Efficacy of Bio-Save 10LP (ESC-10) for control of rhizopus rot of peaches in Canada-Report for 1999.

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EXECUTIVE SUMMARY

Rhizopus rot of peaches is probably being controlled in Canada with an immediate preharvest application of Rovral (iprodione). If this choice of fungicide were to change and it probably will in 2000, with availability of Topas (Orbit, propiconazole), rhizopus rot could become troublesome. Only Botran (dicloran) is registered for postharvest treatment, but it is not being used and is unpopular because of the very visible yellow residue. Our study was undertaken to compare Bio-Save 10LP with another strain of *Pseudomonas syringae* (MA-4), described by Zhou et al. (1999). This report is part of a larger study involving fungicides to be submitted for publication in Plant Disease. Commercially-ripe peaches were wound-inoculated with drops of suspensions of cultured and washed cells of MA-4 and ESC-10, and of a suspension of Bio-Save 10LP. Ten peaches constituted a replicate and each treatment was replicated four times, and both experiments were repeated. Single isolates of *Rhizopus stolonifer* and *R. oryzae* were used at 3 x 10^3 or 1 x 10^4 sporangiospores/ml, co-inoculated with *P. syringae*.

Bio-Save 10LP at 1 x 10^8 CFU/ml gave 85% and 100% control of *R. stolonifer* and *R. oryzae* respectively, using peaches that had been stores (1EC) for 3 days. Repeat experiments were conducted with older peaches which were less effectively protected. However, most fresh-market peaches would be treated and graded one day after harvest, and would be more easily

protected against *Rhizopus* spp. Bio-Save 10LP gave control comparable to that of two fungicides being considered for registration for postharvest uses on fruit namely Medallion (fludioxonil) and Elite (tebuconazole).

INTRODUCTION

In southern Ontario, Canada, rhizopus rot is a serious threat to stone fruit production particularly of peaches, although brown rot caused by *Monilinia fructicola* (Wint.) Honey, is currently the more damaging disease (Biggs & Northover 1985). Rhizopus rot is caused by *Rhizopus stolonifer* (Ehrenb.:Fr.)Vuill. (*R. nigricans* Ehrenb.) and in 1999, *R. oryzae* Went & Prinsen Geerlings (*R. arrhizus* A. Fischer) was also recovered from rotting, fallen peaches on the Niagara peninsula (Northover, unpublished). Besides affecting stone fruits (Luepschen et al 1971, Ogawa et al 1995, Sholberg & Ogawa 1983) these species also cause serious production losses of strawberries and raspberries (Dennis 1983), sweet potatoes (Martin 1964) and cantaloupes (Wade & Morris 1982), often necessitating the preventative use of fungicides.

Several groups of fungicides when applied preharvest have worsened the incidence of postharvest rhizopus rot, although this is not apparent in inoculated laboratory studies. A probable explanation is that the complex fungal and bacterial microflora on the fruit surface may provide some biological control of *Rhizopus* spp on wounded tissue. Fungicides may interfere with the biocontrol process resulting in an increase in rhizopus rot. This view is supported by Zhou et al. (1999) who showed that non-pathogenic isolates of *Pseudomonas syringae* from the apple phyllosphere were very effective for suppressing *R. stolonifer* in wound-inoculated peaches. Species of *Rhizopus* have been controlled biologically by *Candida sake* (Vinas et al 1996) and *Enterobacter cloacae* (Wilson et al 1987) and this topic was reviewed by Droby and Chalutz (1994).

The objectives of the present series of laboratory studies were to determine: the efficacies of two isolates of *P. syringae* for controlling rhizopus rot; and the relative ease of control of *R. stolonifer* and *R. oryzae*.

MATERIALS AND METHODS

Harvested peaches were cooled quickly and stored at 1EC for 3-16 days prior to their experimental use. Peaches were graded and packed on their sides in 20 or 21-cell Panta-Pak trays (50 cm x 30 cm x 7 cm, Richter Mfg., Vasalia, CA, USA) placed inside plastic incubator boxes (52 cm x 36 cm x 14 cm deep) closed with a lid.

