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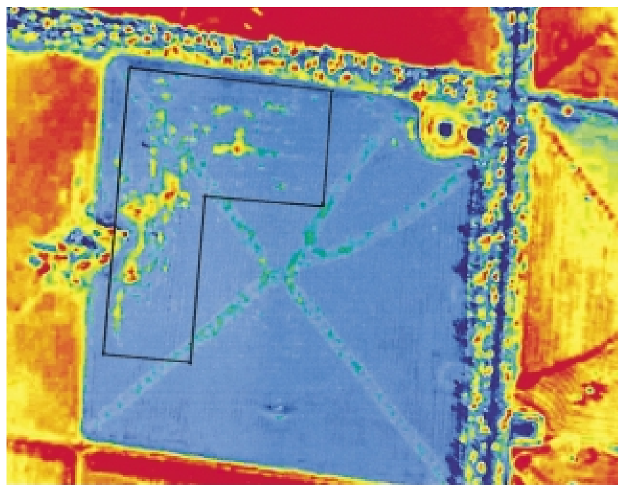

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Saprophytic microorganisms with potential for biological control of *Botrytis cinerea* on Geraldton waxflower flowers

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Abstract. Saprophytic bacteria, yeasts and filamentous fungi were isolated from Geraldton waxflower flowers and screened to identify potential antagonism towards *Botrytis cinerea*. Isolates from other sources (e.g. avocado) were also tested. Isolates were initially screened *in vitro* for inhibition of *B. cinerea* conidial germination, germ tube elongation and mycelial growth. The most antagonistic bacteria, yeasts and fungi were selected for further testing on detached waxflower flowers. Conidia of the pathogen were mixed with conidia or cells of the selected antagonists, co-inoculated onto waxflower flowers, and the flowers were sealed in glass jars and incubated at 20°C. The number of days required for the pathogen to cause flower abscission was determined. The most antagonistic bacterial isolate, *Pseudomonas* sp. 677, significantly reduced conidial germination and retarded germ tube elongation of *B. cinerea*. None of the yeast or fungal isolates tested was found to significantly reduce conidial germination or retard germ tube elongation, but several significantly inhibited growth of *B. cinerea*. *Fusarium* sp., *Epicoccum* sp. and *Trichoderma* spp. were the most antagonistic of these isolates. Of the isolates tested on waxflower, *Pseudomonas* sp. 677 was highly antagonistic towards *B. cinerea* and delayed waxflower abscission by about 3 days. *Trichoderma harzianum* also significantly delayed flower abscission. However, as with most of the fungal antagonists used, inoculation of waxflower flowers with this isolate resulted in unsightly mycelial growth.

Introduction

Geraldton waxflower (*Chamelaucium uncinatum* Schauer) is one of Australia's most economically important cut flower exports (Seaton *et al*. 1993) and is increasingly important in the international cut flower industry (Joyce 1988). It is grown extensively in Australia and accounts for about 50% of the value of native flower exports (Joyce and Wearing 1996).

Postharvest bud and flower abscission is one of the major problems experienced during transport, handling and marketing of waxflower (Joyce 1993). Abscission is often caused by wound-induced endogenous ethylene production brought about by infection of flower tissues by fungal pathogens (Joyce 1993). *Botrytis cinerea* Pers.:Fr. has been implicated as the primary fungal pathogen responsible for wound-induced endogenous ethylene production (Tomas *et al*. 1995).

Present chemical control measures are somewhat inefficient, since many or all floral stages from the shiny bud through to pink hypanthium may be present on the same plant at any one time (Olley *et al*. 1996). Accordingly, developing buds may not receive adequate protection against subsequent challenge by fungal pathogens, unlike open flowers where exposed reproductive structures are more likely to receive fungicidal treatment. In addition, the development of fungicide resistance has resulted in the exacerbation of many diseases. Strains of *B. cinerea* that are resistant to benomyl have been isolated from waxflower (Taylor *et al*. 1996). Moreover, there is significant public unease over the use of pesticides and their effect on the environment, which has led to consumer resistance (El-Ghaouth *et al*. 1992; Prusky and Keen 1993).

