TESTING BACTERIAL AND FUNGAL ISOLATES FOR BIOLOGICAL CONTROL OF *FUSARIUM CULMORUM*

K.S.H. BOYD-WILSON, L.J. MAGEE,
J.K. HACKETT and M. WALTER

*The Horticulture and Food Research Institute of New Zealand Ltd., Canterbury
Agriculture and Science Centre, Lincoln, New Zealand*

ABSTRACT

Micro-organisms isolated from composts were assayed *in vitro* for inhibition of mycelial growth and conidium germination of *Fusarium culmorum*. Eleven bacterial isolates inhibited mycelial growth, and of these seven also inhibited germination of conidia. However, inhibition of mycelial growth was only reproducible for three bacteria. Five fungal isolates were found to have antagonistic effects on *F. culmorum* mycelial growth. Of these five, three isolates gave reproducible results and also suppressed germination of *F. culmorum* conidia. An oat seedling bioassay was developed to determine the potential of microbial antagonists for use as seed treatments against *F. culmorum* in soil inoculated with the pathogen. Three bacterial isolates were selected for methodology development for *in vivo* evaluations. The effect of *F. culmorum* inoculation technique, growth substrate and assessment method were investigated.

Keywords: *Fusarium culmorum*, biological control, *in vitro* testing, bioassay.

INTRODUCTION

Fusarium culmorum (W.G. Smith) Sacc. infects a wide range of host plants and is an ubiquitous soilborne pathogen with preference for temperate environmental conditions (Gordon 1960). This fungus causes pre-emergence seedling blight, root and foot rot and head blight in cereals, brown patch of turf, foot rot of asparagus, carnation, leek and pea, and storage rots of apple, potato, sugar beet and bulbs (Booth and Waterson 1964). In cereals, *F. culmorum* can cause disease from sowing through to harvest, especially in dry, warm soils. It is the most damaging pathogen of wheat, causing roots to darken and dry, with plants easily broken off at ground level (Wiese 1977).

Crop rotation, pathogen-free seed, removal of plant debris and fungicide seed treatments are several management strategies that can be used for control of *Fusarium* diseases. More recently, research has been directed towards developing biological control methods for these diseases (Hervas *et al.* 1998; Calistru *et al.* 1997). In greenhouse experiments, wheat seedlings have been protected from root diseases by seed treatment with fungal antagonists from the genera *Trichoderma*, *Fusarium*, *Aspergillus*, *Nigrospora*, *Penicillium* and *Chaetomium* (Teperi *et al.* 1998). Suppressive soils can give biological control of *Fusarium* diseases (Alabouvette 1999) and composts have been found to suppress *Fusarium* spp. (Hoitink *et al.* 1993, Filippi and Pera 1987).

The objective of this research was to isolate micro-organisms from disease suppressive composts (Walter *et al.* 1995) for *in vitro* evaluation of their ability to inhibit mycelial growth and conidium germination of *F. culmorum*. A glasshouse bioassay was developed to assess selected antagonists for their potential to control *F. culmorum* when applied as seed treatments.

MATERIALS AND METHODS

Bacteria and fungi were isolated onto Difco Potato Dextrose Agar (PDA) from five *Fusarium* disease-suppressive composts (M. Walter, unpubl. data) using the dilution plating technique of Wakelin *et al.* (1998). In total, 112 bacterial and 29 fungal strains were isolated and stored at 4°C on PDA slopes.

Three *F. culmorum* isolates were used; Fus15 and Fus16b were from cereal hosts and PR305 was from tomato. Single conidium isolates were prepared (Windels 1992) and stored on carnation leaf agar slopes (Burgess *et al.* 1988) at 4°C. In mycelial inhibition assays, the isolates were grown for 1 week on oatmeal agar (OA) at 20°C with a photoperiod of 12 h. In all assays, plates were incubated under these conditions.

Mycelium inhibition assays

To test for inhibition of mycelial growth of *F. culmorum*, each bacterial isolate was spot-inoculated at four equidistant points around the edges of three PDA plates containing a centrally placed 7 mm mycelial plug (mycelium side down) from one of the three *F. culmorum* isolates. Plates were assessed after 6 days using a scale from 1-4 where 1=bacterial colony completely overgrown by *Fusarium*, 2=overgrown but bacterial colony visible, 3=grown to the edge of the bacterial colony and 4=inhibition zone.

