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Bacillus megaterium shows potential for the biocontrol of septoria tritici blotch of wheat

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ABSTRACT

Control of septoria tritici blotch (STB) of wheat caused by the fungal pathogen *Mycosphaerella graminicola* is heavily reliant on fungicide application. Rapid emergence of fungal isolates resistant to Quoine outside Inhibitors (QoI) and with reduced sensitivity to triazole-based fungicides necessitates the development and adoption of alternative control strategies. A collection of bacteria originating from barley leaves and grain, oat chaff and wheat rhizospheres and leaves were screened for their ability to control STB. A total of seven bacteria were further assessed under controlled environmental conditions and these inhibited STB development by up to 92%. Three of these bacteria [*Pseudomonas fluorescens* (strains MKB21 and MKB91) and a *Bacillus megaterium* (strain MKB135)] were assessed for their abilities to control STB on adult wheat plants in small-scale field trials, conducted both in 2004 and 2006. In these trials only *B. megaterium* (strain MKB135) consistently retarded STB development (by up to 80%). Additional in vitro seedling studies showed that both *B. megaterium* cell wall components and its culture filtrate and *P. fluorescens* strain MKB91 culture filtrate were capable of inhibiting disease development (by 62, 36 and 52%, respectively). While none of the three bacteria directly inhibited fungal growth in dual culture plate assays, in dual liquid culture assays volatiles produced by these bacteria reduced fungal biomass production by $\geqslant$43%. Thus the ability of the most promising bacterium, *B. megaterium* strain MKB135, to reduce STB severity may be the result of a combination of different mechanisms.

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1. Introduction

Septoria tritici blotch (STB) is currently one of the most important foliar disease of wheat (*Triticum aestivum* L.) worldwide (Eyal, 1999; Hardwick et al., 2001; Bearchell et al., 2005). The causal pathogen *Mycosphaerella graminicola* (Fuckel) J Schroeter in Cohn (anamorph: Septoria tritici Roberge in Desmaz) attacks wheat leaves, causing necrotic blotches which can result in significant yield losses (Forrer and Zadoks, 1983). The increased incidence and severity of this disease over the last three decades has been attributed to several factors, including a lack of adequate disease resistance among the majority of commercial wheat cultivars, changes in wheat cultivation practices and environmental conditions, compounded by decreasing fungicide sensitivity among *M. graminicola* populations (Bayles, 1991; Polley and Thomas, 1991; Bearchell et al., 2005; Mavroeidi and Shaw, 2005; Fraaije et al., 2005, 2007; Zhan et al., 2006). In recent years, fungicides have been largely relied upon to reduce STB and associated yield losses, however the recent emergence of resistance/reduced sensitivity among *M. graminicola* populations to the Quoine outside Inhibitors (QoIs) and the DMI fungicides (Fraaije et al., 2005; Kildea et al., 2006; Leroux et al., 2007) highlights the urgent need for improved integrated control strategies. Such practices should incorporate cultural control measures, host resistance, chemical application and biological control.

Nolan and Cooke (2000) speculated that the slow infection process and the long latency period associated with *M. graminicola* pathogenesis of wheat may provide a window of time during which microorganisms, and/or their biochemical products might disrupt STB development. Previous studies by Levy et al. (1988, 1989, 1992) identified fluorescent pseudomonads capable of suppressing STB development on seedlings. Investigations revealed that the antifungal activity of *P. fluorescens* strains LEC1 and PFM2 observed was provided to an extent by antibiotics (1-hydroxyphenazine and chlororaphin, and 2,4-diacetylphoroglucinol, respectively), produced by the bacteria (Levy et al., 1989, 1992). The ability of some pseudomonads to produce hydrogen cyanide (HCN) also contribute to their antifungal capabilities and

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Flaishman et al. (1996) presented evidence that HCN produced by the bacterium *Pseudomonas putida* strain BK8661 can suppress STB development. Research in Argentina by Perellò et al. (2002) has identified a range of microorganisms isolated from the wheat phylloplane (*Paecilomyces lilacinus*, *Nigrospora sphaeric*, *Cryptococcus* sp. and *Bacillus* sp.) capable of hampering *M. graminicola* spore germination and STB development on wheat seedlings. Perellò et al. (2006) showed that a number of *Trichoderma* spp. isolated from different sources including wheat phylloplanes were able to control STB through out the growing season under field conditions, although levels of control were dependent on treatment type (seed dressing or foliar application) and wheat cultivar. Such examples offer hope that biological control of STB is achievable; however to the best of our knowledge no biological agents have yet been registered for the control of this disease.

An important prerequisite for any biocontrol agent is that it is adapted to the environment in which it is to be used. As part of a research project assessing alternatives to the use of chemical fungicides in Irish cereal systems this study focused on identifying possible biocontrol agents for STB disease control. The potential of 141 bacteria isolated from Irish cereal fields (rhizospheres, leaves, grains and weeds) to reduce STB development on both wheat seedlings and mature plants was evaluated. Three bacteria (two *Pseudomonas fluorescens* and a *Bacillus megaterium*) were assessed for their ability to inhibit STB disease development on adult plants in small-scale field trials. *Bacillus megaterium* strain MKB 135 showed most promise for disease control and its potential antifungal modes of action were investigated.

2. Materials and methods

2.1. Plant, fungal and bacterial material

The winter wheat (*Triticum aestivum* L.) cultivar (cv) Equinox, the spring wheat cvs Baldus and Raffles and the spring barley (*Hordeum vulgare* L.) cv Fractal (kindly supplied by Goldcrop Ltd., Cork, Ireland) were used in this study. Equinox, Baldus and Raffles are susceptible to STB under Irish climatic conditions (DAF, 2002, 2007).