Biological control offers an alternative to chemical control. Bacterial, yeast and fungal antagonists can provide excellent control of *B. cinerea*. Success has been reported for a range of crops including grapes and strawberries (Elad 1994; Sutton 1995). *Pseudomonas* sp., *Aureobasidium pullulans* (de Bary) G. Arnaud and *Gliocladium roseum* Bainier are examples of antagonistic microorganisms that can inhibit germination and growth of *B. cinerea* (Janisiewicz and Roitman 1988; Elad *et al*. 1994; Sutton *et al*. 1997).

Saprophytic microorganisms can inhibit *B. cinerea* through various mechanisms. They may, for example,

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compete aggressively for space and nutrients (Blakeman 1993). Carbohydrates, amino and organic acids, mineral elements and leaf waxes found on the aerial surfaces of plants provide nutrition for saprophytes (Blakeman and Atkinson 1981). Augmentation of saprophyte populations depletes available exogenous nutrients and, thereby, inhibits germination and subsequent growth and development of pathogens, such as *B. cinerea* (Brodie and Blakeman 1975). Other mechanisms of biological control include direct parasitism, production of antibiotics and/or the induction of host plant resistance (Sutton *et al*. 1997).

Performance of microbial antagonists *in vitro* can be poorly correlated with their performance in the field. Janisiewicz (1987) isolated >800 potential microbial antagonists from apple trees and screened them for antagonism towards the blue mould pathogen *Penicillium expansum* Link. While many of the isolates performed well *in vitro*, they did not inhibit decay on apple fruit. Nonetheless, *in vitro* studies involving inhibition of conidial germination and mycelial growth are still useful as a screening procedure for selection of potential antagonists.

Saprophytic microorganisms including bacteria, yeasts and filamentous fungi were isolated from the surface of waxflower flowers and screened for potential antagonism against *B. cinerea*. It was hypothesised that effective antagonists would inhibit pathogen-induced abscission of harvested waxflower flowers from their pedicels. Tests were conducted on harvested waxflowers (*in planta*) as well as *in vitro*. Inhibition of *in vitro* conidial germination and mycelial growth of *B. cinerea* by bacteria, yeasts and fungi were measured in addition to effects on flower abscission.

Materials and methods

Flower materials

Flowering waxflower cv. 'CWA Pink' stems were harvested at 0800 hours from plants growing on Ebonybrook farm near Gatton (27°33′S,152°17′E), south-east Queensland. They were transported about 1 h by road, standing upright in a container of distilled water, to the laboratory in Brisbane.

Isolation of potential antagonists

Flowers were cut from their stems, weighed in groups of 10 (about 1 g) and washed in plastic bags containing 20 mL of phosphate buffered saline (PBS, pH 7.2) and 0.02% (v/v) Tween-80 for 90 s using a Stomacher Lab-Blender 80 (Stirling, 1995). Washed samples were serially diluted in PBS to provide a 4-fold dilution series. Ten replicates of 10 individual flowers per replicate were used.

Aliquots (100 μL) of each dilution were placed in the centre of each separate 90 mm agar plate and spread using a sterile glass spreader. All cultures were incubated at 25°C in darkness.

Bacteria were grown on half-strength tryptic soy agar (half TSA; i.e. the normal concentration of tryptic soy broth was halved). The agar therefore consisted of 15 g tryptic soy broth, 18 g agar and 1 L distilled water. Cycloheximide (75 mg/mL) was added to the half TSA (593 μg/mL agar) to inhibit fungal growth. Yeasts were grown on malt yeast extract agar (MYE). MYE consisted of 3 g yeast extract, 3 g malt extract, 5 g bacto peptone, 10 g D-glucose, 20 g agar and 1 L distilled water. The MYE medium was adjusted to pH 3.8 by the addition of HCL to acidify the medium thereby inhibiting fungal growth. Filamentous fungi were grown on half-strength streptomycin potato dextrose agar (half SPDA; i.e. the normal concentrations of potato extract and D-glucose were halved). Half PDA consisted of 2 g potato extract, 10 g D-glucose, 15 g agar and 1 L distilled water. Where appropriate, streptomycin sulfate (10 mg/mL) was added to the half SPDA (50 μg/mL agar) to inhibit bacterial growth.