Each fungal antagonist was tested against the three *F. culmorum* isolates for mycelial inhibition. Seven millimetre mycelial plugs (mycelium side down) from both an antagonist and a *F. culmorum* isolate, were placed on opposite sides of one OA and one PDA plate. Plates were assessed after 14 days using a scale from 1-5 where 1=antagonist completely overgrown by *F. culmorum*, 2=antagonist growing over *Fusarium* with *F. culmorum* mycelium collapsed, 3=mycelia intergrowing, 4=mycelia grown up to each other and stopped and 5=inhibition zone. Experiments were repeated for the most antagonistic bacterial and fungal isolates to evaluate reproducibility (referred to as Test 1 and Test 2).

Conidium germination inhibition assays

Bacterial and fungal isolates found to inhibit mycelial growth of *F. culmorum* were further tested for their ability to reduce colony formation from *F. culmorum* conidia using a germination inhibition assay. Sterile distilled water containing 0.01% Tween 20 (Tween water) was poured over 2-week-old sporulating plates of *F. culmorum* grown on OA at room temperature (18-25°C) under a light bank (TLD 58W/33 Philips, 12 h photoperiod). Conidia were harvested with a glass rod then the solution was filtered into a centrifuge tube using a sterile funnel lined with sterile lens tissue (Whatman 105). The test tubes were centrifuged at 1500 g for 2 min. The supernatant was decanted, centrifuge tubes filled with Tween water, shaken and centrifuged again. This washing procedure was repeated twice. Numbers of conidia in each *Fusarium* suspension were adjusted after haemocytometer counts to reach a final conidium concentration of 10^3 and 10^4 conidia/ml. Conidium suspensions of the three *Fusarium* isolates were then mixed at a ratio of 1:1:1.

Bacteria affecting mycelial growth of *F. culmorum* were selected and grown overnight in 10 ml potato dextrose broth (Difco) on an orbital shaker (120 rpm) at room temperature. A 10-fold dilution series (to 10^{-6}) was prepared for each isolate in sterile Tween water. For enumeration, three 10 ml droplets of each bacterial dilution were pipetted onto a PDA plate. After 1-2 days bacterial colony forming units (CFU) were counted.

For germination inhibition, three 10 ml droplets of each bacterial dilution were pipetted onto three PDA plates and allowed to dry. On one plate bacterial droplets were overlaid with 5 ml of the *F. culmorum* mix containing 10^3 conidia/ml; on the second plate with 5 μ l of the *F. culmorum* mix containing 10^4 conidia/ml and on the third plate with 5 μ l Tween water (bacterial control). *Fusarium culmorum* controls were set up by applying a series of 10 μ l Tween water droplets onto PDA plates, allowing them to dry and then overlaying the droplets with 5 μ l of each concentration of the *F. culmorum* mix as well as the three single *F. culmorum* isolate suspensions at both concentrations. Plates were assessed after 4 days for the presence of *F. culmorum* mycelium using a scale from 1-5 where 1=no *Fusarium* mycelium visible within the

bacterial droplet, 2=*Fusarium* mycelium visible within the droplet, 3=*Fusarium* mycelium visible outside the droplet (sparse), 4=*Fusarium* mycelium visible outside the droplet (medium) and 5=*Fusarium* mycelium visible outside the droplet (vigorous).

Fungal antagonists affecting *F. culmorum* mycelial growth were further evaluated in the germination inhibition assay by preparing conidium suspensions of 10^8 , 10^7 , 10^6 , 10^5 and 10^4 conidia/ml from three week old test fungi (antagonists). Five 10 ml droplets of each antagonist conidium suspension were pipetted onto two PDA plates for each dilution and allowed to dry. The droplets were overlaid with a 5 ml droplet of the *F. culmorum* inoculum as described for the bacterial germination inhibition assay. Antagonist controls consisted of five droplets of each antagonist conidium suspension overlaid with Tween water. *Fusarium culmorum* controls consisted of Tween water droplets overlaid with the *F. culmorum* conidium suspensions. Plates were assessed after 4 days for *F. culmorum* mycelium using a scale from 1-5 where 1=no *Fusarium* mycelium visible; 2=sparse *Fusarium* mycelial growth; 3=medium *Fusarium* mycelial growth; 4=vigorous *Fusarium* mycelial growth and 5=*Fusarium* covering the plate. Experiments were only conducted once.

