed, field consistency remains the biggest challenge for biocontrol solutions (Parnell et al., 2016; Murphy et al., 2018). It has been suggested that endophytes could provide a more reliable field performance (Collinge et al., 2019; Silva et al., 2018). Their evolutionary history makes them natural competitors for the same biological niche. Furthermore, their endophytic life-style would make them less exposed to environmental factors and will provide a source of nutrients as they deliver their effect.

We have shown that potential candidates colonise wheat spikes and that two days of endophyte establishment are enough for a biocontrol effect to be observed. Similar results were obtained by Comby et al. (2017), who observed an increase in endophyte biomass in wheat spikelets treated with potential biocontrol agents as they reduced FHB symptoms. We observed that endophytes only provided their effect a few days after inoculation. This suggests an endophytic lifestyle and could represent a potential improvement in field performance.

Finally, the screening method to select potential biocontrol candidates has a direct influence on the expected mode of action of such promising microorganisms. Screening procedures often involve tests for direct competition *in vitro*, production of secondary metabolites, utilisation of specific carbon sources and antimicrobial enzyme activity. Such approaches are high throughput and successful in identifying promising microorganisms with specific features, like antibiotic-producing bacteria, but often there is no correspondence between these features and disease control *in planta*.

The mode of action of endophytic fungi in disease control is diverse. It could be direct through antibiosis, indirect via competition or plant-mediated defence activation (Latz et al., 2018). Microorganism selection using indirect approaches that do not involve the plant can lead to poor choices and inefficient use of microorganism collections. In this study, we observed no direct correlation between pathogen inhibition in plates and the capacity to reduce disease symptoms *in planta*. Moreover, there was a clear correlation between the performance in the detached-spikelet test and in greenhouse assay. The reduction in symptoms was associated with a reduction in fungal biomass, meaning that endophyte treatment has a direct effect on fungal activity and not only on the appearance of visible symptoms.

In vitro tests are high throughput, but, in essence, artificial and do not reproduce natural conditions. Using a classical dual-culture selection, the promising organisms of this study would have been discarded. We have used a detached-spikelet assay trying to replicate a more realistic scenario whilst maintaining high throughput and reproducibility. We were therefore able to test several isolates in a relatively short time and coupled this approach with a pre-selection for industrial properties such as no pathogenicity, high spore production and low safety risks (Köhl et al., 2011). It is recommendable to implement this type of *in planta* screening methods when searching for potential biocontrol agents as these could provide a greater array of modes of action (Latz et al., 2018).

In conclusion, naturally occurring fungal endophytes are a potential source of new biocontrol agents. We isolated fungi from healthy looking spikes in fields with high FHB incidence and identified four fungal isolates that were able to reduce FHB symptoms in wheat. This is the first report of naturally occurring biocontrol of FHB provided by *Sarocladium strictum*, *Anthracocystis floculossa* and *Penicillium olsonii*. The role of the plant microbiome in plant health has only recently gained interest. In order to exploit fully the diversity in biotechnological solutions for agriculture, we must refine our methods and rethink our understanding of plant–microbe interactions.

CRediT authorship contribution statement

Edward C. Rojas: Conceptualization, Methodology, Investigation,

Formal analysis, Writing - original draft. **Birgit Jensen:** Methodology, Writing - review & editing, Supervision. **Hans J.L. Jørgensen:** Methodology, Writing - review & editing, Supervision. **Meike A.C. Latz:** Resources, Writing - review & editing. **Pilar Esteban:** Investigation, Validation, Formal analysis. **Yuwei Ding:** Investigation, Validation, Formal analysis. **David B. Collinge:** Funding acquisition, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2020.104222.

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