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Improvements to the Pesta Formulation to Promote Survival and Dispersal of *Pseudomonas fluorescens* BRG100, Green Foxtail Bioherbicide

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ABSTRACT

A modified pesta granule was developed for *Pseudomonas fluorescens* BRG100, a bioherbicidal bacterium for grass weeds, green foxtail (*Setaria viridis*) and wild oat (*Avena fatua*). This study reports: i) the effect of formulation water activity (a_w) on survival of *P. fluorescens* BRG100 and, ii) the effect of starch on disintegration and dispersal of a green fluorescent protein transformant of *P. fluorescens* BRG100 from pesta in laboratory sand columns. The long-term refrigerated storage stability of *P. fluorescens* BRG100 was examined in pesta granules dried to different a_w. Drying pesta to 0.3 a_w stabilized the population of *P. fluorescens* BRG100 for 16 months at 8.5 $\log_{10}$ cfu/g. When pesta was dried to 0.8 a_w, *P. fluorescens* BRG100 population decreased to 7.3 $\log_{10}$ cfu/g over six months. The impact of starch addition (corn, pea, rice and potato) to pesta and concentration (13% and 26%, wt/wt) on the disintegration rate of pesta granules was determined with laser diffractometry. The order of fast to slow disintegration following starch amendment was pea>potato>corn> rice. Increasing pea, potato and corn starch content from 13 to 26% promoted faster disintegration of pesta, conversely, increasing rice starch content decreased disintegration. Half-life disintegration profiles were determined with pea starch amended pesta (26% w/w) being most rapid, 0.8 minute, rice starch (26% w/w) amended pesta was slowest, 4 minutes and non-amended pesta, 2.5 minutes. *P. fluorescens* BRG100*gfp* was detected 2 hr earlier in the middle and bottom sections of the sand columns from corn starch amended (26%) pesta than from non-amended pesta. The ability to produce pesta granules with different disintegration and bioherbicide release characteristics provides the formulator with the potential to design pesta that insures the active ingredient is delivered to the pest when it is most susceptible.

Keywords: bacteria, biocontrol, granule disintegration, green fluorescent protein

INTRODUCTION

Development of robust formulations for bioherbicidal bacteria is a key step towards advancing this technology into integrated pest management systems. The primary role of the formulation is to shelter; maintain population stability and efficacy of the biocontrol agent. However, it must also be inexpensively produced and readily adapted to current agricultural practices. Types of bioherbicide formulations include soil applied granules, crop seed coatings, wettable powders, invert and water-oil-water emulsions for spray application (**Table 1**). The decision on formulation type is dependent on the biocontrol agent's mode of action and the stage at which the host plant is most susceptible to the agent. Granular and seed treatment formulations usually are best suited for pre-emergent weeds while water-in-oil emulsions or wettable powder formulations are most appropriate for spray application for post-emergent weed management.

Granular formulations, composed of matrices of natural products, pesta, sodium alginate and starch, i.e. "stabilize", provide protection for bioherbicidal microorganisms from harsh environmental conditions and anthropogenic sources (Walker and Connick 1983; Connick *et al.* 1991; Quimby *et al.* 1999). The pesta formulation, a matrix of durum wheat, kaolin, sucrose and the bioherbicide culture, has been adapted by many researchers for studying weed management in laboratory and field experiments (Connick *et al.* 1996). **Table 1** lists some of the studies with fungal and bacterial bioherbicides in pesta and alginate granule formulations, seed treatments and foliar applied formulations. For more details on formulation development for microbial biopesticides see Burges (1998).

Under best management practices, crop yield losses in western Canada due to weeds were estimated to be $612 million (Swanton *et al.* 1993). Green foxtail, *Setaria viridis*, is one of the most abundant grass weeds in the North American plains, reduces the potential yield in grains, oilseeds and pulse crops, and is considered the world's worst weed in agriculture (Dekker 2003). Dependence on chemical pesticides to control green foxtail has led to the development of herbicide resistant populations. Chemical herbicide resistance is an immediate concern to western Canadian producers and estimates for growers to control herbicide-resistant wild oat and green foxtail with alternative herbicides exceeds $4 million annually (Beckie *et al.* 1999a, 1999b). One of the benefits of providing a biocontrol alternative for weed control is to slow the selection of chemical herbicide resistance in weeds.

Fluorescent and non-fluorescent pseudomonads were frequently isolated from the rhizosphere of weeds and several strains were reported to possess weed suppressive activity (Kremer *et al.* 1990). *Pseudomonas fluorescens* and *P. syringae* pv. *tabaci* and *tagetis* have been reported to be potential biocontrol agents for weeds (Boyetchko 1997; Brinkman *et al.* 1999; Daigle *et al.* 2002; Zidack and Quimby 2002; Zdor *et al.* 2005). Unlike most fungi and *Bacillus* sp, some of which are the active ingredients in registered biocontrol products, i.e. Contans WG® and Serenade Max® respectively, *Pseudomonas* sp. are non-spore forming bacteria and therefore maybe more challenging to formulate.

This study reports of the effect of pesta a_w on survival of *P. fluorescens* BRG100 and examines disintegration of pesta and dispersal of a green fluorescent protein transformant of *P. fluorescens* BRG100 from pesta in laboratory sand columns.

Received: 25 August, 2009. Accepted: 1 October, 2010.

Original Research Paper

Table 1 Granular, seed treatment and liquid formulations for bioherbicidal microorganisms.

