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# Microencapsulation: a Strategy for Formulation of Inoculum

R. S. WINDER[1], J. J. WHEELER[2], N. CONDER[1], I. S. OTVOS[1], R. NEVILL[3] AND L. DUAN[4]

[1]*Natural Resources Canada, Canadian Forest Service, Pacific Forestry Centre, Victoria, BC V8Z 1M5, Canada;* [2]*Prometheus Enterprises Inc., Surrey, BC V3S 8T3, Canada;* [3]*Formerly: Pacific Forestry Centre, Currently: Appraisal, Consulting, Research and Training, Inc., Urban Forestry, P.O. Box 1540 Lodi, CA 95241-1540, USA;* [4]*Inner Mongolia Forestry College, Xinjian Dongjie 275, Huhhot 010019, P.R. China*

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*A non-toxic phase separation method was developed for microencapsulation of inoculum used in biological control. Aqueous sodium alginate or gelatin and agar was mixed with inocula of various biopesticides and emulsified in a mixture of corn oil,* n*-hexadecane, and lecithin. Gelatin and agar globules gelled in the emulsion; alginate globules gelled after settling into a lower phase of aqueous* $CaCl_2$*. A layer of gelatinous material thus surrounded the inoculum as 'capsules'. Mixing with* n*-hexadecane reduced the specific gravity and surface tension of the oil, allowing aqueous extraction of the capsules. Successful extraction of alginate capsules depended upon lecithin (*$>0.17\%$*),* n*-hexadecane (*$>30\%$*), and* $CaCl_2$ ($>0.01$ *M) concentrations. Alginate-encapsulated macroconidia of* Fusarium avenaceum *caused* $23 \pm 3\%$ *leaf area damage to seedlings of marsh reed grass, versus* $4 \pm 3\%$ *for unformulated controls. In green foxtail seedlings, gelatin and agar-encapsulated conidia of* Bipolaris sorokiniana *caused 21.3 vs. 7.9 lesions per plant for encapsulated versus unformulated conidia. Mortality of Douglas-fir tussock moth larvae caused by a nuclear polyhedrosis virus was delayed when 23 polyhedral inclusion bodies (PIB) were incorporated into alginate capsules, but it proceeded normally for 2.3 PIB/ capsule, where efficacy was also higher versus positive controls. Microencapsulation enhances the activity of biological control agents and protects them from adverse conditions.*

**Keywords:** *fungi, virus,* Calamagrostis canadensis, Setaria viridis, Orgyia pseudotsugata, *bioherbicide, bioinsecticide, biopesticide, biocontrol, biological control*

Correspondence to: R. Winder. Tel: +1-250-363-0773; Fax: +1-250-363-0797; E-mail: rwinder@pfc.cfs.nrcan.gc.ca

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## INTRODUCTION

Formulation can be an important factor in the success of microbial pesticides (Auld & Morin, 1995). Simple improvements in the performance of biological agents can be achieved with water-soluble adjuvants, oils, and emulsions (e.g., Connick *et al*., 1991a; Bateman *et al*., 1993; Barnes & Moore, 1997; Green *et al*., 1998; Ibrahim *et al*., 1999). These materials have been used to improve efficacy by providing nutrients, protection from desiccation, etc., but they also have some limitations. Aqueous formulations may require a substantial amount of adjuvant material, and these materials may become diluted with rain or heavy dew. Oil formulations and emulsions also may require substantial amounts of material, and the material can be phytotoxic.

Agents can also be encapsulated in macroscopic ( $> 0.5$ mm diam.) granules of alginate, starches, and other materials (e.g., Walker & Connick 1983; Boyette and Walker, 1985; Connick *et al*., 1991b; McGuire *et al*., 1994; Shasha & McGuire, 1998), or in microscopic powders (Winder, 1999a). To encapsulate phytopathogens with alginate, for example, clay and sodium alginate are mixed with inoculum and the material is allowed to drip into a $CaCl_2$ solution, where it immediately gels into ca. 5-mm diameter beads that harden when dried (Walker & Connick, 1983). These large beads can improve persistence in the soil, but they are not easily applied to foliage. Some insect pathogens like viruses and bacteria need to be ingested to cause infection. The size of formulated inoculum particles is therefore important for these agents. Relatively small granules (500–800 μm) used to encapsulate insect pathogens can only be ingested by larger insects. Microscopic powders, on the other hand, are difficult to produce without also grinding or crushing the incorporated inoculum.