Glasshouse bioassay

Three bacterial isolates were selected for methodology development for *in vivo* evaluations. The effect of *F. culmorum* inoculum concentration, growth substrate and assessment method were investigated.

Bacterial isolates were inoculated onto PDA and grown for 10 days at 20°C at 12 h photoperiod. To each plate 10 ml of sterile potassium phosphate buffer were added and the surface of the plate was rubbed with a glass rod to harvest the bacteria. The bacterial suspensions were centrifuged for 20 min at 16 000 g and the pellet re-suspended in potassium phosphate buffer. Bacteria were enumerated as described above by dilution plating.

Mechanically dressed organic-grown oat seeds (80) were surface sterilised in a 0.3% solution of sodium hypochlorite solution (pH 6.5) for 5 min, thoroughly rinsed in tap water, left to dry and then placed into the test bacterial suspensions amended with 0.1 g of methyl-cellulose (BDH) and mixed for 10 min. The seeds dried overnight in a laminar-flow cabinet. The control treatment consisted of a seed-coating with phosphate buffer amended with methyl-cellulose. Bacterial loading on the seeds was determined by washing seeds in phosphate buffer and dilution plate counts of the washing solutions.

F. culmorum soil inoculum was prepared by soaking oat seeds (volume of approximately 100 ml) overnight in water, autoclaving the seeds and inoculating with 20 ml *F. culmorum* (Fus16b) conidium suspension ($>10^6$ conidia/ml). The seeds were incubated for 1 week and used as soil inoculum. Conidium loading on seeds was determined by washing seeds in phosphate buffer and haemocytometer counts of the washing solutions.

Two glasshouse bioassays were conducted using one (non-sterile soil) or three growth substrates (standard potting mix, non-sterile soil and sterile soil). The soil (Wakanui silt loam) was collected from Lincoln University field research area and sterilised by autoclaving.

Polythene planter bags (PB 1½) were filled with the growth substrates. The effects of *F. culmorum* inoculation technique and inoculum density on seedling infection were evaluated in non-sterile soil. The first inoculation method consisted of placing one inoculum seed at a depth of 25-30 mm, covering this with 1-3 mm soil and planting the surface sterilised oat seed on top. The other methods consisted of mixing zero, four, eight or 16 inoculum oat seeds into the soil and planting oat seeds at a depth of 25-30 mm. For each treatment two planter bags were used and nine surface sterilised oat seeds were planted in each bag.

In experiments evaluating growth substrate and bacterial seed coating on *F. culmorum* disease development, the first inoculation method was used. Nine surface sterilised seeds were planted into two to four replicate bags/treatment. Controls consisted of autoclaved inoculum seeds plus oat seeds coated with methyl-

cellulose (nil control) and inoculum seeds plus oat seeds coated with methyl-cellulose only (*Fusarium culmorum* control) for each growth substrate.

In all experiments planter bags were examined weekly for seedling emergence, browning of leaf tips and wilting of seedlings. After 6 weeks the seedlings were removed. The subcoronal internode and approximately 20 mm of stem base of each seedling was removed, surface sterilised in 1% sodium hypochlorite solution for 30 s, rinsed in sterile water, dried, cut in half and plated onto PDA. The plates were assessed after 1 week for the presence of *Fusarium*.

Data was analysed using analysis of variance. Assessment methods were compared using Pearson's Correlation Coefficient.

RESULTS

Mycelial inhibition

After 6 days *F. culmorum* controls completely covered the plates. Eleven bacterial isolates (Code nos 18, 22, 26, 28, 33, 34, 62, 76, 89, 94 and 100) from 120 tested were found to inhibit (score 4) mycelium growth of at least one *F. culmorum* isolate in at least one of the duplicate tests. Of these, three (isolate nos 28, 89 and 100) inhibited all *F. culmorum* isolates in both tests and two bacteria (isolate nos 33 and 62) inhibited two of the *F. culmorum* isolates consistently in both tests.