Formulation	Bioherbicide	Weed host	Reference
Granule			
Pesta	*Alternaria cassiae*	Sicklepod (*Senna obtusifolia*)	Connick *et al.* 1991
Pesta	*A. crassa*	Jimsonweed (*Datura stramonium*)	Connick *et al.* 1991
Pesta	*Colletotrichum truncatum*	Hemp sesbania (*Sesbania exaltata*)	Connick *et al.* 1991
Pesta	*Fusarium lateritium*	Velvetleaf (*Abutilon theophrasti*)	Connick *et al.* 1991
Pesta	*Alternaria* sp.	Swamp dodder (*Cuscuta gronovii*)	Daigle *et al.* 1998
Pesta	*F. oxysporium* Foxy 2	*Striga* spp.	Elzein *et al.* 2004
Pesta	*Colletotrichum truncatum*	Hemp sesbania	Boyette *et al.* 2007
Pesta	*Pseudomonas fluorescens*	Leafy spurge (*Euphorbia esula*)	Brinkman *et al.* 1999
Pesta	*Alternaria alternata*, *Trematophoma lignicola*	(*Amaranthus retroflexus)*	Lawrie *et al.* 2001
Pesta	*F. oxysporum* f.sp. *orthoceras*	*Orobanche cumana*	Shabana *et al.* 2003
Pesta	*F. oxysporum* f.sp. *orthoceras*	*Orobanche cumana*	Müller-Stover and Sauerborn 2007
Pesta	*Pseudomonas fluorescens* BRG100	Green foxtail (*Setaria viridis*), wild oat (*Avena fatua*)	Daigle *et al.* 2002
Pesta	*P. fluorescens* G2-11	Green foxtail, velvet leaf	Zdor *et al.* 2005
Pesta, alginate	*F. oxysporum* f.sp. *orthoceras*	*Orobanche*	Müller-Stover *et al.* 2004
Alginate	*A. cassiae*, *A. macrospora*, *F. lateritum*, *C. malvarum*, *Phyllosticta* sp.	None indicated	Walker and Connick 1983
Pesta, Rice-alginate prill	*F. oxysporum* f.sp. *erythroxyli*	Coca (*Erythroxylum coca*)	Bailey *et al.* 1998
Barley granules	*Phomopsis convolvulus*	Bindweed *Convolvulus arvensis* L	Vogelgsang *et al.* 1998
Alginate microencapsulate - gelatine or agar	*F. avenaceum*	Marsh reed grass *Calamagrostis canadensis*	Winder *et al.* 2003
Starch (Stabileze process)	*C. gloeosporioides*, *F. oxysporum*	None indicated	Quimby *et al.* 1999
Starch (Stabileze)	*Pseudomonas syringae*	None indicated	Zidack and Quimby 2002
Seed coating			
	Fusarium nygamai	*Striga*	Zahran *et al.* 2008
Liquid			
Invert emulsion	*C. truncatum*	Hemp sesbania	Boyette *et al.* 1991, 1993
Invert emulsion	*Alternaria cassiae*	Sicklepod	Walker and Boyette 1985
Invert emulsion	*Ascochyta pteridis*	Bracken *Pteridium*	Womack *et al.* 1996
Water/oil/water (WOW)	*C. orbiculare*	Bathurst burr *Xanthium spinosum*	Auld *et al.* 2003
Water, 0.1% Tween 80	*C. truncatum*	Scentless chamomile	Graham *et al.* 2006
Water, surfactants	*Myrothecium verrucaria*	Sicklepod	Weaver *et al.* 2009

MATERIALS AND METHODS

Bioherbicidal *Pseudomonas fluorescens* strains

Pseudomonas fluorescens BRG100 was selected from a collection of over 400 bacteria strains originally isolated from the rhizosphere of downy brome, green foxtail and wild oat growing in fields in Alberta and Saskatchewan, Canada (Boyetchko 1997). This strain showed superior suppression of root growth in laboratory assays with green foxtail and wild oat relative to the other strains from the collection.

Pseudomonas fluorescens BRG100 and *P. fluorescens* BRG100 green fluorescent protein (*gfp*) were grown in M9 medium: Na_2HPO_4 3 g, KH_2PO_4 3 g, NH_4Cl 1 g, NaCl 2 g, sucrose 2 g, $MgSO_4 \cdot 7H_2O$ 1.23 g; $CaCl_2 \cdot 2H_2O$ 0.015 g, $ZnSO_4 \cdot 7H_2O$ 0.06 g, molasses 200 ml, dH_2O 800 ml.

Transformation of *P. fluorescens* BRG100 with the green fluorescence gene

Escherichia coli S17-λ1 that had been previously transformed with the Tn5 mini-transposon suicide plasmid pAG408, which harbours the *gfp* gene, was used to transform *P. fluorescens* BRG100 (Suarez *et al.* 1997). *E. coli* pAG408 was cultured on LB agar containing 50 mg/L kanamycin, 30 mg/L gentamycin (Sigma-Aldrich, St. Louis, MO, USA), and 50 mg/L ampicillin (Sigma-Aldrich) and incubated at 23°C for 72 hrs. *P. fluorescens* BRG100 was cultured on LB agar and incubated at room temperature for 72 hrs. Single colonies of the two bacterial strains were added to 50 ml of LB broth (Difco™, Becton, Dickinson and Co., Sparks, MD, USA) and the flasks were placed on the rotary shaker at 150 rpm for 24 hrs at 23°C. One ml of broth from each flask was added to three 1.5 ml centrifuge tubes and washed twice with 1 ml of 0.9% NaCl.