***Rhizopus* cultures.** Single isolates of *R. stolonifer* (DAOM 225707) and *R. oryzae* (DAOM 178621) were obtained from the Canadian Fungal Culture Collection, Ottawa, and were used throughout this study. Cultures were grown on potato dextrose agar (PDA, Difco. Detroit, MI, USA) slants in large glass tubes (200 mm long, 24 mm wide) in diffuse light at 23 EC for 10 days to allow abundant sporangial development. Sporangiospores (spores) were obtained by adding 10 ml of sterile water to a tube, shaking it vigorously for 10 sec., and measuring the spore concentration of the resulting suspension with a hemacytometer. The spore concentration was adjusted by dilution to a double strength of 6×10^3 spores/ml for both species, except that in later experiments, *R. oryzae* was prepared at a double strength of 2×10^4 spores/ml to increase fruit infection to >90 %. For each experiment, the identity of each culture was confirmed by growth (*R. oryzae*) or absence of growth (*R. stolonifer*) at 36 EC on PDA (Inui et al. 1965).

Biocontrol agents. MA-4 (*P. syringae*) of phyllosphere origin (Zhou et al. 1999) was stored in 30 % glycerol at -70 EC, and cultured in potato dextrose broth (PDB, Difco, Detroit, MI, USA) shaken at 150 rpm at 22 EC in darkness for 3-4 days. The cells were harvested by

centrifugation at 6500 g for 5 min. at 4 EC. The pellet was resuspended in sterile water. The bacterial concentration was determined with a spectrophotometer (DU 640, Beckman Instruments) at 420 nm. The bacterial cell concentration was determined from a calibration curve that related absorbance to the number of colony forming units (CFU) per ml, from a dilution plating series.

Bio-Save 10LP (*P. syringae* strain ESC-10) was provided by EcoScience Produce Systems Corp., Orlando, FL, USA, as a freeze-dried coarse powder in a sealed container. A fresh culture of ESC 10 was prepared in the same manner used for MA-4. Additionally, a freshly received frozen supply of Bio-Save 10LP (9×10^{10} CFU/g) was suspended in sterile water at 23 EC for 40 min. before dilution to twice the final required concentration, prior to combining with equal volumes of *Rhizopus* spore suspensions.

Inoculation protocol and disease evaluation. The upper side of each peach was punctured once with a 5 mm pointed probe, 4 mm wide at its base which had a 12 mm diameter collar, to limit the depth of penetration. Within 2 min. of wounding, each site received a 38 Fl drop from a Pasteur pipette. The drop consisted of a spore suspension of *R. stolonifer* or *R. oryzae* mixed with an equal volume of a suspension of a biocontrol agent, fungicide or of water (check). Peaches on one half of the tray were inoculated with *R. stolonifer* and the remaining 10 fruits were inoculated with *R. oryzae*. Only one concentration of *P. syringae* was used in each tray. Each treatment was replicated four times within each experiment, and the incubator boxes were randomly distributed in a walk-in incubator at 24 EC. Peaches were incubated long enough to allow abundant sporangial development on lesions, and permitting a visual distinction from sporulating brown rot lesions arising from preharvest infections.

The number of peaches with lesions >60 mm in diameter centered on the inoculation site, with sporangial development, in each replicate of 10 peaches was expressed as a percentage, and averaged over the four replicates per experiment. Percentage values were transformed to arcsin/% and examined by ANOVA and Fisher=s Protected LSD test for significant (P=0.05) separation of paired means. Data for each species of *Rhizopus* were analysed separately.

Effect of concentration of *P. syringae* from three preparations on the control of rhizopus rot. The efficacies of *P. syringae* against rhizopus rot of peaches, wound-inoculated separately with both species of *Rhizopus* were examined in four factorial experiments. Three preparations of *P. syringae* (MA-4 culture, ESC 10 culture and Bio-Save 10LP) were compared at final concentrations of 0 (check), 1 x 10^7, 3 x 10^7 and 1 x 10^8 CFU/ml. A fifth concentration of 3 x 10^8 CFU/ml was added to each of the repeat experiments conducted with both pathogens. The final concentration of *Rhizopus* spores in the inoculation drop was 3 x 10^3 spores/ml, except for the second *R. oryzae* experiment where the concentration was increased to 1 x 10^4 spores/ml to elevate the level of infection in the check. Harrow Beauty peaches stored at 1 EC for 3 days were used for the first experiments with both *Rhizopus* spp, and the peaches used in both second experiments had been cold-stored for10 days.

