SEED COATING WITH BIOCONTROL STRAIN *PSEUDOMONAS FLUORESCENS* F113

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ABSTRACT

Pseudomonas fluorescens strain F113, which has biocontrol capacity against the plant pathogenic fungus *Pythium ultimum*, was formulated and applied to onion seed using patented biopolymer technology developed at AgResearch, Lincoln. Initial loadings on seeds ranged between 8.6×10^6–1.1×10^7 bacteria/seed. In a preliminary screening of four different formulations, shelf life studies indicated that most formulations maintained high cell numbers on seed stored at 4°C for up to 70 days. Bacterial numbers declined on seed stored at 20°C, but significant numbers of bacteria remained viable after 70 days storage. In a second experiment, the two formulations giving best shelf-life at 20°C were monitored for stability at 20°C for 4 weeks. Packaging material had a significant effect on bacterial survival on seed. Germination of freshly treated seeds was not affected by seed treatment. The study has demonstrated the potential to treat seed with fluorescent pseudomonads with biocontrol capability.
Keywords: biological control, seed treatment, seed germination, shelf-life, formulation.

INTRODUCTION

The use of selected microbial antagonists for control of soil-borne phytopathogens has been widely studied but there are few commercial products yet available. A key constraint to commercialisation is the availability of effective formulations and delivery systems that ensure the long-term viability of the inoculant and its biocontrol activity (McIntyre & Press 1991). Application of microbial antagonists, for example *Pseudomonas* spp., to seed provides an ideal delivery system as it introduces inoculum to the rhizosphere where plant pathogens such as *Pythium* and *Rhizoctonia* are active, causing seed rots in the spermosphere and seedling damping-off. While bacteria can be applied directly to the seed surface, vegetative bacterial cells are very susceptible to the physical and chemical stresses associated with seed preparation, such as desiccation or temperature changes. In addition, seeds of many species need to be coated with additional material to ensure ease of handling and sowing. Because of the potential shown by some *Pseudomonas* isolates for control of intractable soil-borne phytopathogens, there have been several attempts at development of seed coating techniques, with mixed levels of success (Slininger et al. 1996; Shah-Smith & Burns 1997; Moenne-Loccoz et al. 1999).

Pseudomonas fluorescens F113 inhibits *Pythium ultimum* under laboratory conditions (Shanahan et al. 1992) and reduced severity of damping-off of sugar beet in soil microcosms prepared with soil naturally infested with *Pythium* spp. (Fenton et al. 1992). In this study, *P. fluorescens* F113 was formulated and applied to onion seed using techniques developed at AgResearch, Lincoln. Bacterial shelf life was measured at regular intervals and germination of treated seeds was assessed in a pot trial.

MATERIALS AND METHODS

Bacterial isolate and production of inocula

Pseudomonas fluorescens F113Rif (Carroll et al. 1995) is a spontaneous rifampicin-resistant mutant of strain F113. *Pseudomonas fluorescens* F113Rif was grown in liquid medium (40 g raw sugar, 10 g yeast extract, 2 g urea and 2 g NPK /litre). Cultures were grown for 72 h at 27°C with shaking (200 rpm). Numbers of colony forming units (CFU) in broth and on seed were determined by serial dilution plating onto Luria Bertani (LB) agar (Sambrook et al. 1989). Plates were incubated for 24-48 h at 30°C before colonies were counted.

Formulation and seed treatment

Five hundred ml of freshly grown cells were concentrated by centrifugation at 8000 rpm for 25 min at 16°C. The supernatant was removed to give a cell paste (approximately 4.9×10^{10} cfu/g) which was used in the formulations. The cell concentrate was formulated with various biopolymers into gel formulations using technology developed at AgResearch, Lincoln (NZ Patent No. 506484). Gels were coated onto clean onion seeds, with additives to obtain a flowable product. Three different biopolymers and additives were used in combination to prepare the biomatrix gels.