One ml of the *E. coli* pAG408 culture was poured into a 50 ml centrifuge tube, containing 1 ml of *P. fluorescens* BRG100. The tubes were vortexed and the contents were filtered using a Nalgene membrane filter device (Nalgene™, Rochester, NY, USA) with a pore size of 0.20 µm. The filters were removed and placed on LB agar for 24 hrs and then washed twice with 1 ml of 0.9% NaCl. One hundred µl of each of the two combined cultures was added to LB agar containing 50 mg/L kanamycin. The plates were incubated at 23°C for 48 hrs and examined under UV light, 366 nm, for the presence of *gfp* fluorescing colonies. Putative *P. fluorescens* BRG100*gfp* transformants were transferred to selective citrate agar: $MgSO_4 \cdot 7$, H_2O 0.2 g, $NH_4(H)_2PO_4$ 1 g, Na citrate 2 g, NaCl 5 g, kanamycin 50 mg, gentamycin, 30 mg, dH_2O 1000 ml. *E. coli* pAG408 lacks the ability to utilize citrate.

Preparation of pesta formulations of *P. fluorescens* BRG100 and *P. fluorescens* BRG100gfp

Stationary phase cultures of *P. fluorescens* BRG100, 60 hrs old, and *P. fluorescens* BRG100*gfp* from M9 medium were mixed with 150 g oat flour, 40 g maltose, and 10 g peat to prepare the pesta granular formulation (Diagle *et al.* 2002). Pesta dough was prepared by combining ingredients in a Viking Professional food processor (Viking, Greenwood, MS, USA) with 85 ml of broth culture containing *P. fluorescens* BRG100 or *P. fluorescens* BRG100*gfp* (final concentration: 9 $\log_{10}$ cells/g) or water (control). The dough was extruded through a 1.2 mm 25% dome die using a Fuji Paudal MG55 granulator (LCI Corp., Charlotte, NC, USA). Extrudate was transferred into a Fuji Paudal Marumerizer, model QJ-230 (LCI Corp., Charlotte, NC, USA) and spheronized producing pesta granules approximately 1.2 mm in

diameter. Pesta granules were transferred to a Sherwood Scientific fluidized bed drier (Sherwood Scientific Ltd., Cambridge, UK). A water activity meter (Aqua Lab, Decagon Devices, Pullman, WA, USA) was used to determine the a_w of the pesta. Gravimetric moisture determination was not conducted. Adjustments in the drying regime were made to produce pesta with 0.3, 0.5 and 0.8 a_w. The batches of pesta were stored in paper bags (Unisource Inc., Saskatoon, SK, #20 heavy) at 6°C. Pesta was enumerated for *P. fluorescens* BRG100 monthly for 6 months then every 2nd month to 16 months as described below.

For experiments requiring different titres of *P. fluorescens* BRG100, stationary phase cultures were concentrated by continuous flow centrifugation (Benchtop Centrifuge CEPA model LE, Carl Padberg Company, Lahr, Germany) and reconstituting the accumulated bacterial cells into the spent broth at 1/5th, 1/10th and 1/20th the original volume. Treatments included pesta with initial titres of *P. fluorescens* BRG100 of 8.4, 8.8, 9.1, and 9.5 $\log_{10}$ cfu/g. Pesta was enumerated for *P. fluorescens* BRG100 monthly for 6 months as described below. These concentrated broths were used to prepare pesta as described above.

In an unrelated study on granular formulation development for bioherbicides, starch amendment showed potential for altering the granule disintegration rate (Hynes and Bailey, unpublished). As a result, the role of starch type and concentration was explored. Seventy-five and and 37.5 g of corn, pea, potato and rice starches displaced an equivalent amount of oat flour from the recipe described above. Pesta dough preparation, extrusion, spheronization, and fluidizing bed drying were carried out as described above.

Pesta particle sizing

Particle size was determined by laser diffractometry in a Mastersizer 2000 (Malvern Instruments, Worcestershire, UK) and reported as volume mean diameter (VMD) or De Brouckere mean diameter, $D[4,3] = \Sigma d^4/\Sigma d^3$, where volume has a d^4 dependence and surface area a d^3 dependence (Rawle 2009). VMD was reported in µm. Approximately 250 mg of pesta was added to 170 ml of water in a 250 ml separatory funnel, the weight of pesta added varied in order to achieve the 10 to 20% laser obscuration parameter needed for particle size analysis. The contents of the separatory funnel were circulated (Masterflex console drive Model 77521-40 with an Easy load II L/S Model 77200-62 pump head) through particle size analysis cell. The initial measurement of particle size was determined 15 sec after the pesta was introduced into the Mastersizer and additional measurements were conducted every 30 sec for 9 min. The change in VMD of pesta was calculated from the slope of the line from time 0 to 1 min for the pesta with 26% pea starch, whereas for most of the other pesta preparation the change in VMD was calculated from 0.5 min to 2-3 min. Starch amended and non-amended pesta disintegration rates were determined from the linear portion of the disintegration curves. The 50% disintegration time or half-life of pesta was also determined from the linear part of the line. Pesta swell time was calculated from the time of granule introduction into the particle size analyser to the initial indication of particle disintegration.

Enumeration of *P. fluorescens* BRG100 in pesta

Enumeration of *P. fluorescens* BRG100 and *P. fluorescens* BRG100*gfp* in pesta were determined by serial dilution and spreading diluents on Plate Count Agar (PCA) usually within 48 hrs of pesta preparation (0 time) and 1 to 16 months on monthly intervals (1-6 months) and then every second month (8-16 months). The protocol was as follows. Ten grams of pesta granules were added to dilution bottles containing 90 ml sterile 0.1M $MgSO_4$ solution. The pesta was left to soak for 2 min. It was then homogenized at 15,000 rpm for 20 sec with the Heidolph DIAX 900 homogenizer with a 20 mm diameter saw-tooth generator probe (Rose Scientific Ltd., Edmonton, AB, Canada). The mixture was allowed to settle for 3 min and diluted using 0.1 M $MgSO_4$ solution to 10^{-4} and 10^{-5}. The suspension was then plated using the Spiral Biotech Autoplate 4000 on PCA plates. Two 10 g samples were plated and 5 plate replicates were made for each sample. The plates were then enumerated using the computerized program CIA-BEN (Advanced Instruments Inc., Spiral Biotech, Norwood, MA). This experiment was repeated 2 times.