Isolates were subcultured within a week to obtain pure cultures of the various bacterial, yeast and fungal isolates. Single spore cultures of fungal isolates were obtained and then agar discs bearing the organisms were stored under sterile water. Fungal isolates from waxflower were identified to genus level, by observing conidia under the light microscope, according to the descriptions of Barnett (1960).

Besides microbes from waxflower flowers, the following microbial agents were evaluated against *B. cinerea*: *Talaromyces* sp. from Dr Lindy Coates, DPI, Brisbane; *Trichoderma harzianum* Rifai from Dr Andrew Rath, Abbott Laboratories, Sydney; *Gliocladium roseum* from Dr John Alcorn, DPI, Brisbane; and, *Bacillus* spp. (9 isolates), *Enterobacter* sp., *Pseudomonas* sp., *Aureobasidum* spp. (3 isolates), an Actinomycete, 2 white yeasts and 3 pink yeasts from Dr Marcelle Stirling, Biological Crop Protection, Brisbane.

Effect on Botrytis cinerea *conidial germination and growth*

Botrytis cinerea (BRIP 25227) and saprophytic fungal isolates were grown on half SPDA at 25°C under alternating 12 h near-UV light. Bacterial isolates were grown on half TSA at 25°C in darkness for 1–2 days before all experiments. Yeast isolates were grown on MYE at 25°C in darkness for 3–4 days before use. Conidia of *B. cinerea* and fungal isolates were obtained by washing 14–21-day-old colonies growing on half SPDA with sterile distilled water and dislodging the conidia with a sterile glass rod. The conidia were filtered through 2 layers of sterile gauze. The resultant conidial suspension was adjusted to a concentration of 5×10^5 conidia/mL using a haemocytometer. Bacteria and yeasts were removed from culture and placed in sterile distilled water using a sterile inoculating loop. The concentration of yeasts and bacteria was standardised to 0.8 absorbance unit at 600 nm using a Varian DMS 100S spectrophotometer.

The *B. cinerea* conidial suspension was mixed with the conidial or cell suspension of fungal, bacterial or yeast isolates in a ratio of 0.5 mL to 0.5 mL. Aliquots (100 μL) of the suspensions were placed on water agar in Petri dishes by means of a micropipette and spread evenly with a sterile glass rod. Conidial germination and germ tube length were determined after the dishes were incubated for 24 h at 25°C in darkness.

Germination percentage was determined by counting the number of germinated and non-germinated conidia in a field of view at ×100 magnification (about 30 conidia). Two replicate Petri dishes and 5 samples (i.e. 5 fields of view) per Petri dish were used. Conidia were considered germinated when the germ tube was longer than the diameter of the conidia. Germ tube length was measured on 10 randomly selected *B. cinerea* conidia on each of 2 replicate Petri dishes.

To determine the effect of potential antagonists on mycelial growth of *B. cinerea*, a 5 mm diameter agar disc from a 7-day-old *B. cinerea* culture was placed at the centre of a 90 mm diameter Petri dish containing half PDA. Agar discs from saprophytic fungal cultures were placed onto the medium near opposite edges of the Petri dish, about 40 mm from the *B. cinerea* plug (Fig. 1). Bacteria and yeasts were spread over an area of 5 mm diameter on the agar with a sterile inoculating loop. Two replicate half PDA plates were used for each microorganism. Inhibition of fungal growth was assessed by measuring the inhibition zone (mm) and colony diameter (mm) (Fig. 1) after incubation at 25°C for 7 days in darkness. By this time, *B. cinerea* in the control plates had reached the edge of the plate. Bacteria or yeast isolates that did not produce an inhibition zone but caused the fungus to grow sparsely were described as non-zonal competitors.