A preliminary un-replicated screening of several formulations was carried out to select promising formulations for further more detailed assessment. In the preliminary screening, four types of formulation (NF 596, 598, 600 and 602) were prepared using the patented technology, with formulations containing either a single biopolymer (NF 596, 598) or a combination of biopolymers (600, 602). Treated seeds were packed in thick, gas transferable bags (TGT bags, 120 µm thick; Convex Plastic Limited, Hamilton). Each batch of treated seed was divided into two bags (25 g treated seed/bag) with one bag being stored at 4°C and the other at 20°C. The shelf life of bacteria on stored seeds was assessed at fortnightly intervals for 2 months, as described below. Bags were sampled under aseptic conditions, resealed and stored at the appropriate testing temperature.

The two seed treatments that gave the longest shelf life in the preliminary screening experiment (NF 600 and NF 602) were used in a second replicated experiment. Freshly prepared cell paste was formulated and applied to seeds, which were then stored in two types of bags: gas transferable bags, as used in the previous experiment, and high barrier aluminium foil bags (Convex Plastic Limited, Hamilton). On each sampling date, four replicate bags were sampled from each treatment. *Pseudomonas fluorescens* surviving on seed stored at 20°C were enumerated at 0, 2 and 4 weeks. The water activity (a_w) of NF 600-treated seeds was measured immediately after treatment and after one month's storage using an Aqualab Water Activity Meter Series 3 model TE. Water activity is a measure of the free unbound water that is available to micro-organisms.

A further two batches of seed were also treated with the same biopolymers and additives used in NF 600 and NF 602, but without *P. fluorescens* F113. These seeds were used as control treatments in the germination experiment described below.

Survival of formulated *P. fluorescens* F113 on stored onion seeds

On each sampling date, 1 g samples of seeds from each bag were transferred to sterile tubes containing 9 ml sterile buffer (1 g/litre tetra-sodium pyrophosphate [BDH Laboratory Supplies Pool, England] and 1 ml/litre Tween® 80). The tubes were shaken in a wrist-hand shaker at the highest speed for 10 min to release the cells into a homogeneous suspension. Appropriate serial dilutions of this suspension were plated in duplicate on LB agar plates.

Germination of onion seed

Germination of treated seeds prepared in the second experiment was measured in a greenhouse pot trial, using a randomised block design with five treatments and four blocks. The treatments consisted of a 2 × 2 factorial (2 formulations × ± *P. fluorescens* F113) plus 1 (bare seed). Ten seeds from each treatment were sown into each of four replicate pots (150 mm diameter × 150 mm depth) containing approximately 1250 g of Wakanui silt loam soil with a layer of peat (5 mm) on the surface of the soil. Seeds

were sown on the same day they were treated. The temperature in the glasshouse was maintained at 22°C, with 13:11 h light:dark. Seedlings were counted 14 days after sowing.

Statistical analysis

Bacterial numbers (CFU/g seed) from the shelf life studies of the replicated laboratory assay were $\log_{10}$ transformed before analysis of variance. Percentage germination data was subjected to analysis of variance using GenStat.

RESULTS

Seeds treated with *P. fluorescens* formulated in biopolymers in the preliminary screening experiment carried an initial average concentration of 3.8×10^8 CFU/g seed (range: $6.5 \times 10^7 - 7.3 \times 10^8$), where 1 g contains approximately 176 seeds. Bacterial numbers on seeds remained fairly stable in all four treatments stored at 4°C, with an average of 1.4×10^8 CFU/g seed after 70 days (Fig. 1). Persistence of bacteria on seed stored at 20°C was more variable, with bacterial numbers declining in all treatments. Although survival of bacteria was reduced at 20°C, treatments NF 600 and NF 602 had $>10^5$ CFU/g of seed after 70 days. In the other two treatments stored at 20°C, no viable bacteria were recovered from seed after 42 days storage.