P. fluorescens BRG100gfp dispersal from pesta in laboratory sand columns

P. fluorescens BRG100*gfp* was prepared in pesta with and without 26% wgt/wgt corn starch as described above in preparation of pesta formulations. Lexan polycarbonate columns, 75 mm by 40 mm, were filled with 35 g of sand (Inland Aggregates, Saskatoon, SK. The bottom of the column was fitted with a nylon disc covering two holes in the #7 rubber stopper to permit drainage. 150 mg of pesta containing *P. fluorescens* BRG100*gfp* was evenly distributed on top of the sand column. An additional 10 grams of sand was placed over the sample which was then topped with filter paper disc (Whatman #2). Sterile distilled water was pumped, 7 ml in 3 min, to saturate the columns. Treatments included sand columns inoculated with i) corn starch amended pesta with *P. fluorescens* BRG100*gfp*, ii) non-amended pesta with *P. fluorescens* BRG100*gfp*, iii) non-inoculated sand columns. Each treatment was replicated 2 times and the experiment was conducted 2 times. After 2 and 4 hrs incubation at 22°C, the top and bottom rubber bungs of the columns were removed and the sand core was carefully deposited onto a plexiglass template. Ten g of sand, wet weight, was removed from the columns at 3 depths, 0-6 mm, 15-21 mm and 27-33 mm, and transferred to 90 ml 0.1 M $MgSO_4$ in 200 ml dilution bottles for enumeration of *P. fluorescens* BRG100*gfp*. Bottles were shaken for 2 min before transferred of 1 to 9 ml of 0.1 M $MgSO_4$. Ten-fold serial dilution series was carried out and 100-µl aliquots of the diluents were spread on to nutrient broth agar media. The presence of *P. fluorescens* BRG100*gfp* was confirmed under UV light and colony enumeration was carried out after 48 hrs.

Statistical analysis

The data represent treatment means and standard errors of the means. Statistical analysis was performed with SAS using GLM. A *t*-test (Fisher's protected LSD test) was used to determine whether means were significantly different ($P<0.05$). R^2 values were determined from linear regression analysis. All experiments were conducted at least twice.

RESULTS

Effect of pesta water activity on survival of *P. fluorescens* BRG100

The titre of *P. fluorescens* BRG100 ($\log_{10}$ cfu/g pesta) decreased significantly in pesta granules with a_w of 0.3, 0.5 and 0.8 over 5 months storage at 15 °C (r^2= 0.9184, 0.9926, 0.9951, respectively, $P \leq 0.05$, **Fig. 1**), however, the smallest change in titre occurred at a_w of 0.3 and the greatest change occurred at a_w of 0.8 The titre of *P. fluorescens* BRG100 decreased the least, 0.19 $\log_{10}$ cfu/g pesta/month, with the initial a_w of pesta at 0.3, as compared to 0.27 and 0.51 $\log_{10}$ cfu/g/month with initial a_w of 0.5 and 0.8, respectively. Pesta was not sealed from the environment and, consequently, the a_w of the pesta treatments changed slightly for initial a_w of 0.3 and 0.5 over the duration of the experiment; 0.3 to 0.37, 0.5 to 0.43 and 0.8 to 0.6.

The titre of *P. fluorescens* BRG100 ($\log_{10}$ cfu/g pesta) decreased significantly over 16 months (r^2= 0.8823, $P \leq 0.05$) (**Fig. 2**).Shelf-life studies indicated that of the titre of *P. fluorescens* BRG100 in pesta decreased 0.85 $\log_{10}$ cfu/g. From sampling time 0 (9.4 $\log_{10}$ *P. fluorescens* BRG100

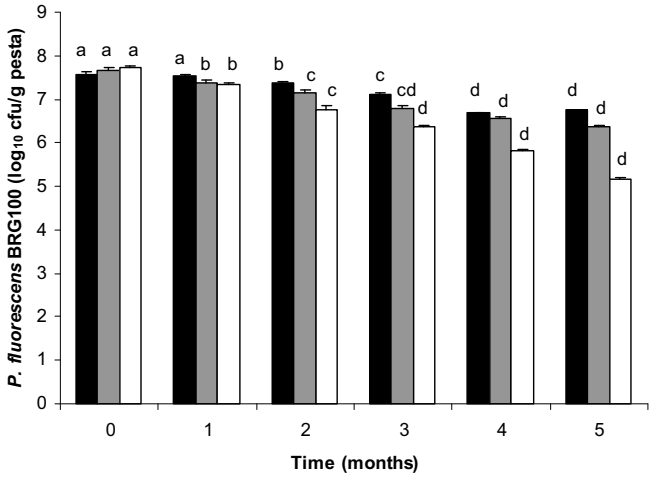

Fig. 1 Changes in the titre of *P. fluorescens* BRG100 in pesta granules with a_w of 0.3 (black), 0.5 (light grey) and 0.8 (white) stored at 15 °C. Analysis of valance was conducted within each a_w data series over the experiment duration and columns with different letters indicate significant differences in $\log_{10}$ cfu/g pesta of *P. fluorescens* BRG100, (r^2= 0.9184, 0.9926, 0.9951, respectively, $P \leq 0.05$). The data sets include error bars - standard error of the mean, n=5 replicates/treatment.

cfu/g pesta) to 4 months (8.9 $\log_{10}$ *P. fluorescens* BRG100 cfu/g pesta), the titre of *P. fluorescens* BRG100 steadily and significantly ($P \leq 0.05$) declined. Counts of *P. fluorescens* BRG100 at 3 and 5 month sample periods were not significantly different from each other, as were the counts from 6 and 10 month, 8 and 10 months and 8 and 12 month sample periods, but were significantly different from all other sample periods (**Fig. 2**).