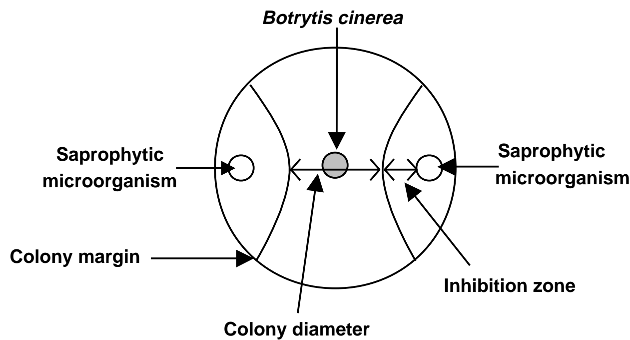

Figure 1. Relative position of *Botrytis cinerea* and potential microbial antagonists on half PDA plates used in mycelial growth inhibition tests.

Effect on flower abscission

Necrotic bracteoles clinging to fully open waxflower flowers were removed. Flowers with green, nectiferous hypanthiums were selected, detached from the stems, surface sterilised in 0.15% NaOCl (sodium hypochlorite) for 60 s, rinsed in sterile distilled water, and dried on blotting paper to remove surface moisture. Individual flowers were then placed in plastic stands within sterile 125 mL glass jars containing 10 mL sterile distilled water (Fig. 2). Each flower was inoculated with *B. cinerea* (BRIP 25227) alone or in combination with a fungal, yeast or bacterial antagonist.

The specific treatments were as follows: 1, control; 2, inoculation with *B. cinerea*; 3, *Bacillus* sp. 480; 4, *Bacillus* sp. 480 and *B. cinerea*; 5, *Pseudomonas* sp. 677; 6, *Pseudomonas* sp. 677 and *B. cinerea*; 7, control; 8, inoculation with *B. cinerea*; 9, *Aureobasidium* sp. 274; 10, *Aureobasidium* sp. 274 and *B. cinerea*; 11, *Aureobasidium* sp. 468; 12, *Aureobasidium* sp. 468 and *B. cinerea*; 13, control; 14, inoculation with *B. cinerea*; 15, *Epicoccum* sp.; 16, *Epicoccum* sp. and *B. cinerea*; 17, *Fusarium* sp.; 18, *Fusarium* sp. and *B. cinerea*; 19, *Trichoderma harzianum*; and 20, *Trichoderma harzianum* and *B. cinerea*.

The *B. cinerea* conidial suspension was mixed with the conidial or cell suspension of the fungal, bacterial or yeast isolates in a ratio of 0.5 mL to 0.5 mL. The resultant suspension was applied by placement of 2.5 µL aliquots on the base of all 5 petals as well as onto the stigma (Fig. 2). The flowers were enclosed inside glass jars and incubated in the dark at 20°C for 14 days, or until the flowers abscised. Glass jars containing the waxflowers were dropped (impact energy = 0.245 J) each day from a height of 20 mm to determine the number of days to flower abscission. Ten replicate flowers were used for each treatment.

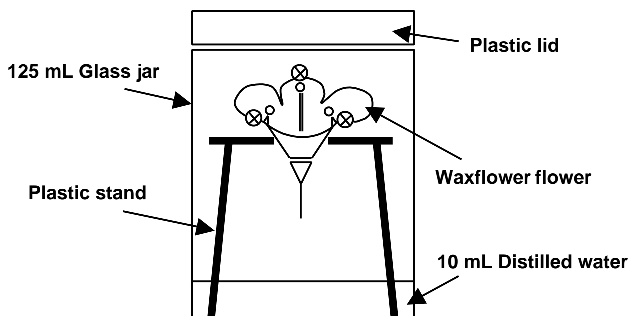

Figure 2. Diagrammatic representation of glass jars used to incubate Geraldton waxflowers after inoculation with *Botrytis cinerea*. The circles enclosing crosses mark the inoculation points.