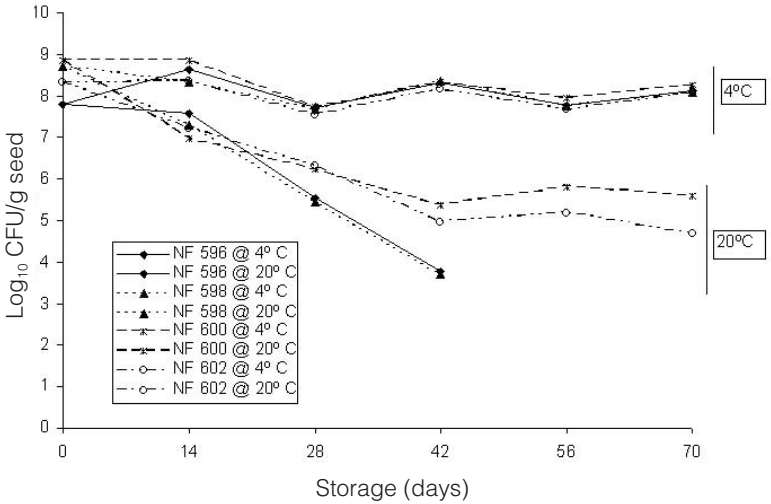

FIGURE 1: Numbers of bacteria ($\log_{10}$ CFU/g seed) in four formulations of *Pseudomonas fluorescens* F113 on treated onion seed stored at 4 and 20°C for 70 days.

The two formulations that gave best bacterial survival on treated seed in the first experiment (NF 600 and NF 602) were assessed in a second experiment. The initial bacterial loadings differed between the two treatments ($P<0.01$): seeds treated with NF 600 carried 3.0×10^9 CFU/g seed, while seeds treated with NF 602 carried 9.0×10^8 CFU/g seed (Table 1). The bacterial populations showed a similar rate of decline as was seen for seeds stored at 20°C in the first experiment. After 14 days of storage at 20°C, the bacterial loadings on the seeds did not differ significantly between biopolymer treatments but a significant effect of packaging was seen, with bacteria

surviving best in foil bags (Table 1). The effect of packaging material was also apparent after 28 days of storage. Bacteria formulated with NF 600 had 79% greater survival in foil bags than in gas transferable bags after storage for one month and 59% improvement in bacterial survival was seen in NF 602-treated seeds in foil bags.

There was a difference in a_w of NF 600-treated seeds stored for one month in the two types of packaging. After one month at 20°C in gas transferable bags, a_w of NF 600-treated seeds had declined from 0.982 to a_w=0.574, while in foil bags a_w was maintained at the higher level of 0.831.

TABLE 1: Numbers of bacteria (log_{10} CFU/g seed) in two formulations (NF 600 and NF 602) of *Pseudomonas fluorescens* F113 on treated onion seed directly after formulation (0 days) or stored for 14 or 28 days at 20°C in two types of packaging material.

Formulation and packaging[1]	0 days	14 days	28 days
NF 600 (TGT bag)	9.450	7.664	5.321
NF 600 (Foil bag)	9.450	8.145	6.179
NF 602 (TGT bag)	8.948	7.666	5.808
NF 602 (Foil bag)	8.948	8.272	6.013
LSD (P<0.05)	0.191	0.328	0.455
Significance of contrasts[2]:			
NF 600 versus NF 602	P<0.01	ns[3]	ns
Bag type	-	P<0.001	P<0.01
Interaction	-	ns	P<0.05

[1]TGT=thick gas transferable bags and Foil = aluminium foil bags.
[2]Contrasts for the main effects of formulation or bag type and for the interaction.
[3]ns=not significant.

Germination rates did not vary significantly between the two biopolymer treatments, nor did the addition of *P. fluorescens* F113 have any significant effect on the percentage germination measured at 14 days after sowing (Table 2).

TABLE 2: Germination after 14 days (%) of treated onion seeds that were sown immediately after treatment with two seed coating formulations (NF 600 and NF 602), either with or without *Pseudomonas fluorescens* F113.