After 14 days incubation *F. culmorum* and antagonist controls completely covered PDA and OA plates, with all fungal colonies sporulating profusely. Five fungal isolates (nos 113, 114, 120, 140 and 141) were found to have an antagonistic effect (score 2 or 5) on *F. culmorum* mycelial growth on both agar substrates in Test 1. Of these five, only three (isolate nos 113, 114 and 120) showed reproducible effects (score 2) in Test 2, with the fungal antagonist isolates 140 and 141 producing inhibition zones (score 5) in Test 1 only. Inhibition of *F. culmorum* mycelial growth by antagonistic fungi was affected by agar type ($P < 0.05$).

Germination inhibition

In all *Fusarium culmorum* controls, *F. culmorum* colonies were formed from each droplet. Of the eleven bacterial isolates showing some effect on *F. culmorum* mycelial growth, nine isolates (nos 18, 22, 26, 28, 62, 76, 89, 94 and 100) also affected *F. culmorum* germination (score 1 or 2) in at least one of the duplicate experiments. *Fusarium culmorum* conidium concentration and bacterial concentration affected *F. culmorum* germination ($P < 0.05$). Bacterial inhibition of *F. culmorum* conidium germination was different between tests, with higher bacterial concentrations required to inhibit *F. culmorum* germination in Test 2. Only bacterial isolate 89 consistently suppressed *F. culmorum* conidia germination at both pathogen concentrations in both tests.

In tests for the effects of fungal antagonists on *F. culmorum* conidium germination, all antagonists and *Fusarium culmorum* controls formed colonies covering plate surfaces within 4 days. Of the five fungal antagonist isolates affecting *F. culmorum* mycelial growth, three isolates (nos 113, 114 and 120) also affected *F. culmorum* conidium germination (score 1), with fungal isolate 120 requiring the least conidia to suppress *F. culmorum* germination at the two concentrations tested. Both *F. culmorum* conidium concentration and fungal antagonist concentration affected ($P < 0.05$) *F. culmorum* germination.

In summary, from the *in vitro* assays on *F. culmorum* mycelial growth and germination inhibition, two bacterial isolates (nos 89 and 62) consistently inhibited *F. culmorum* mycelial growth and spore germination. Similarly, three antagonistic fungi (isolate nos 113, 114 and 120) consistently affected *F. culmorum* mycelial growth and spore germination. Three bacterial isolates (62, 89 and 100) were selected for development of plant bioassays. Best performing bacterial and fungal isolates are currently identified to species level but this information is confidential.

Glasshouse bioassays

Average *F. culmorum* concentration on oat seeds used as inoculum was 1.9×10^6 conidia/seed. The different soil inoculation methods used for *F. culmorum* did not affect ($P > 0.05$) oat seedling emergence ($> 80\%$). There was no difference ($P > 0.05$)

between inoculation methods on *F. culmorum* disease development as assessed by browning of seedling tips, however increasing the amount of seed inoculum mixed into the soil increased wilting ($P < 0.05$). The number of dead plants was highest (38%) in pots where inoculum seed and oat seed were planted together compared to pots where inoculum seeds were mixed into the non-sterile soil ($P < 0.05$). Colonisation of subcoronal internode and stem base by *F. culmorum* was not affected ($P < 0.05$) by the *F. culmorum* inoculation method.

Growth substrate had no effect on seedling emergence ($P > 0.05$). However, seedling emergence was different ($P = 0.05$) between uninoculated (nil control) and *F. culmorum* inoculated (*F. culmorum* control) growth substrates, averaging 78% and 58% respectively. In contrast to the observed seedling emergence effect, there were no differences ($P > 0.05$) between the two control treatments for browning of tips, wilting, and colonisation of the subcoronal internodes and stem bases of seedlings. No colonisation of subcoronal internodes and stem bases, wilting or browning of tips of seedlings were observed in the nil controls of the sterile soil.

Whilst the type of growth substrate did not affect seedling emergence, it affected browning of tips, wilting, and plant tissue colonisation of oat seedlings ($P < 0.01$), with increased wilting of seedlings grown in non-sterile soil. Seedlings grown in potting mix expressed approximately four times as much browning of tips than seedlings grown in non-sterile or sterile soil. Seedlings grown in sterile soil had the highest colonisation of *F. culmorum* on the subcoronal internodes and stem bases.