RESULTS

Effect of concentration of P. syringae from three preparations on the control of rhizopus rot. In the first experiment with *R. stolonifer*, the ESC 10 culture gave slightly better overall suppression of rhizopus rot than MA-4 culture and Bio-Save 10 LP (Table 4, Experiment 1). At a concentration of 1 x 10^8 CFU/ml**,** disease incidence was reduced from 100 % to 11 %. In the second experiment, the ESC 10 culture again gave the best overall suppression, with the

freeze dried preparation being the worst (Table 1, Experiment 2). However, at 1 x 10^8 CFU/ml the disease incidence of 27 % was similar for the three preparations and rot was not significantly improved by increasing the concentration of *P. syringae* to 3 x 10^8 CFU/ml (Table 1, Experiment 2).

Against the *R. oryzae*, the three preparations were equally effective at a bacterial concentration of 1 x 10^8 CFU/ml, and the rot incidence was reduced from 38 % to only 1 % (Table 2, Experiment 1). In the repeat experiment, both of the freshly prepared cultures gave an overall better suppression of rhizopus rot than Bio-Save 10LP (Table 2, Experiment 2). Nevertheless, at 1 x 10^8 CFU/ml, the rot incidence was reduced from 93 % to 8 % with no difference between the preparations. There was no significant further improvement in rot suppression with a *P. syringae* concentration of 3 x 10^8 CFU/ml.

DISCUSSION

In our account of the biological control of rhizopus rot, we provided the first report of the postharvest control of *R. stolonifer* with freshly produced cells of *P. syringae* isolates MA-4 and NSA-6 (Zhou et al. 1999). In the present study, freshly prepared cells of ESC-10 *(P. syringae*) were slightly more potent that those of MA-4. A freeze dried product of ESC-10 (Bio-Save 10LP) suspended in water at 1 x 10^7 CFU/ml showed weak activity, but it was as effective as the freshly prepared cells of ESC-10 and MA-4 when used at 1 x 10^8 CFU/ml, and co-inoculated with *Rhizopus* inocula. *R. stolonifer* was less well suppressed in the second experiment with Harrow Beauty peaches that had been stored for 7 days longer than those used in the first experiment. The greater difficulty of controlling disease in the more senescent fruits was attributed primarily to a progressive loss of natural host resistance, and a greater susceptibility of the older peaches to rhizopus rot.

Under normal commercial conditions, peaches for the fresh market are placed in cold storage a few hours after harvest, to slow ripening. The next day, the cooled and firmer fruits are washed, defuzzed, waxed and graded. A postharvest treatment could be applied between defuzzing and grading, and because the peaches have been stored for only one day, rhizopus rot might be controlled with lower concentrations of biocontrol agent, than those determined in this study.

Several other microorganisms have been effective for controlling rhizopus rot of various crops (Droby & Chalutz 1994). The yeasts *Pichia guilliermondii* (Ben-Arie et al 1991, Chalutz et al 1991), *Candida sake* (Vinis et al 1996) and *Kloeckera apiculata* (McLaughlin et al 1992), endophytic bacteria (Pratella et al 1994) and the bacterium *Enterobacter cloacae* (Wilson et al 1987), have been examined for their efficacies against rhizopus rot of tomato and grape, apple and pear, grape, apple and peach, respectively.

The postharvest treatment of peaches with fludioxonil, tebuconazole or *P. syringae*, primarily against rhizopus rot, would be an excellent strategy to complement preharvest treatments that are more effective against brown rot. However, fludioxonil is considered prone to segregating fungicide resistant pathogens, and should be used in a fungicide resistance avoidance strategy as recommended by FRAC (Anon 1998), involving other fungicides or biological control agents. The excellent activity of *P. syringae* against rhizopus rot in our studies, suit it well for use in such a strategy for the postharvest control of rhizopus rot.