Experimental design and statistical analyses

Effects of antagonistic bacteria, yeasts and fungi on conidial germination, germ tube elongation, mycelial inhibition and colony diameter of *B. cinerea* were evaluated using a 1-way ANOVA (Genstat 5, Release 4.1). Treatment effects on the number of days to flower abscission were evaluated using a 2-way ANOVA (isolate × inoculation). Means were separated using the least significant difference test ($P = 0.05$).

Results

Isolates from waxflower flowers

Six different bacteria, 2 yeasts and 9 filamentous fungi were isolated from waxflower flowers. The majority of the bacterial isolates appeared similar in terms of culture characteristics to the *Bacillus* sp. isolates obtained from Dr Marcelle Stirling, Biological Crop Protection, Brisbane. One pink and 1 white yeast were isolated from waxflower, but neither was identified at genus level. A diverse range of filamentous fungi were isolated, these being *Alternaria* sp., *Aspergillus* sp., *Cladosporium* sp., *Epicoccum* sp., *Fusarium* sp., *Nigrospora* sp., *Penicillium* sp., *Pestalotiopsis* sp., *Phoma* sp. and *Trichoderma* sp.

Effect of isolates on conidial germination and growth of Botrytis cinerea

Overall, *Pseudomonas* sp. 677 was the most antagonistic bacterial isolate (Table 1) and significantly reduced conidial germination and germ tube elongation of *B. cinerea*. *Bacillus* sp. 480 and bacterial isolates 2 and 3 also reduced conidial germination. Bacterial isolates 2 and 3 significantly affected germ tube length, but *Bacillus* sp. 480 did not. Conversely, *Bacillus* sp. 75 significantly reduced germ tube length, but did not affect conidial germination. The most antagonistic bacteria in terms of mycelial inhibition were *Bacillus* spp., especially isolates 480 and 553 and bacterial isolate 2. These produced inhibition zones greater than 10 mm. Neither *Bacillus* sp. 933 nor bacterial isolate 4 produced large inhibition zones. However, they both competed with *B. cinerea* by rapidly growing outwards from the edge of the agar plate (non-zonal inhibition). Neither of these isolates was overgrown by the pathogen, even when incubated for a further 2 weeks.

None of the yeasts tested significantly reduced either *B. cinerea* conidial germination or germ tube length (data not shown). The yeasts did not greatly inhibit *B. cinerea* mycelial growth. However, *Aureobasidium* sp. 140, 274 and 468, as well as pink yeast 373, all produced small inhibition zones and significantly reduced *B. cinerea* mycelial growth (Table 2).

Conidial germination and germ tube elongation were not significantly reduced by any of the fungal isolates tested (data not shown). Mycelial growth of *B. cinerea* was suppressed substantially by *Fusarium* sp. and *Nigrospora* sp.

Table 1. Relative ability of bacterial isolates to inhibit *Botrytis cinerea* conidial germination and germ tube elongation after incubation for 24 h at 25°C on water agar and mycelial growth after incubation for 7 days at 25°C on half-strength potato dextrose agar

Total number of observations for germination percentage was 10 (2 replications × 5 samples) and germ tube length was 20 (2 replications × 10 samples)
Means within each column followed by the same letter are not significantly different at $P = 0.05$
Actinomycete 202, *Bacillus* spp., *Enterobacter* sp., and *Pseudomonas* sp. were obtained from Dr Marcelle Stirling, Biological Crop Protection; all other bacteria were isolated from waxflower
Significance: germination %, $P = 0.018$; germ tube length, $P = 0.003$; inhibition zone, $P<0.001$; colony diameter, $P<0.001$

