EVALUATION OF MICRO-ORGANISMS FOR BIOCONTROL OF GREY MOULD ON LETTUCE

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ABSTRACT

A range of micro-organisms, including saprophytic yeasts, bacteria and fungi, were evaluated for control of *Botrytis* grey mould on whole lettuce plants. In a controlled environment (18–22°C, 85–90% relative humidity and 12 h photoperiod), lettuce leaves were spray inoculated with antagonists (10⁷ fungal spores/cells/ml or 10⁸ bacterial cells/ml), allowed to dry and challenged by spray inoculation with the pathogen (10⁶ spores/ml). After 7 days, the mean *Botrytis* lesion areas were assessed. Four fluorescent *Pseudomonas* spp. (isolates LC8, PF13, PF14 and PF15) significantly reduced lesion areas by an average of 79% on whole lettuce plants in two experiments. An *Ulocladium* sp. (U13) significantly reduced lesion areas by 94% in one of two experiments.

Keywords: *Botrytis cinerea*, *Epiccocum*, *Ulocladium*, *Pseudomonas*, antagonist.

INTRODUCTION

The causal agent of grey mould, *Botrytis cinerea* Pers., is a ubiquitous necrotrophic pathogen and can infect almost every plant part on a wide range of plants worldwide (Rosslenbroich & Stuebler 2000). In New Zealand, the fungus can cause significant economic losses on grape, strawberry, tomato, lettuce and kiwifruit. Current control relies heavily on the use of chemical fungicides but due to the pathogen's highly variable heterokaryotic nature, there is a high risk of resistance developing (Fletcher 1984). Fungicides used to control the pathogen are rapidly losing their efficacy since resistant strains are now widespread throughout the world, including New Zealand. This coupled with the current public concern over pesticide residues on foodstuffs and the expanding demand for organically grown vegetables and fruits, highlights the need for an alternative to chemical control. Biological control of this ubiquitous fungal pathogen has been shown to be effective in many crops (Elad et al. 1996) and can offer an attractive alternative to chemical control.

In vitro biological control screens are generally poor predictors of the performance of micro-organisms in the field (Andrews 1992) and, therefore, the objective of this study was to develop a standardised, efficient screen that would evaluate the biocontrol potential of micro-organisms in the whole plant environment. Lettuce was chosen as the model system since *B. cinerea* can cause significant loss to the crop both pre and post harvest.

METHODS

Test micro-organisms

There were two separate trials of different antagonistic micro-organisms. Each trial consisted of two identical experiments, the second experiment being carried out ten days after the first one. Each experiment was a randomised complete block design, with 14 treatments in Trial 1 and 10 treatments in Trial 2. All treatments were replicated four

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times. In all experiments the pathogen only control was sprayed with aqueous Tween 80 solution (1 drop per 1000 ml) instead of antagonist or fungicide.

In Trial 1 seven fungal antagonists and three bacterial antagonists with known biocontrol activity against other fungal pathogens were selected from the Lincoln University culture collection. These were *Cladosporium* spp. (ER3 and YNA) and *Pseudomonas marginalis* (GA8-PS4) from *Camellia japonica* flowers (R. van Toor, unpubl. data), *Trichoderma harzianum* (C52) and *T. longipile* (6sr4) from lettuce (Rabeendran et al. 1998), *Ulocladium* spp. (U13 and U16) and *Epicoccum* sp. (E21) from grape leaf (Stewart et al. 1998), *Bacillus subtilis* (PT69) and *Paenibacillus polymyxa* (18-25) from soil (Wakelin 2001). The performance of the selected micro-organisms was compared to two commercial biocontrol products and a chemical fungicide, recommended for control of *Botrytis*. The commercial product Trichodex (provided by Makhteshim Agan Chemical works Ltd., Be'er Sheva, Israel), containing over 5×10^8 Colony Forming Units (CFU)/g of *T. harzianum* T39, was applied at the recommended rate of 4 g/litre. The commercial product Prestop (provided by Kemira Agro Oy, Porkkalankatu, Helsinki, Finland), containing over 10^7 CFU/g of *Gliocladium catenulatum*, was applied at the recommended rate of 10 g/litre. The fungicide iprodione was applied at the recommended rate of 0.25 g ai/litre (Rovral FLO 15 ml/litre).

The second trial (Trial 2) used one unidentified white yeast (PK10), three unidentified pink yeasts (Y44, Y46 and Y48), three unidentified fluorescent *Pseudomonas* spp. (PF13, PF14 and PF15) isolated from strawberry fruit and *P. fluorescens* (LC8) isolated from *C. japonica* flowers (R. van Toor, unpubl. data).