Microencapsulation of inoculum within polymeric materials may address some of these problems. First, a relatively small amount of non-toxic material can be used as the formulation component. Also, microcapsules can be formed from polymers that do not dilute in rain or heavy dew. Finally, microscopic particles can be efficiently dispersed from the air onto foliage, and even small, young larval stages of insects can consume them more readily than larger beads.

Methods developed for various biomedical and technical applications (e.g., Goosen *et al*., 1985; Sonnleitner & Fiechter, 1986; Mandenius *et al.*, 1987; Svoboda & Ourednicek, 1990; Wong & Chang, 1991; Chang, 1992; Uludag & Sefton, 1993) offer a starting point for microencapsulation of living cells. Current methods include extrusion (Goosen *et al*., 1985; Daigle *et al*., 1997, 1998), spraying (Chang, 1988), or mixing into solutions that gel or polymerize the encapsulating material (Nilsson *et al*., 1983). These techniques have various limitations for large-scale applications; for example, some rely on expensive materials, produce beads that are too large, or have low efficiency or cumbersome methodology.

A relatively easy and reliable method for microencapsulation involves chemical phase separation, wherein polymer capsules can be rapidly harvested from emulsions created in hydrophobic dispersion media (Fong, 1979; Komen & Groenendaal, 1991; Rickey *et al*., 1998). Typically, phase separation methods use agitation with the dispersion medium to emulsify aqueous solutions into microscopic globules. The globules are then allowed to gel or polymerize through a variety of means, and extracted from the oily dispersion medium by evaporation (Kitajima *et al*., 1970, Mitsuru *et al*., 1975; Morishita *et al*., 1976; Misselbrook *et al*., 1989), filter washing (Friend, 1992), or extraction with solvents or detergents (Fong, 1983, 1990; Tice & Gilley, 1995; Ramstack *et al*., 1997). Extraction with solvents is probably the simplest approach; although extant solvent extraction methods involve the use of toxic compounds, there are non-toxic, oil-miscible solvents such as *n*-hexadecane (Crooks *et al*., 1990) that are potentially suitable for microencapsulation of biological control agents.

This is the first report of a non-toxic phase separation method capable of microencapsulating biological control inoculum with various polymers. We tested a phase separation technique that employs *n*-hexadecane and corn oil to microcenapsulate biological control agents with alginate, agar, gelatin and other materials. We also tested the viability of

microencapsulated inoculum in dry environments, and the impact of microencapsulation on the efficacy of several biological control agents in laboratory and greenhouse situations. The overall objective of this research was to develop a general microencapsulation method that could be subsequently adapted and specialized by other researchers to help address their particular formulation requirements.

## MATERIALS AND METHODS

### Capsule Generation

*Dispersion medium.* To microencapsulate inoculum with alginate or agar and gelatin, corn oil (100 mL) was used as the base material for the emulsifying medium. Pure *n*-hexadecane (100 mL) was added to the corn oil to reduce its specific gravity and surface tension and permit the formation of larger ( > ca. 50-μm diam.) aqueous globules. Unbleached soya lecithin (10 μL) was added to this mixture as an emulsifying agent and stabilizer, to allow the slow fusion of smaller ( < ca. 50-μm diam.) globules into globules large enough to settle. The lecithin was a proprietary mixture of phosphatidyl choline, phosphotidyl inositol, phosphatidyl ethanolamine, and phosphatidyl serine (Lot L4455, Swiss Herbal Remedies Ltd., Richmond Hill, Ontario, L4B 4C2 Canada). The mixture of oil, *n*-hexadecane, and lecithin is referred to hereafter as the dispersion medium.