The average concentration of the bacterial isolate nos 62, 89 and 100 on coated oat seeds at the planting date was approximately 1.2×10^6 CFU/seed. Compared to control treatments, bacterial seed coating did not reduce *F. culmorum* colonisation of the subcoronal internodes and stem bases, wilting or browning of tips of seedlings ($P > 0.05$), nor did the bacterial seed coating improve seedling emergence ($P > 0.05$).

Comparison of assessment methods resulted in significant correlations between wilting and browning of tips of seedlings ($r = -0.51$; $P = 0.001$); wilting and colonisation of subcoronal internodes and stem bases ($r = 0.31$; $P < 0.05$); as well as browning of tips of seedlings and colonisation of subcoronal internodes and stem bases ($r = 0.3$; $P < 0.05$).

DISCUSSION

In vitro mycelial and germination inhibition assays were useful screening techniques for assessing potential antagonist activity against three *F. culmorum* isolates. However, the germination inhibition assay did not assess conidium germination of *F. culmorum* isolates directly, but determined their ability to establish colonies from conidia when challenged with bacteria or conidia from fungal antagonists. The duplicate tests also gave a good indication on the reproducibility of results which is essential for assessment of any potential biological control agent. Although the three *F. culmorum* isolates did not differ in pathogenicity to oat seedlings, bacterial and fungal activity inhibiting mycelial growth of *F. culmorum* isolates was isolate-specific. This was expected, as *Fusarium* species are known to be highly variable (Windels 1992) and therefore a mix of isolates was used in this study.

For further evaluation of bacterial antagonists a glasshouse assay was developed to aid selection prior to field evaluations. A wide range of inoculation techniques and disease evaluations have been published and partially reviewed by Windels (1992). *In situ*, biological control agents need to be able to overcome or function within diverse soil parameters such as soil type, disease pressure, temperature and moisture. An assay mimicking 'natural' conditions and a simple assessment method, which also could be used in field trials, was desired. Pathogen inoculum placed close to seeds was found to provide a higher, more even disease pressure than mixing inoculum into the growth substrate. For pathogen inoculum, infested seeds were chosen. This resembles a natural source of infection, providing both mycelial and conidial inoculum.

In this study, growth substrate influenced *F. culmorum* infection of oat seedlings. This is in agreement with Bell *et al.* (2000) who found that growth medium and inoculation method of the biological control agent affected seedling emergence.

Burgess *et al.* (1988) also reported on soil parameters influencing severity of disease caused by *F. culmorum*. This emphasises the need to test potential biological control agents under different environmental and soil conditions.

There was evidence of the presence of *F. culmorum* in the non-sterile soil and potting mix. Seedling disease occurred in all growth substrates for all treatments, except for the nil control treatment of sterilised soil. *Fusarium* species are widespread with saprophytic and pathogenic strains. Assessments did not allow for differentiation of *Fusarium* species. This highlights the need for careful selection of growth substrate and monitoring of 'background' disease levels. Sterilisation of soils by pasteurisation, fumigation or autoclaving usually allows the pathogen to proliferate (Burgess *et al.* 1988). Some soils may be toxic after sterilisation (Armstrong and Armstrong 1975), but the healthy seedlings in the nil control of the sterile soil treatment suggest that autoclaving did not result in phytotoxicity.

Bacterial seed coating did not reduce disease under the conditions tested. Disease pressure may have been too high, the antagonist inoculum level too low, antagonist application inefficient (Bell *et al.* 2000) and/or infection might have been more severe in dry conditions (plants were watered sparingly) (Booth 1971). However, these experiments were not designed to evaluate the interactions between disease pressure and antagonist application method and concentration but to design a simple glasshouse bioassay suitable for future studies.

Symptoms used to evaluate *F. culmorum* wilt are diverse and often controversial. For example, Armstrong and Armstrong (1975) recommended that external wilt symptoms are the best criteria for evaluation, whereas Swanson and Van Gundy (1985) recommended assessments of vascular cross sections of roots. In the present study, all assessments were suitable for disease measurement as shown by their high correlations with each other.

In future studies evaluating biological control agents for *F. culmorum* control on cereals in glasshouse and field assays, we recommend the use of seedling emergence combined with wilting assessments. This will allow the design of larger experiments with more replicates compared to the time-consuming task of root harvesting, washing, surface sterilisation, dissection and final assessments on agar.

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