Formulation and treatment	Germination at day 14 (%)
NF 600 – F113	92.5
NF 600 + F113	87.5
NF 602 – F113	92.5
NF 602 + F113	97.5
Bare seed	(100)[1]
LSD (P<0.05)	15.6
Significance of contrasts[2]:	
NF 600 versus NF 602	not significant
F113 effect	not significant
Interaction	not significant

[1]Bare seed germination in parenthesis excluded from statistical analysis.
[2]Contrasts for the main effects of formulation or F113 and for the interaction.

DISCUSSION

The shelf-life of environmentally sensitive micro-organisms continues to be a challenging and success-limiting step in development of biocontrol products (Paau 1998). While fluorescent pseudomonads have significant potential as biocontrol agents, they are very sensitive to environmental factors, in particular temperature and desiccation. These types of stresses are encountered in seed treatment processes, where seeds are typically air-dried for storage purposes, for example sugar beet seeds are dried to about 9% water content (w/w) prior to storage and shipment (Moenne-Loccoz et al. 1999). This trial has demonstrated the ability of biopolymer coatings to maintain viability of *Pseudomonas* spp. on onion seeds. Bacterial viability was maintained on seed stored at 4°C but was more variable at 20°C. The improved survival at lower temperatures was unsurprising and has been reported previously by Shah-Smith & Burns (1997), in their experiments on survival of *P. putida* on sugar beet seeds. Low temperatures have been shown to extend bacterial survival by reducing metabolic activity (Wessendorf & Lingens 1989).

Further work is required to provide greater protection of bacteria on seeds stored at ambient temperatures. The finding that survival was increased in foil bags, in comparison with thick gas transferable bags, suggests that desiccation is a factor involved in bacterial mortality at 20°C. Preliminary measurements of water activity support this suggestion, since a_w values were lower in gas transferable bags, indicating that moisture had been lost during storage at 20°C. Further work on conditions of storage and packaging materials is likely to lead to improvements in shelf-life of bacteria applied to seeds.

It is difficult to compare results obtained in this study with previous data in the literature as a range of *Pseudomonas* species/strains, and seed coating techniques and materials have been used. The present results compare favourably with one study where numbers of *P. fluorescens* F113 on seeds stored at 12°C declined from an initial count of approximately 1×10^6/sugar beet seed to $10\text{-}10^4$/seed in 20 days (Moenne-Loccoz et al. 1999). The same study demonstrated the potential to improve the tolerance of fluorescent pseudomonads to desiccation and other environmental stresses by varying the conditions under which the bacterial inoculum is produced. Moenne-Loccoz et al. (1999) showed that growing *P. fluorescens* in nutrient-amended vermiculite mix, instead of adding the cells to the unamended mix immediately before drying and pelleting of seed resulted in better survival of the bacteria. Slower drying of seed (20 h instead of 3 h) also enhanced bacterial survival. Onion seeds used in the current experiment were not air-dried because a single flowable seed was obtained immediately after inclusion of additives in the formulations but as discussed above, further experimentation with varying levels of water activity may result in increased survival.

Germination rates of treated seeds tended to be lower than bare seed and further investigation of effect of seed treatment on germination rates is required. Moenne-Loccoz et al. (1999) reported that pelleting of sugar beet seeds resulted in a lower rate of germination compared to unpelleted seed. Similarly Slinginger et al. (1996) found that seed batches inoculated with cells of *P. fluorescens* 2-79 formulated in methylcellulose suffered significant germination losses, which the authors attributed to phytotoxic metabolites produced by the bacterium. They suggested that dilution of the phytotoxic metabolites by harvesting and washing of bacterial inocula may be necessary.

The characteristics of formulations used to deliver bacterial seed inoculants can influence the subsequent behaviour of the inoculated bacteria in the rhizosphere (Moenne-Loccoz et al. 1999). An important next step in the development of seed treatments that incorporate fluorescent pseudomonads will be to determine the effect of seed treatments on colonisation of the roots and protection of seedlings under disease pressure.

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