Inoculum production

Botrytis cinerea was isolated from an infected commercial lettuce crop (Marshlands, Christchurch) and maintained on potato dextrose agar (PDA, Oxoid) at 20°C. Inoculum was produced in the same way as the antagonistic fungi.

Filamentous fungi, yeasts and bacteria were stored on PDA, malt extract agar (MEA, Difco) and nutrient agar (NA, Difco) respectively at -4°C. Filamentous fungi were cultured on PDA at 20°C for 10-29 days and yeasts for 3 days on MEA plates. Sporulation of the fungi was initiated by cultivating under fluorescent light with a 12 h photoperiod regime. All fungal spore suspensions were prepared by flooding individual cultures with 10 ml aqueous Tween 80 solution (1 drop in 1000 ml sterile water) and scraping gently with a sterile loop. The resulting crude suspension was filtered through a layer of Miracloth (Calbiochem) to remove mycelial fragments. Bacteria were cultured in 50 ml of nutrient broth (Difco) in 250 ml Erlenmeyer flasks at room temperature on an orbital shaker at 160 rpm for 24 h. Cells were collected by centrifugation for 10 min at 1000 x g and re-suspended in aqueous Tween-80 solution (1 drop in 1000 ml sterile water). Inoculum concentration was estimated using a haemocytometer and adjusted to 1×10^6 conidia/ml for *B. cinerea*, 1×10^7 spores/ml for other fungi and 1×10^8 cells/ml for bacteria.

Treatment and assessment of plants

Plastic pots (2 litre) were filled with potting mix consisting of 80% composted pine bark, 20% WAP5 washed crusher dust and Osmocote Plus fertiliser at 5 kg/m². Lettuce seeds of the Marksman type (Yates, Australia), a variety of iceberg lettuce partially resistant to powdery mildew, were planted four to a pot with a coverage of no more than 5 mm potting mix. They were grown in a glasshouse with temperatures of 15-25°C (monitored using a Tinytag temperature data logger), with irrigation as needed, but without additional lighting. After three weeks, they were thinned to one per pot and transferred to a controlled environment (18-22°C, 85-90% relative humidity and 12 h photoperiod) for 3 weeks to adapt to these conditions.

At the start of the experiments, small wounds (3 mm diameter) were made on the centre lower section of three medium sized leaves per lettuce plant (1 wound per leaf) using an electric powered soldering iron. Freshly wounded leaves were sprayed with a suspension of antagonist, aqueous Tween 80 solution (pathogen only control) or fungicide

till near run off (leaves were uniformly covered) using a handheld sprayer. After 10 min, a suspension of *B. cinerea* was applied to each plant, 1 spray per wound, using the handheld sprayer. Disease progress was assessed after 7 days by measuring the length and width of disease lesions (mm) spreading from the wound site using a digital calliper. Total lesion area was calculated and analysis of variance (ANOVA) was used to determine differences between means. Fishers Least Significant Difference (LSD) test was used to rank treatments in order of effectiveness.

RESULTS

Trial 1

In experiment 1, iprodione and *Ulocladium* (U13) significantly reduced lesion development compared to the pathogen-only control ($P < 0.05$). These treatments were not different from each other ($P > 0.05$). Percentage reduction in lesion area was 95%, 94% and 78% respectively (Table 1). Other isolates, namely *P. polymyxa* (18-25), *Ulocladium* (U16), *Trichoderma* spp. (C52 and 6sr4) and the commercial product Prestop gave percentage reductions in lesion area ranging from 56% to 74% but these were not significantly different from the pathogen control (Table 1). In experiment 2, no treatment gave a significant reduction in lesion development compared to the pathogen-only control although *Epicoccum* (E21) and *Ulocladium* (U13) again gave the greatest reduction in lesion area of 78% and 41% respectively (Table 1).

TABLE 1: The size of *B. cinerea* lesions (mm²) on lettuce after treatment with various micro-organisms. Results are presented for both experiments in Trial 1.

Treatment	Experiment 1	Experiment 2
Pathogen control	1990 a ¹	514 a ¹
<i>Pseudomonas marginalis</i> (Ga8-PS4)	1420 a	424 a
<i>Paenibacillus polymyxa</i> (18-25)	520 a	750 a
<i>B. subtilis</i> (PT69)	1500 a	873 a
<i>Epicoccum</i> (E21)	441 a	115 a
<i>Ulocladium</i> (U13)	122 b	303 a
<i>Ulocladium</i> (U16)	665 a	387 a
<i>T. harzianum</i> (C52)	875 a	620 a
<i>T. longipile</i> (6sr4)	692 a	1090 a
<i>Cladosporium</i> (ER3)	761 a	1308 a
<i>Cladosporium</i> (YNA)	1340 a	855 a
Trichodex	1180 a	1070 a
Prestop	824 a	459 a
Iprodione	93 b	498 a

¹Means in a column followed by the same letter are not significantly different at $P < 0.05$ according to Fishers Least Significance Difference test.