The titre of *P. fluorescens* BRG100 ($\log_{10}$ cfu/g pesta) decreased significantly in pesta granules with initial titres of 8.4, 9.1 and 9.5 $\log_{10}$ cfu/g (r^2= 0.7344, 0.1095, 0.8781 respectively, $P \leq 0.05$, **Fig. 3**). There was a poor fit of the linear regression line with the data set for the pesta with initial titre of 9.1 $\log_{10}$ cfu *P. fluorescens*/g. The titre of *P. fluorescens* BRG100 of pesta with a starting titre of 8.8 $\log_{10}$ cfu/g pesta did not change significantly from 0 time and the 6-month enumeration.

Effect of starch dispersants on pesta disintegration

The relative effect of corn, pea, potato and rice starch amendment to pesta on disintegration was compared to non-amended pesta. The order of fast to slow disintegration of pesta following amendment with starch type was pea > potato > corn > rice (**Fig. 4**). Non-amended pesta disintegrated faster than pesta amended with rice starch, 26 and 13% and corn starch, 13%. Increasing the amount of pea, potato or corn starch from 13 to 26% in pesta promoted faster disintegration, whereas, increasing the concentration of rice starch in pesta decreased the rate of disintegration.

The decrease in volume mean diameter (VMD) of amended and non-amended pesta is reported in **Table 2**. Pesta amended with 26% pea starch disintegrated most rapidly and unlike all of the other pesta preparations did not increase in VMD or swell during the initial 30–60 sec of particle size data collection. Pesta disintegration from its initial size of about 1200 µm to 200–400 µm varied from 1.5 min with pesta-pea starch (26%) to greater than 10 min for pesta rice starch (26%). Non-amended pesta disintegrated in 7 min.

A representative particle size distribution pattern from granule disintegration studies using laser diffractometry is shown in **Fig 5**. Pesta amended with 37% corn starch disintegrated from a relatively narrow particle size at 0 sec, 900–1200 µm, to a very broad range of particle sizes at 180 sec, 10 to 1000 µm.

Effect of corn starch of *P. fluorescens* BRG100gfp on dispersal from pesta in laboratory sand columns

P. fluorescens BRG100*gfp* was not detected in the middle and bottom sections of the sand columns treated with the non-amended pesta after 2 hr incubation, whereas, in the columns treated with corn starch amended pesta about 2.5 $\log_{10}$ *P. fluorescens* BRG100*gfp* cfu/g sand were detected (**Fig. 6**). After 4 hr incubation 2.4 and 1.1 $\log_{10}$ cfu *P. fluorescens* BRG100*gfp* was detected in the middle and bottom sections of the columns from non-amended pesta; however, from corn starch-amended pesta, 4.3 and 2.8 $\log_{10}$ cfu *P. fluorescens* BRG100*gfp* was detected in the middle and bottom sections of the columns.

DISCUSSION

A formulation shelters and shields a large and efficacious population of bioherbicidal microorganisms from harsh environmental conditions encountered during storage, handling, shipping and delivery to the weed pest. Additional steps can be taken to promote shelf-life of bioherbicides such as reduced storage temperature and modified atmosphere packaging (Teshler *et al.* 2007). Granular formulations, including pesta, with water activity (a_w) of 0.06 to 0.3 promoted the survival of beneficial microorganisms (Mugnier and Jung 1985; Connick *et al.* 1996; Elzein *et al.* 2004). The results of this study suggests that survival of *P. fluorescens* BRG100 was best with a_w of 0.3 as compared to a_w of 0.5 and 0.8 over 5 months at 15°C and 16 months at 6°C.

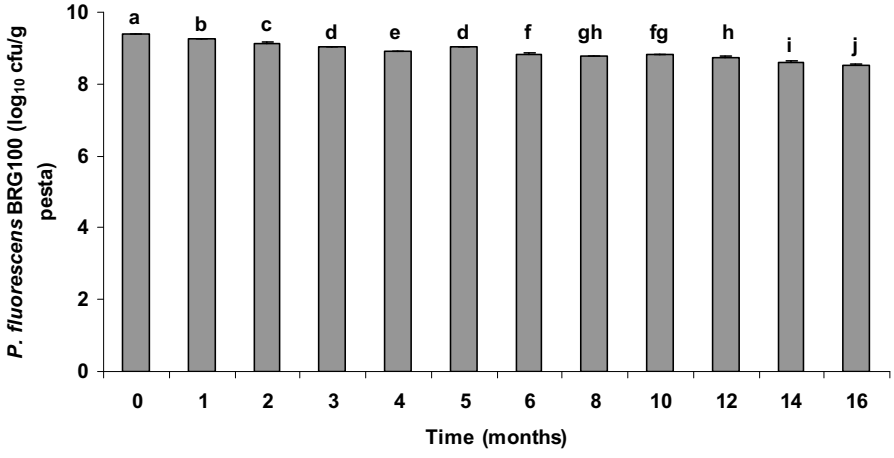

Fig. 2 Changes in the titre of *P. fluorescens* BRG100 in pesta granules over 16 months at 6°C. Analysis of valance was conducted on the a_w data series and columns with different letters indicate significant differences in $\log_{10}$ cfu/g pesta of *P. fluorescens* BRG100, r^2= 0.8823, $P \leq 0.05$. The data represents treatments means, n=5 replicates/treatment.