Mycosphaerella graminicola (Fuckel) J Schroeter in Cohn strains THORN, BACK, ATHY (from the UCD phytopathogenic fungal collection) and 30T (kindly supplied by Eugene O'Sullivan, Teagasc Crops Research Centre, Oak Park, Carlow) were used in this research. Pycnidiospores of the fungal isolates were stored at −70 °C in 10% glycerol (vv^{-1}). Prior to use, isolates were sub-cultured twice on potato dextrose agar (PDA) (Oxide Ltd., UK) and grown at 20 °C under a 12 h dark/near ultraviolet light cycle for 7 days. Pycnidiospores were scraped from the surface of these cultures into a 0.2% Tween 20 sterile distilled water (SDW) solution and filtered through sterile cheesecloth before quantification using a glasstic® haemocytometer (Hycor Biomedical Inc., USA). Spore concentration was adjusted to 1×10^6 ml^{-1} using a 0.2% Tween 20 solution.

The 141 bacterial isolates used in this study were isolated from cereal rhizospheres, leaves, grain and weeds (taken from Irish cereal fields) in 2002, and were purified and morphologically characterized by Khan et al. (2006). Maintenance of bacteria, preparation of bacterial inoculum (log CFU ml^{-1} = 7.0–8.5) in Luria Bertani (LB) broth (Luria and Burrous, 1957) and of the culture filtrates of *P. fluorescens* strains MKB21 and MKB91 and *B. megaterium* strain MKB135 were as described by Khan et al. (2006). Potato dextrose broth (PDB; Oxoid, UK) cultures of *Bacillus megaterium* strain MKB135 in vacuum flasks were prepared using the LB culture conditions described by Khan et al. (2006) (24-h-old 30 ml cultures; log CFU ml^{-1} = 8.5).

Cell walls of *P. fluorescens* strains MKB21 and MKB91, and *B. megaterium* strain MKB135 were extracted according to Van Wees et al. (1997). Bacterial cells were propagated in LB cultures (incubated at 25 °C for 18 h and adjusted to a log CFU ml^{-1} of 8.5) by centrifugation at 4500g for 5 min. Cells were resuspended in 50 mM Tris–HCl plus 2 mM EDTA (pH 8.5), tubes were submerged in iced water and sonicated (Pulsatron KS400, Guyson International Ltd., UK) for 15 s at maximum resonance amplitude. Sonication was repeated eight times. The resulting suspension was centrifuged, firstly at 600g for 20 min to remove intact cells (pellet), and secondly at 8000g for 60 min to collect a cell wall fraction (pellet). Cell walls were resuspended in 10 mM phosphate-buffered saline (pH 7.2) and stored at −70 °C. Prior to use, the cell walls were pelleted by centrifuging (8000*g*, 60 min), washed and resuspended in LB (in a volume equivalent to that of the original LB culture from which walls originated).

2.2. DNA extraction and PCR analysis

The identity of bacteria showing promise as biocontrol agents was confirmed by analysis of one or more DNA sequences. DNA was extracted from the LB-cultured bacterial cells, as previously described (Sessitsch et al., 2003). 16S rDNA, internal transcribed spacer 1 fragment (ITS1), DNA gyrase submit B (gyrB) and RNA polymerase sigma factor (RpoD) gDNA sequences were amplified using primer pairs 0206F/0209R (Pichon et al., 2003), 16F945/23R458 (Guasp et al., 2000), UP-1E/AprU and 70F/70R (Yamamoto et al., 2000), respectively, and the amplification conditions described by the respective authors. PCR products were purified using QIAEX II Agarose Gel Extraction kit (Qiagen, Germany), sequenced (MGW biotech, Germany) and homology determined using the Blastn programme (Altschul et al., 1997) within the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/).

2.3. In vitro septoria tritici blotch seedling tests

All 141 bacteria underwent a preliminary screen to test their ability to control STB of wheat caused by the *M. graminicola* strain THORN. Seeds of the winter cv Equinox were germinated in the dark at 20 °C on moist Whatman No.1 filter paper (Whatman International Ltd., England) for 48 h. Germinating seeds were placed in 6 × 7 × 6 cm pots (2 per pot) containing John Innes Compost No.2 (Westland Horticulture, Co. Tyrone) and pots were placed in a controlled environment chamber (75% relative humidity under a 12 h day/night cycle at 20/12 °C, respectively). After three weeks growth, i.e. approximately growth stage (GS) 13 (Zadoks et al., 1974), plants were removed from the contained environment and the youngest leaf (3rd leaf) of the plants was inoculated until runoff (approximately 10 ml) with a *M. graminicola* pycnidiospore suspension (described above) or 0.2% Tween 20 (control plants), using a hand-held sprayer. The plants were returned to the controlled environment chamber; growth conditions were as above except relative humidity was adjusted to 95% to promote *M. graminicola* infection. Forty-eight hours post-fungal inoculation, plants were again removed, the third leaves inoculated to run-off (±10 ml) with a bacterial isolate (log CFU ml^{-1} = 7.0) or LB broth (control plants) (one isolate per pot) and then returned to the controlled environment. Treatments included: negative controls (0.2% Tween 20 + either LB or a bacterial isolate), positive controls (*M. graminicola* + LB) and *M. graminicola* + a bacterial isolate. Disease assessments were made on the third leaves 28 days post-fungal inoculation; disease was measured as the percentage of leaf area that was both diseased (i.e. blotched) and bore pycnidia. The pre-screen included 2 plants (1 pot) per bacterial treatment and the test was not repeated.

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