Isolate	Germination (%)	Germ tube length (μm)	Inhibition zone (mm)	Colony diameter (mm)
No. of replicates (*n*)	2	2	2	2
Control	96.4ab	184.0a	0h	90.0a
Actinomycete 202	98.4a	159.0ab	6.5de	51.0cd
Bacillus sp. 75	96.2ab	105.0bcd	4.0efg	60.5b
Bacillus sp. 78	91.2abc	127.0abc	0.0h	90.0a
Bacillus sp. 301	91.9abc	128.5ab	6.0def	48.5cde
Bacillus sp. 359	85.0abcde	140.0ab	0h	90.0a
Bacillus sp. 480	73.0de	129.5ab	16.8a	28.0g
Bacillus sp. 544	89.4abc	141.0ab	7.0d	43.5ef
Bacillus sp. 553	92.0abc	158.5ab	12.0b	40.5f
Bacillus sp. 638	87.5abcd	137.0ab	8.3cd	46.5cde
Bacillus sp. 933	97.0ab	137.5ab	1.5gh	60.5b
Enterobacter sp. 632	81.8bcde	70.0cde	2.8g	60.0b
Pseudomonas sp. 677	71.2e	31.5e	3.0g	60.5b
Bacterial isolate 1	82.1bcde	137.0ab	8.3cd	45.5def
Bacterial isolate 2	80.2cde	111.0bc	10.5bc	40.0f
Bacterial isolate 3	70.7e	53.0de	0h	90.0a
Bacterial isolate 4	92.3abc	147.0ab	3.3fg	52.0c

and moderately by *Pestalotiopsis* sp., *Talaromyces* sp. and *Gliocladium roseum*. *Epicoccum* sp. produced the largest inhibition zone (Table 3). Several other fungi sporulated profusely, including *Aspergillus* sp., *Cladosporium* sp. and *Penicillium* sp. They grew sporadically over the agar plate making assessment of *B. cinerea* growth difficult. Both *Trichoderma* sp. isolates also significantly inhibited growth of *B. cinerea*. While neither isolate produced an inhibition zone, each grew rapidly and markedly reduced mycelial growth (Table 3).

Effect of potential antagonists on flower abscission

Several bacterial (*Bacillus* sp. and *Pseudomonas* sp.), yeast (*Aureobasidium* spp.) and fungal (*Epicoccum* sp., *Fusarium* sp. and *Trichoderma* sp.) isolates were tested *in vivo*. These isolates had no significant effect on the number of days to flower abscission when inoculated alone (Table 4). *Botrytis cinerea* significantly reduced the number of days to flower abscission in all 3 experiments. *Pseudomonas* sp. 677 was the most antagonistic microorganism. This bacterium significantly delayed flower abscission by 3 days when co-inoculated with *B. cinerea* compared with inoculation with *B. cinerea* only. Likewise, *Trichoderma harzianum* significantly delayed waxflower flower abscission. However, *Aureobasidium* sp. 274 and 468 had no effect of waxflower flower abscission (Table 4).

Discussion

The result achieved with *Pseudomonas* sp. 677 warrants its further investigation as a potential biological control agent for preharvest or postharvest application against *B. cinerea* on waxflower. Although *Bacillus* sp. 480 and isolates of *Aureobasidium* sp. also performed well *in vitro*, they did not significantly delay waxflower flower abscission. It was anticipated that yeast isolates might provide adequate control of *B. cinerea* on waxflower, since yeasts have been used with success against *B. cinerea* on other crops (Elad *et al*. 1994; Mercier and Wilson 1994). However, as was found by Janisiewicz (1987) during screening of microbial antagonists for the control of *Penicillium expansum* (blue mould) on apples, there were discrepancies between the *in vitro* and *in vivo* results.