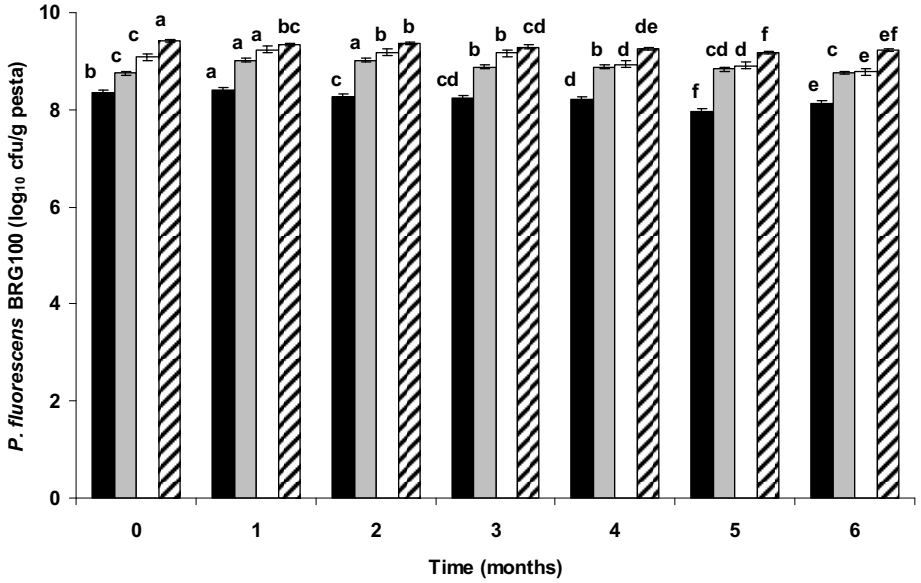

Fig. 3 Changes in the titre of *P. fluorescens* BRG100 in pesta granules with initial titres of 8.4 (black, 8.8 (light grey), 9.1 (white), and 9.5 (striped) $\log_{10}$ cfu *P. fluorescens* BRG100/g. Analysis of valance was conducted within each data series over the experiment duration and columns with different letters indicate significant differences in $\log_{10}$ cfu/g pesta of *P. fluorescens* BRG100, r^2= 0.7344, 0.1095, 0.8781 respectively, P≤0.05. The data represents treatments means, n=5 replicates/treatment.