ACKNOWLEDGMENTS

We greatly appreciate the assistance of Barry Kemp for the management of peach orchards and supply of fruit, and for the technical support of Cheryl Homeyer, Karin Schneider, Noella Gray, Lisa Trang and Todd Larmet. We also extend our thanks to Lucy Grant (EcoScience Produce Systems Corp.) for the generous and timely supply of research samples.

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Table 1. Effect of concentration of three preparations of *Pseudomonas syringae* on the percentage incidence of rhizopus rot of Harrow Beauty peaches, wounded and co-inoculated with *Rhizopus stolonifer* in two experiments[1].

P. syringae preparation	Concentration of *P. syringae* (CFU/ml)					
	0	1 x 10^7	3 x 10^7	1 x 10^8	3 x 10^8	Mean
Experiment 1						
MA-4 (culture)	100 C a[2]	38 AB a	48 B b	10 A a	-	49 b
ESC 10 (culture)	100 C a	25 B a	18 AB a	8 A a	-	38 a
B-S 10 LP (freeze dried)	100 C a	48 B a	45 B b	15 A a	-	52 b
Mean	100 C	37 B	37 B	11 A		
Experiment 2						
MA-4 (culture)	100 D a	70 C b	45 B ab	23 A a	28 AB a	53 b
ESC 10 (culture)	100 C a	35 B a	43 B a	25 AB a	18 A a	44 a
B-S 10LP (freeze dried)	100 C a	95 C c	73 B b	33 A a	25 A a	65 c
Mean	100 D	67 C	54 B	27 A	24 A	

[1] The peaches were harvested on 24 August, and used for experiments 1 and 2 after storage at 1EC for 3 and 10 days respectively. The final spore concentrations for *R. stolonifer* in both experiments was 3 x 10^3 spores/ml.

[2] Within the same experiment, means in the same column with no lower case letter in common, and means in the same row with no upper case letter in common, differed significantly (P=0.05) using Fisher=s Protected LSD test for the separation of paired means. The ANOVA for each experiment gave Probability (P) values for the main effects of preparation (prep.), concentration (conc.) and the interaction (prep. x conc.): Expt. 1, prep. P= 0.0267, conc. P= 0.0001, prep.x conc. P= 0.5136. Expt. 2, prep. P= 0.0001, conc. P=0.0001, prep.x conc. P=0.0003.

Table 2. Effect of concentration of three preparations of *Pseudomonas syringae* on the percentage incidence of rhizopus rot of Harrow Beauty peaches, wounded and co-inoculated with *Rhizopus oryzae* in two experiments[1].

P. syringae preparation	Concentration of *P. syringae* (CFU/ml)					
	0	1 x 10^7	3 x 10^7	1 x 10^8	3 x 10^8	Mean
Experiment 1						
MA-4 (culture)	38 B a[2]	3 A ab	0 A a	0 A a	-	10 a
ESC 10 (culture)	38 B a	0 A a	8 AB a	3 A a	-	12 a
B-S 10LP (freeze dried)	38 B a	20 AB b	5 A a	0 A a	-	16 a
Mean	38 B	8 A	4 A	1 A	-	
Experiment 2						
MA-4 (culture)	93 B a	8 A a	10 A a	5 A a	10 A b	25 a
ESC 10 (culture)	93 B a	3 A a	8 A a	5 A a	0 A a	22 a
B-S 10LP (freeze dried)	93 C a	63 B b	53 B b	13 A a	5 A ab	45 b
Mean	93 D	25 C	24 BC	8 AB	5 A	

[1] The peaches were harvested on 24 August, and used for experiments 1 and 2 after storage at 1EC for 3 and 10 days respectively. The final spore concentrations for *R. oryzae* used in experiments 1 and 2 were 3 x 10^3 and 1 x 10^4 spores/ml, respectively.

[2] Within the same experiment, means in the same column with no lower case letter in common, and means in the same row with no upper case letter in common, differed significantly (P=0.05) using Fisher=s Protected LSD test for the separation of paired means. The ANOVA for each experiment gave Probability (P) values for the main effects of preparation (prep.), concentration (conc.) and the interaction (prep. x conc.): Expt. 1, prep. P= 0.5289, conc. P= 0.0001, prep.x conc. P= 0.5650 . Expt. 2, prep. P= 0.0001, conc. P=0.0001, prep.x conc. P=0.0003.