*Alginate encapsulation method.* A schematic outlining the general process of microencapsulation and extraction of microcapsules from the dispersion medium is given in Figure 1. An aqueous mixture of 1% (w/v) sodium alginate (450 μL) and inoculum (absent in controls) was added to 10 mL of dispersion medium, and mixed using a mechanical vortexer (30 s) to emulsify them. The resulting emulsion was poured onto an aqueous solution of 0.01 M $CaCl_2$, thus forming a mixture of two immiscible solutions. The mixture was allowed to stand for 5 min. This allowed the smaller alginate globules to fuse into larger globules and sink into the $CaCl_2$ solution. Exchange of sodium and calcium resulted in teardrop-shaped 'capsules' that consisted of inoculum surrounded by a layer of gelatinized alginate. Capsules were harvested from the $CaCl_2$ solution by vacuum filtration with a 0.45 μm Teflon® filter.

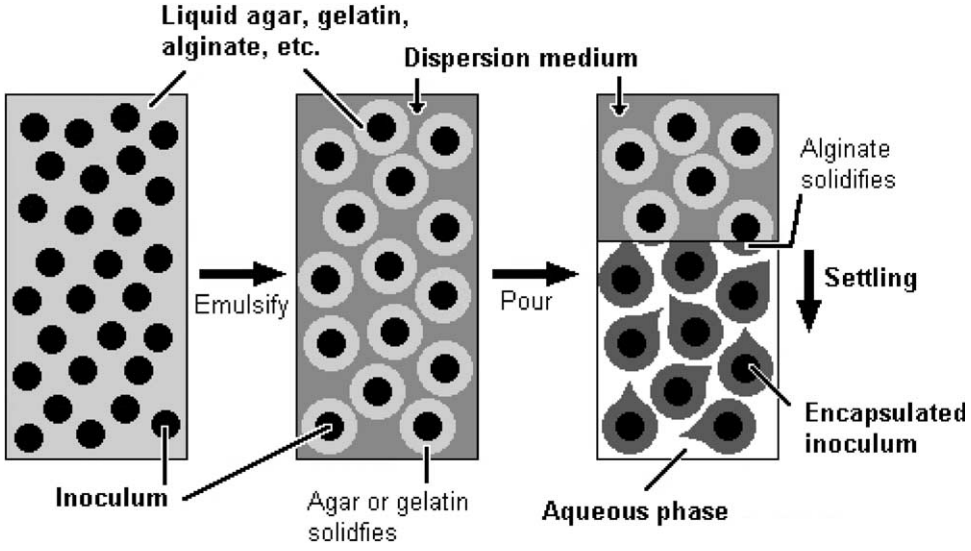


FIGURE 1. Outline of a general process for microencapsulation of inoculum using chemical phase separation.

*Gelatin and agar capsules.* The alginate method was modified to microencapsulate inoculum with various other types of gels. In this modified method, a mixture of agar and gelatin was used to limit the migration of lipiphilic inoculum into the dispersion medium. A molten aqueous mixture (1:1) of agar (10 g/L) and gelatin (10 g/L) was allowed to cool to ca. 40°C and inoculum was mixed with 5 mL of the lukewarm material. The resulting suspension was poured into 100 mL of dispersion medium and immediately agitated briskly before cooling and gelation occurred. After 1 min, the globules formed spherical capsules consisting of inoculum surrounded by a layer of gelatinized agar and gelatin. Capsules were extracted by pouring the emulsion onto 25 mL of water, gently agitating, and allowing the mixture to stand 10 min. The capsules settled into the water as the water phase settled below the oil.