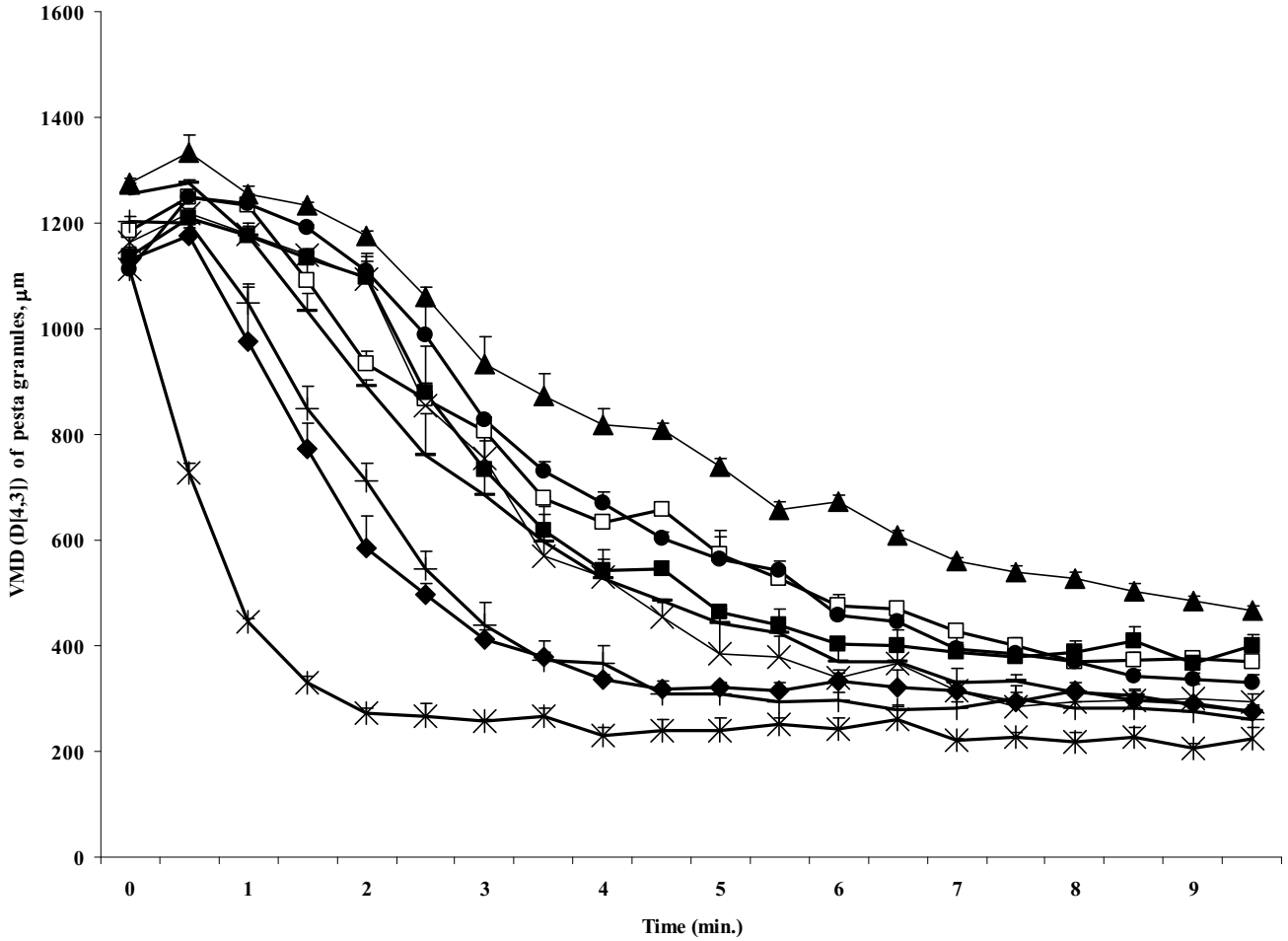

Fig. 4 Effect of starch on disintegration of pesta over time. Pea starch 26% ✳, 13% +, potato starch 26% ◆, 13% ■, corn starch 26% X, 13% •, rice starch 26 ▲ 13 ▬, and no starch □. The bars represent the standard error of the mean, n=3 replicates/treatment.

When the a_w was adjusted initially to 0.8 and 0.5, the titre of *P. fluorescens* BRG100 declined over the five month when stored at 15°C. In this study pesta was not sealed from the environment since we were also interested in collecting information on a_w stability in a practical container. The a_w changed the least when initially established at 0.3 and 0.5 (a_w ±0.07) and the greatest when set at 0.8 (decrease of a_w 0.2). This was likely due to the relative humidity of the temperature controlled room the pesta was stored in. Recently we have dried pesta containing *P. fluorescens* BRG100 to a_w 0.2 and seen similar titre stability after six months storage (data not presented). These results suggest that some of the difficulties that up until now have hindered development of non-spore forming bacteria as biopesticidal

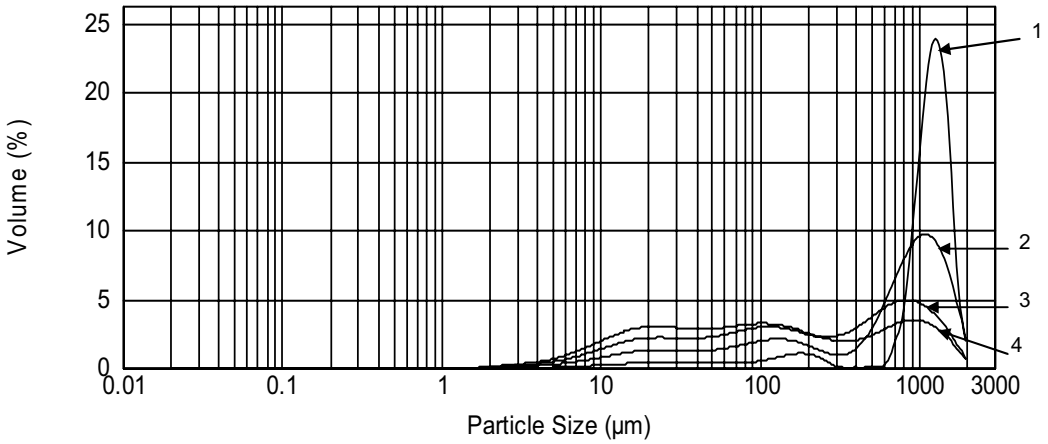

Fig. 5 Particle size distribution (µm) of 37% corn starch amended pesta granules using laser diffractometry (Mastersizer 2000). Time: sec, at 0, (1), 60, (2), 120 (3) and 180 (4).

Table 2 Effect of pea, potato, corn and rice starch amendment on disintegration time of pesta.

Pesta starch amendment (%)	Pesta disintegration rate $\Delta D[4,3]$ µm/sec	50% pesta disintegration in size (min)	Pesta swell time (min)
No starch	-2.52	2.5	1
Pea (26)	-8.51	0.8	0
Pea (13)	-5.07	1.5	0.5
Potato (26)	-5.09	1.3	0.5
Potato (13)	-3.52	1.5	1
Corn (26)	-3.60	2	1
Corn (13)	-2.34	3	1
Rice (26)	-1.73	4	0.5
Rice (13)	-2.84	2.5	0.5

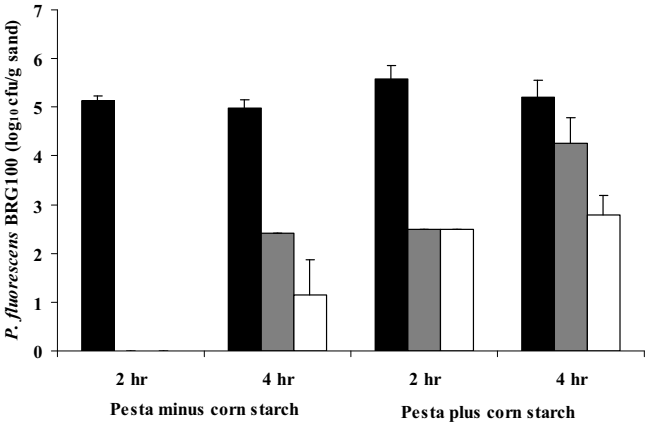

Fig. 6 Effect of corn starch amendment to pesta, 26%, on the dispersal of *P. fluorescens* BRG100*gfp*. Top (black), middle (light grey) and bottom (white) bars, of the laboratory sand columns. The data sets include error bars - standard error of the mean, n=5 replicates/treatment.

products in granular formulations maybe overcome by drying pesta granules to 0.3 a_w.

