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

Russell K. Hynes* · Susan M. Boyetchko

ABSTRACT

A modified pesta granule was developed for *Pseudomonas fluorescens* BRG100, a bioherbicalic 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 0.8 a_w stabilized the population of BRG100 and, ii) the effect of starch on disintegration and dispersal of a green fluorescent protein transformant of *P. fluorescens* BRG100 from non-amended pesta. The ability to produce pesta granules with different disintegration and bioherbicide release characteristics predetermined 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 bioherbicial 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 treatments formulations are usually 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; Quinby et al. 1999). The pesta formulation, a matrix of durum wheat, kaolin, sucrose and the bioherbicidal 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 (Kremet 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 Sere-nade Max® respectively, *Pseudomonas* sp. are non-spore forming bacteria and therefore maybe more challenging to formulate.

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

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Bioherbicide</th>
<th>Weed host</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granule</td>
<td>Pesta</td>
<td>Alternaria cassiae</td>
<td>Sicklepod (Senna obtusifolia)</td>
</tr>
<tr>
<td></td>
<td>Pesta</td>
<td>A. crassa</td>
<td>Jimsonweed (Datura stramonium)</td>
</tr>
<tr>
<td></td>
<td>Pesta</td>
<td>Colletotrichum truncatum</td>
<td>Hemp sesbania (Sesbania exaltata)</td>
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<tr>
<td></td>
<td>Pesta</td>
<td>Fusarium lateritium</td>
<td>Velvetleaf (Abutilon theophrasti)</td>
</tr>
<tr>
<td></td>
<td>Pesta</td>
<td>Alternaria sp.</td>
<td>Swamp dodder (Cuscuta gronovii)</td>
</tr>
<tr>
<td></td>
<td>Pesta</td>
<td>F. oxysporum Sp. Foxy 2</td>
<td>Striga</td>
</tr>
<tr>
<td></td>
<td>Pesta</td>
<td>Colletotrichum truncatum</td>
<td>Hemp sesbania</td>
</tr>
<tr>
<td></td>
<td>Pesta</td>
<td>Pseudomonas fluorescens</td>
<td>Leafy spurge (Euphorbia esula)</td>
</tr>
<tr>
<td></td>
<td>Pesta</td>
<td>Alternaria alternata, Trematopha lonicola</td>
<td>(Amaranthus retroflexus)</td>
</tr>
<tr>
<td></td>
<td>Pesta</td>
<td>F. oxysporum Sp. orthoceras</td>
<td>Orobanche cumana</td>
</tr>
<tr>
<td></td>
<td>Pesta</td>
<td>F. oxysporum Sp. orthoceras</td>
<td>Orobanche cumana</td>
</tr>
<tr>
<td></td>
<td>Pesta</td>
<td>Pseudomonas fluorescens BRG100</td>
<td>Green ftoxial (Setaria viridis), wild oat (Avena fatua)</td>
</tr>
<tr>
<td></td>
<td>Pesta</td>
<td>P. fluorescens G2-11</td>
<td>Goosefoot, velvet leaf</td>
</tr>
<tr>
<td></td>
<td>Pesta, alginic phase</td>
<td>F. oxysporum Sp. orthoceras</td>
<td>Orobanche</td>
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<td></td>
<td>Alginic</td>
<td>A. cassiae, A. macrospora, F. lateritum, C. malvarum, Pythiostica sp.</td>
<td>None indicated</td>
</tr>
<tr>
<td></td>
<td>Pesta, Rice-alginic phase</td>
<td>F. oxysporum Sp. eteroxylol</td>
<td>Coca (Erythroxylum coca)</td>
</tr>
<tr>
<td></td>
<td>Barley granules</td>
<td>Phomopsis convolvulata</td>
<td>Bindweed</td>
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<tr>
<td></td>
<td>Alginic phase microencapsulate</td>
<td>F. avenaceum</td>
<td>Marsh reed grass</td>
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<tr>
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<td>starch (Stabilize process)</td>
<td>C. gloeosporioides, F. oxysporum</td>
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<td></td>
<td>starch (Stabilize)</td>
<td>Pseudomonas syringae</td>
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<td>Seed coating</td>
<td>Fuxarium nygamai</td>
<td>Striga</td>
<td>Zahran et al. 2008</td>
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<td>Liquid</td>
<td>Invert emulsion</td>
<td>C. truncatum</td>
<td>Hemp sesbania</td>
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<td>Alternaria cassiae</td>
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<td>Invert emulsion</td>
<td>Ascochyta pteridis</td>
<td>Bracken Pteridium</td>
</tr>
<tr>
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<td>water/oil/water (WOW)</td>
<td>C. orbiculare</td>
<td>Bathurst burr</td>
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<td></td>
<td>Water, 0.1% Tween 80</td>
<td>C. truncatum</td>
<td>Scintless chamomile</td>
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<tr>
<td></td>
<td>Water, surfactants</td>
<td>Myrothecium verrucaria</td>
<td>Sicklepod</td>
</tr>
</tbody>
</table>

**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: Na₂HPO₄ 3 g, KH₂PO₄ 3 g, NH₄Cl 1 g, NaCl 2 g, sucrose 2 g, MgSO₄.7H₂O 1.23 g, CaCl₂.2H₂O 0.015 g, ZnSO₄.7H₂O 0.06 g, molasses 200 ml, dH₂O 800 ml.