**Effect of Chemical Components on Alginate Capsules**

Several completely randomized experimental designs were used to assay the effects of the components of the dispersion medium on capsule size and precipitation. The effects of lecithin on capsule size and number were compared in dispersion media containing either 0, 0.17, 0.33, or 0.5% (v:v) lecithin. The diameter of 14 capsules from each treatment was determined with a hemacytometer under a compound microscope; three experiments were conducted. The effects of oil on capsule number and size were compared in dispersion media containing 0 to 100% (v/v) oil in 10% increments. The diameter of 50 capsules from each treatment was determined as in the lecithin trial; three experiments were conducted. The interactive effects of oil and alginate on capsule number and size were compared in dispersion media combining 30, 50, or 70% (v/v) oil with 0.5, 1.0, 1.5, or 2.0% (w/v) alginate. The diameter of 14 capsules from each treatment was determined as in the lecithin trial; three experiments were conducted. Encapsulation was also compared by using either 0.01 or 1.0 M $CaCl_2$ to solidify the alginate. The diameter of 14 capsules from each treatment was determined as in the lecithin trial; three experiments were conducted. Data from all of the experiments on chemical effects were subjected to analysis of variance (Statsoft Inc., 1995).

**Encapsulation of Bioherbicide Inoculum**

*Microencapsulation of* Fusarium avenaceum *in alginate.* We used the alginate method to microencapsulate macroconidia of *Fusarium avenaceum* (Fr.:Fr.) Sacc. (Hypocreaceae: Hypocreales), a potential fungal agent for control of several weed species (Winder, 1999a). Conidia of *F. avenaceum* isolates PFC70 and PFC2562 were produced on potato dextrose agar using previously described methods (Winder, 1999a). To compare the viability of encapsulated and unformulated spores at various humidity levels, conidial germination of this isolate was assayed in an experiment using a completely randomized block design. Conidia were scraped from the surface of a plate culture with a scalpel, and half were mixed with 1% sodium alginate, while the other half (unformulated controls) were suspended in the $CaCl_2$ solution. The suspensions were filtered through two layers of cheesecloth to remove any clumps of hyphae or agar, and the conidia mixed with alginate were encapsulated. Blocks in the experiment consisted of a series of small glass chambers with controlled relative humidity (RH) of 5.5, 20, 33, 67, or 85% (Winston & Bates, 1960). Drops containing encapsulated conidia and controls were placed individually on glass slides positioned in the chambers, and incubated for 24 h at 20°C before germination was assessed with a microscope. The experiment was replicated three times. The activity of encapsulated and unformulated conidia of *F. aveaceum* isolate PFC2562 was tested on host plants, using a completely randomized experimental design. Seedlings of the host, marsh reed grass (*Calamagrostis canadensis* [Michx.] Beauv.), were grown in Petri dishes (36 seeds/plate) containing water agar (Winder, 1997). Plant assays (Oleskevich *et al*., 1998) were conducted using the marsh reed grass plate cultures, where seedlings within a plate were treated as subsamples. All plants within a plate (control) were inoculated with $10^6$ conidia/mL using an

artist's #5 paint brush to coat seedlings. In another plate, all plants were inoculated in the same fashion, using the same number of conidia concentrated into a suspension of $10^5$ capsules/mL. Plates were incubated with covers unsealed to prevent condensation. After 48 h, the mean RH inside the plates was measured with a humidity sensor (digital humidity/temperature meter 35519-043, VWR, Edmonton, Alberta, Canada), and the percentage of shoot damage was visually assessed for each seedling. This experiment was repeated eight times. Mean damage per plate was used to calculate treatment means and standard errors.

*Microencapsulation of* B. sorokiniana *in gelatin and agar.* The effect of microcapsulation with gelatin and agar on conidia of fungal weed pathogens was evaluated in a $2 \times 2 \times 2$ factorial test of *Bipolaris sorokiniana* (Sacc.) Shoemaker (Pleosporaceae: Dothidiales) on seedlings of green foxtail (*Setaria viridis* [L.] Beauv.). The fungal strain employed in this test was an isolate routinely used for formulation comparisons; fungal cultures and seeds of the host were provided by Dr. Gary Peng (Ecological Pest Management Section, Agriculture and Agri-Food Canada, 107 Science Place, Saskatoon, SK). Inoculum dosages employed in the test were intended to deliver non-lethal effects, in order to resolve treatment differences. All combinations of contrasting inoculum dosage (0 or $1 \times 10^5$ conidia/mL), microencapsulation (0 or $1 \times 10^4$ capsules/mL), and dew period (0 or 18 h) were tested.