Contamination of pesta at 0.8 a_w was believed to be a leading contributor to the decline in the titre of *P. fluorescens* BRG100. Frequently *Rhizopus* sp was observed growing on the surface of batches of pesta with *P. fluorescens* BRG100 prepared at 0.8 a_w. Lowering a_w of foods is a strategy used by the food industry to increase the shelf life of their products. Since dehydration to a low a_w (~0.3) did not appear harm the bioherbicidal bacterial cells from this and other studies with fungal spores and conidia (Connick *et al.* 1996; Elzein *et al.* 2004), a similar strategy can be considered for preserving bacteria in pesta. Generally, growth of bacterial contaminants was inhibited at 0.7 a_w (Mugnier and Jung 1985); however, Mossel *et al.* (1995) reported that Gram-negative bacteria usually failed to grow if the a_w was below 0.95. The minimum a_w at which fungal contaminants grew was 0.61 (Beuchat 1983). Although steps can be taken to minimize contamination of pesta in the laboratory, large scale production of pesta for bioherbicides will likely have to rely on reducing the a_w or some other economically viable means to suppress the growth of contaminants.

Starch has been typically used as a filler and carrier for active ingredients in pharmaceutical formulations. Chemically modified starch such as sodium starch glycolate is widely used in oral pharmaceuticals as a disintegrant in capsule and tablet formulations. However, a chemically modified starch such as sodium starch glycolate would not be an economical choice as a dispersant for an agricultural product. Chan *et al.* (2006) reported optimum protease (subtilisin) activity in a granular detergent occurred for granules formulated with 10% corn starch and attributed this to starch enhanced disintegration.

This study reports a variety of disintegration patterns following modification of pesta with different native starch types and concentrations. Amylose and amylopectin are two major components of starch and the concentration of each has a critical role in starch functionality (Hoover 2001; Li and Yeh 2001; Lindeboom *et al.* 2004). Starch swelling is influenced by amylose content, starch granule size and amylopectin exposure to excess water (Li and Yeh 2001). Amylose, the lower molecular weight molecule influences water movement into the starch and exposes the amylopectin, the highly branched, high molecule weight molecule to excess water (Copeland *et al.* 2009). Specifically, amylose reduces the crystalline nature of the amylopectin promoting movement of water into starch. Starch granules added to room temperature water, as in the case of experiments reported here, swell slightly, as much as 15% depending on starch type. The size of starch determines its swelling functionality with larger granules having greater swelling power to smaller granules. The size of starches used in this study averaged from 6.4 µm in rice, 17.8 µm in corn, 19.9 µm in pea and 38.3 µm for potato (Li and Yeh 2001). Swelling is also determined by amylose content of starch, i.e. low amylose content results in low swelling ability. Pea starch amended pesta (26%) promoted most rapid disintegration with a half-life of 0.8 minutes, whereas rice starch amended pesta (26%) slowed disintegration, with a half-life of greater than 4 minutes, relative to the other starch amended and non-amended pesta granules. The greater amylose content in pea, potato and corn starches, 28, 23 and 25%, as compared to that of rice starch, 16%, may have contributed to the difference observed in the disintegration of the pesta granules (Li and Yeh 2001). However, as Copland *et al.* (2009) suggests, structural variations in the molecular architecture of amylopectin also contributes to starch functionality. Optimization of pest management may require quick or a slow release of the biopesticide from a granular formulation. Biopesticide dispersion from a granular formulation

is an important characteristic, since biopesticides must interact with the pest when it is most susceptible. Manipulating the disintegration rate of the granules with addition of starch will result in different dispersion characteristics of the biopesticide prompting delivery to the target in a timely manner. Relative granule disintegration rates are reported in this study using laser diffractrometry. It is not possible to predict granules disintegration rates in soil at this time. However, in laboratory experiments corn starch-amended pesta had completely disintegrated whereas, non-amended pesta was found in moist (39% water) soil after 7 days (Hynes and Hupka, unpublished). Therefore available moisture promotes corn starch amended pesta over that of non-amended pesta.

Dispersal of the active ingredient from a granular inoculant is critical for bioherbicide performance. Successful management of weed pests will depend on a formulation that promotes survival and dispersal of the bioherbicide so that the weed is colonized rapidly and adequately (Ramadan *et al*. 1990; Comeau *et al*. 1993). Addition of starch to pesta improved dispersal of the bioherbicide as compared to non-amended pesta. Additional research is underway examining the effect of the other starches on dispersal of *P. fluorescens* BRG100*gfp*. Greenhouse studies examining dispersal and movement of *P. fluorescens* BRG100*gfp* from pesta to the roots of green foxtail in soil are required.

CONCLUSIONS

Parameters establishing greater survival of the grass weed bioherbicide, *Pseudomonas fluorescens* BRG100, in pesta have been described. Pesta dried to a a_w of 0.3 significantly ($P \leq 0.05$) promoted survival of *P. fluorescens* BRG100 as compared to pesta at a_w of 0.5 and 0.8. Starch type and concentration added to pesta greatly affected the disintegration of the granules. The order of fast to slow disintegration following starch amendment was pea > potato > corn > rice. Corn starch-amended pesta promoted greater dispersion of *P. fluorescens* BRG100*gfp* in sand columns that non-amended pesta.

ACKNOWLEDGEMENTS

We thank Dan Hupka, Karen Sawchyn, Jon Geissler, Kent Chen and Breann Kozun for their work on this project, Dr. Peter Chang for enlightening discussions on starch functionality and Caressa Caldwell for preparing *P. fluorescens* BRG100*gfp*.

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