As noted, the performance of microbial antagonists on plant organs in biocontrol assays does not necessarily mean that the same antagonists will perform well under field conditions (Tatagiba *et al*. 1998). The level of control

Table 2. Relative ability of yeast isolates to inhibit *Botrytis cinerea* mycelial growth after incubation for 7 days at 25°C on half-strength potato dextrose agar

Means within each column followed by the same letter are not significantly different at $P = 0.05$
Total number of observations for germination percentage was 10 (2 replications × 5 samples) and germ tube length was 20 (2 replications × 10 samples)
Aureobasidium spp., pink yeasts and white yeasts were obtained from Dr Marcelle Stirling, Biological Crop Protection; all other yeasts were isolated from waxflower.
Significance: inhibition zone, $P = 0.009$; colony diameter, $P<0.001$

Isolate	Inhibition zone (mm)	Colony diameter (mm)
No. of replicates (*n*)	2	2
Control	0b	90.0a
Aureobasidium sp. 140	3.0a	56.0b
Aureobasidium sp. 274	1.8a	56.0b
Aureobasidium sp. 468	2.3a	53.0bc
Pink yeast Q6	0b	90.0a
Pink yeast Q34	0b	90.0a
Pink yeast 373	2.3a	49.5c
White yeast 268	0b	90.0a
White yeast 711	0b	90.0a
Yeast isolate 1	0b	90.0a
Yeast isolate 2	0b	90.0a

Table 3. Relative ability of fungal isolates to inhibit *Botrytis cinerea* mycelial growth after incubation for 7 days at 25°C on half-strength potato dextrose agar

Means within each column followed by the same letter are not significantly different at $P = 0.05$
Total number of observations for germination percentage was 10 (2 replications × 5 samples) and germ tube length was 20 (2 replications × 10 samples).
All fungal isolates were obtained from waxflower, with the exception of *Talaromyces* sp., which was obtained from Dr Lindy Coates, DPI, and *Trichoderma harzianum*, which was obtained from Dr Andrew Rath, Abbott Laboratories
Gliocladium roseum was not included in conidial germination and germ tube elongation experiments due to poor sporulation in culture
Significance: inhibition zone, $P<0.001$; colony diameter, $P<0.001$

Isolate	Inhibition zone (mm)	Colony diameter (mm)
No. of replicates (*n*)	2	2
Control	0e	90.0a
Alternaria sp.	0.8d	47.0c
Aspergillus sp.	0e	15.5f
Cladosporium sp.	0e	55.0b
Epicoccum sp.	5.0a	50.0bc
Fusarium sp.	2.3c	33.0d
Gliocladium roseum	0.0e	48.5c
Nigrospora sp.	1.0d	33.5d
Penicillium sp.	0e	26.0e
Pestalotiopsis sp.	0e	45.0c
Phoma sp.	0e	50.0bc
Talaromyces sp.	3.3b	44.5c
Trichoderma sp.	0e	23.0e
Trichoderma harzianum	0e	33.5d

achieved by the antagonists in the biocontrol assays on waxflower might well have been enhanced by stable environmental conditions inside the jars used in these experiments. Constant temperature and high relative humidity should favour rapid germination and colonisation of the waxflowers by the antagonists. It is unlikely that environmental conditions would be so favourable during normal postharvest handling. Moreover, environmental conditions in the field are even less favourable. Leaf and flower surfaces can be hostile environments to microbial antagonists (Elad 1990). Fluctuating temperature and relative humidity, the absence of free water, and other variables, such as leaf exudates, wind and UV radiation, can all hamper colonisation by microorganisms (Elad 1990). Yeasts are believed to be relatively well adapted to cope with environmental fluctuations (Blakeman 1993). However, the isolates tested failed to control *B. cinerea* on waxflower, even under seemingly favourable conditions.

Sutton *et al.* (1997) suggest that the filamentous fungi *Gliocladium roseum* is an effective and versatile *B. cinerea* antagonist. The *Gliocladium roseum* isolate tested in these experiments displayed a degree of antagonism towards *B. cinerea*. However, this isolate was problematical, like the *Talaromyces* sp., *Nigrospora* sp. and *Pestalotiopsis* sp. isolates, because of poor sporulation. Therefore, the isolate of *Epicoccum* sp. that produced the largest inhibition zone *in vitro* was selected for further testing *in vivo*. *Epicoccum purpurascens* was found to prevent grey mould disease, notably lesion formation on bean leaves and petals, under a range of environmental conditions (Hannusch and Boland 1996). However, not all *Epicoccum* species are saprophytic. Some are characterised as being opportunistic plant pathogens (Bruton *et al*. 1993). For example, *Epicoccum nigrum* has been reported to infect melon fruit and cause red discolouration of the skin (Bruton *et al*. 1993). The fungus was also found to be pathogenic towards cucumber, tomato, apple and pear (Bruton *et al*. 1993). Further pathogenicity testing of the isolate obtained from waxflower flowers would be necessary to ensure it is saprophytic. High concentrations of *Epicoccum* sp. conidia may also have harmful effects on plant growth. It is believed that the dark coloured conidia can interfere with light reception (Zhou and Reeleder 1991).