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

*Escherichia coli* S17-1λ 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: MgSO₄.7H₂O 0.2 g, NH₄(H₂)PO₄ 1 g, Na citrate 2 g, NaCl 5 g, kanamycin 50 mg, gentamycin, 30 mg, dH₂O 1000 ml. *E. coli* pAG408 lacks the ability to utilize citrate.

Preparation of pesta formulations of *P. fluorescens* BRG100 and *P. fluorescens* BRG100/gfp

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 log₁₀ cells/g) or water (control). The dough was extruded through a 1.2 mm 25% dome die using a Fuji Paudal Marumerizer, model QJ-230 (LCI Corp., Charlotte, NC, USA) and spheronized producing pesta granules approximately 1.2 mm in diameter.
Pesta water activity and disintegration. Hynes and Boyetchko
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 165°C, 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 bioherbicidal, 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 diffraactometry in a Mastersizer 2000 (Malvern Instruments, Worcestershire, UK) and reported as volume mean diameter (VMD) or De Brouckere mean diameter, D(4,3) = 4/3πD^3, where volume has a d^3 dependence and surface area a d^2 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 1 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 BRG100gfp 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^-3 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 BRG100gfp 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 BRG100gfp 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 BRG100gfp, ii) non-amended pesta with P. fluorescens BRG100gfp, 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 BRG100gfp. 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 BRG100gfp 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 ≤ 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 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 ≤ 0.05) (Fig. 2). Shelf-life studies indicated that 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
The titre of *P. fluorescens* BRG100 of pesta with a starting titre of 8.8 log\(_{10}\) cfu/g pesta (r\(^2\) = 0.9184, 0.9926, 0.9951, respectively, P ≤ 0.05). The data sets include error bars - standard error of the mean, n=5 replicates/treatment.}

\[\text{Effect of corn, pea, potato and rice starch dispersants on pesta disintegration}\]

*P. fluorescens* BRG100 (log\(_{10}\) cfu/g pesta) did not change significantly from 0 time and the cfu/g pesta did not change significantly from 0 time and the titres of *P. fluorescens* BRG100, r\(^2\) = 0.8823, 0.9926, 0.9951, respectively, P ≤ 0.05. The data sets include error bars - standard error of the mean, n=5 replicates/treatment.

**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* BRG100gfp 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* BRG100gfp cfu/g sand were detected (Fig. 6). After 4 hr incubation 2.4 and 1.1 log\(_{10}\) cfu *P. fluorescens* BRG100gfp 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* BRG100gfp 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 (Teslher 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. 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 ≤ 0.05). The data sets include error bars - standard error of the mean, n=5 replicates/treatment.](image1)

![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 ≤ 0.05. The data represents treatments means, n=5 replicates/treatment.](image2)
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 (\(a_w \pm 0.07\)) 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
products in granular formulations maybe overcome by drying pesta granules to 0.3 aw. Contamination of pesta at 0.8 aw 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 aw. Lowering aw of foods is a strategy used by the food industry to increase the shelf life of their products. Since dehydration to a low aw (~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 aw (Mugnier et al. 1996; Elzein et al. 1985); however, Mossel et al. (1995) reported that Gram-negative bacteria usually failed to grow if the aw was below 0.95. The minimum aw 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 aw 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 amylpectin 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 amylpectin exposure to excess water (Li and Yeh 2001). Amylose, the lower molecular weight molecule influences water movement into the starch and exposes the amylpectin, the highly branched, high molecular weight molecule to excess water (Copeland et al. 2009). Specifically, amyllose reduces the crystalline nature of the amylpectin 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. Pesta 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 Copeland et al. (2009) suggests, structural variations in the molecular architecture of amylpectin 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 diffraclometry. It is not possible to predict granules disintegration rates in soil at this time. However, in laboratory experimentation 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.

Dispersion 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 dispersion of the bioherbicide as compared to non-amended pesta. Additional research is underway examining the effect of the other starches on dispersion of P. fluorescens BRG100gfp. Greenhouse studies examining dispersal and movement of P. fluorescens BRG100gfp 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<sub>0</sub> of 0.3 significantly (P < 0.05) promoted survival of P. fluorescens BRG100 as compared to pesta at a<sub>0</sub> 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 disperion of P. fluorescens BRG100gfp in sand columns that non-amended pesta.

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REFERENCES


Comeau Y, Greer CW, Samson R (1993) Role of inoculum preparation and density on the bioremediation of 2,4-D-contaminated soil by bioaugmentation. Applied Microbiology and Biotechnology 38, 681-687


Li JJ, Yeh AI (2001) Relationships between thermal, rheological characteristics and swelling power for various starches. Journal of Food Engineering 50, 147-148


Müller-Störer D, Sau bern J (2007) A commercial iron fertilizer increases the survival of Fusarium oxysporum f. sp. orthocarpus propagules in a wheat flour-kaolin formulation. Biocontrol Science and Technology 17, 597-604


plant growth of *Convolvulus arvensis*. Weed Research 38, 175-182