Trial 2

Four isolates of fluorescent *Pseudomonas* (LC8, PF13, PF14 and PF15) gave a significant reduction in *Botrytis* lesion area compared to the pathogen control in both experiments. Lesion areas were reduced by 60% to 84% in experiment 1 and by 62% to 96% in experiment 2 (Table 2). Isolate PF13 showed greatest suppression with lesion areas ranging from 159 to 513 mm² which was equivalent to a lesion area reduction of 76% and 96%, respectively compared to the pathogen control (Table 2).

TABLE 2: The size of *B. cinerea* lesions (mm²) on lettuce after treatment with various micro-organisms. Results are presented for both experiments in Trial 2.

Treatment	Experiment 1	Experiment 2
Pathogen control	2120 a ¹	3810 a
Unidentified yeast (PK10)	1110 a	4010 a
Unidentified yeast (Y44)	1560 a	2830 a
Unidentified yeast (Y46)	1720 a	2620 a
Unidentified yeast (Y48)	1740 a	3890 a
<i>T. harzianum</i> (C52)	1430 a	3990 a
<i>P. fluorescens</i> (LC8)	601 b	371 b
Fluorescent <i>Pseudomonas</i> (PF13)	512 b	159 b
Fluorescent <i>Pseudomonas</i> (PF14)	349 b	1430 b
Fluorescent <i>Pseudomonas</i> (PF15)	845 b	222 b

¹Means in a column followed by the same letter are not significantly different at $P < 0.05$ according to Fishers Least Significance Difference test.

DISCUSSION AND CONCLUSIONS

This study evaluated 18 micro-organisms, two commercial biocontrol products and a fungicide for the suppression of *B. cinerea* lesions on whole lettuce plants in a controlled environment. Four fluorescent *Pseudomonas* spp. significantly reduced *Botrytis* lesion development compared to the pathogen only control. Fluorescent *Pseudomonas* spp. have been shown to antagonise a wide range of fungal and bacterial pathogens on a wide range of plants. For example, an isolate marketed as BlightBan has been commercialised for the control of bacterial pathogens on aerial plant surfaces (Wilson 1997). Another *P. fluorescens* isolate (PB92BB10E) was found to suppress *B. cinerea* on whole petunia plants by an average of 77% across seven different trials (Gould et al. 1996). Swadling & Jeffries (1996) identified a strain of *P. fluorescens* that was equally or more effective than the fungicide dichlofluanid for controlling grey mould in a strawberry field trial. Past research has shown that antagonistic activity of fluorescent *Pseudomonas* spp. is based on fungistatic and/or germicidal activity (Swadling & Jeffries 1998; Gould et al. 1996).

The *Epicoccum* sp. (E21) reduced lesion areas by an average of 78% in both experiments compared to the pathogen only control, although this difference was not statistically significant. An *Epicoccum* sp. has previously been shown to suppress *B. cinerea* on bean (Hannusch & Boland 1996). It was found that biocontrol efficacy was independent of the atmospheric environment when evaluated at temperatures ranging from 20–28°C and relative humidities ranging from 90–100%. Under these conditions, the isolate reduced grey mould by 100%. Mechanisms of action were attributed to competition for nutrients or antibiosis (Hannusch & Boland 1996).

Ulocladium (U13) reduced lesion area significantly in experiment 1 but not experiment 2 of Trial 1. An *Ulocladium atrum* isolate suppressed the sporulation of *B. cinerea* on strawberry, onion, cyclamen and geranium (Kohl & Fokkema 1998) by competitively excluding the pathogen from colonising senescent tissue. Both the *Epicoccum* and *Ulocladium* isolates used in this study had already been shown to suppress *B. cinerea* infection and sporulation on sterilised grape rachis (Fowler et al. 1999). The species identities of the most successful isolates from this study are not yet known.

In experiment 1 of Trial 2, the results were confounded by an unusually low infection level in the pathogen control. This could have been due to the *B. cinerea* isolate losing its aggressiveness after frequent propagation onto PDA. Prior to the second experiment, the pathogen was re-isolated back from diseased lettuce leaves in order to maintain its aggressiveness.

It is concluded that *Epicoccum* (E21), *Ulocladium* (U13) and the four fluorescent *Pseudomonas* spp (LC8, PF13, PF14 and PF15) offer the most potential for the antagonism of *B. cinerea*. These isolates will now be assessed for the control of *B. cinerea* on whole plants in a large-scale field trial. Research will also aim to identify the mode of action of the most successful isolates as this information may help to optimise their biocontrol efficiency in the field.

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