As expected, *Trichoderma harzianum* was the most antagonistic fungus *in vivo* against *B. cinerea*. A commercial formulation of *T. harzianum* (Trichodex) is available and has been used for the control of *B. cinerea* on other crops, including grapes and strawberries (Elad 1994; Kovach 1996; Burgess and Keane 1997). However, *Trichoderma* spp. have been reported to be ineffective biocontrol agents in dry

Table 4. Relative ability of two bacterial isolates, two yeast isolates and three fungal isolates to inhibit waxflower cv. 'CWA Pink' flower abscission from petioles when they were incubated at 20°C and >95% relative humidity

Row and column means for bacterial, yeast and fungal isolates followed by the same letter are not significantly different at $P = 0.05$

Isolate	Days to flower abscission Control	Inoculated	Row means ($n = 20$)
No. of replicates (n)	10	10	
Bacterial isolates[A]			
Control	12.8	6.1	9.5b
Bacillus sp. 480	13.4	8.1	10.8ab
Pseudomonas sp. 677	14.7	10.7	12.7a
Column means ($n = 30$)	13.6a	8.3b	
Yeast isolates[B]			
Control	13.8	7.5	10.7a
Aureobasidium sp. 274	15.0	8.6	11.8a
Aureobasidium sp. 468	14.0	9.8	11.9a
Column means ($n = 30$)	14.3a	8.6b	
Fungal isolates[C]			
Control	13.0	5.9	9.5b
Epicoccum sp.	12.9	9.3	11.1ab
Fusarium sp.	12.0	7.2	9.6b
Trichoderma harzianum	13.2	13.2	13.2a
Column means ($n = 40$)	12.8a	8.9b	

[A]Significance: isolate, $P = 0.006$; inoculation, $P<0.001$; isolate × inoculation, n.s.
[B]Significance: isolate, n.s.; inoculation, $P<0.001$; isolate × inoculation, n.s.
[C]Significance: isolate, $P = 0.005$; inoculation, $P<0.001$; isolate × inoculation, $P = 0.023$.

conditions (Köhl *et al*. 1995). Because of these characteristics, the search for more durable antagonists microbes is ongoing.

Unsightly mycelium associated with surface colonisation by fungal isolates can be a problem with using filamentous fungi as microbial antagonists. Thus, most fungal antagonists may be impractical as postharvest biological control agents for *B. cinerea* on waxflower flowers. In contrast, such fungal antagonists may be beneficial if employed for competitive exclusion of *B. cinerea* in the field before harvest. Preharvest conditions should not be conducive to proliferation. In this context, future research could determine if the more promising fungal antagonists, such as *Trichoderma harzianum* and *Gliocladium roseum* can reduce preharvest colonisation of waxflower by *B. cinerea*.

In conclusion, future developments in biological control of *B. cinerea* on waxflower might entail an integrated pre- and postharvest biological control approach. Saprophytic fungal antagonists could be used in the field to colonise necrotic material and reduce *B. cinerea* sporulation. Fungi, such as *Trichoderma* spp., could also be used to destroy survival structures like sclerotia (Elmer and Köhl 1998). Antagonistic yeasts and bacteria, such as *Pseudomonas* 677, might then be employed after harvest to colonise flowers. Their direct competition with *B. cinerea* should prevent infection of healthy tissues (Elmer and Köhl 1998).

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