Green foxtail seedlings were grown in $8 \times 8$-cm plastic pots in a temperature-controlled greenhouse, using methods previously reported for *C. canadensis* (Winder, 1999a). Temperatures during the trial were maintained at 25°C (day) and 20°C (night), and humidity was continuously monitored. Plants were inoculated 2 months subsequent to planting (eight- to nine-leaf stage), with five seedlings per pot. Conidia were generated on 200 g of autoclaved puffed wheat in a sterile plastic bag, using methods and conditions previously reported for *F. avenaceum* (Winder, 1999b). After 2 months of incubation at 20°C, the contents of the bag were added to 600 mL distilled water in a blender, homogenized for 1 min, and strained twice through a double layer of cheesecloth.

Two sets of conidia were harvested onto paper filters from 25 mL of the culture extract using vacuum filtration. The first set was scraped from the paper filter and microencapsulated using the agar/gelatin method, resulting in a 25 mL suspension of encapsulated conidia in water. The second set was suspended in 25 mL water without formulation, and served as a positive control for capsules. A third suspension (25 mL) was formed consisting of agar/gelatin capsules without inoculum, and served as a negative control for inoculum. Immediately after encapsulation, the suspensions were applied to separate sets of plants with a hand sprayer, resulting in ca. 50% leaf area wetted. One set of plants was also treated with 25 mL of water only, which served as a negative control for capsules. Plants were randomly arranged, with one set of inoculated plants and controls receiving a dew period through overnight (18 h) placement inside a large plastic bag. Another set of inoculated plants and controls did not receive the dew period. All combinations of inoculum presence, capsule presence, and dew presence were thus represented in the treatments. The number of dark brown necrotic lesions occurring on each plant was counted 48 h subsequent to inoculation. The experiment was replicated 18 times, and the mean number of lesions per pot was subjected to analysis of variance (Statsoft Inc., 1995). To test the rehydration properties of the dried capsules, all plants were misted with water until run-off after the lesions were counted, and were incubated for 96 h at ca. 80–90% RH in the absence of dew. The subsequent incidence of lesions per plant (pot mean) was again counted and subjected to analysis of variance (Statsoft Inc., 1995).

**Encapsulation of Bioinsecticide Inoculum**

A completely randomized experimental design was employed to assess the effect of microencapsulation on the efficacy of microencapsulated *Orgyia pseudotsugata* [McDunnough] (Lymantriidae: Lepidoptera) multicapsid nuclear polyhedrosis virus (*Op* MNPV) (Baculoviridae). *Op* MNPV is currently registered as TM Biocontrol-1® both in the United

States and Canada, and as Virtuss® in Canada for control of Douglas-fir tussock moth (DFTM; *O. pseudotsugata*) (Otvos *et al*., 1998). DFTM larvae were taken from a laboratory-reared colony (38 generations). The insects were reared on artificial diet (Thompson & Peterson, 1978) in Petri dishes (100 × 15 mm), 10 larvae/dish, at 25 ± 1°C, 50–60% RH with a 16-h diurnal photoperiod. Due to the large size of the experiment, dispersion media used in the basic encapsulation method were recycled to conserve materials, encapsulating lower viral doses before higher ones. Recycling caused variable efficiency in capsule production, but actual dosages remained uncorrected to ensure timely inoculation.

Viral inoculum used in the experiment consisted of *Op* MNPV polyhedral inclusion bodies (PIB) in form of a dry powder, obtained from lot 10 of the TM Biocontrol-1 product, stored at −10°C prior to use. Unformulated PIB were suspended in distilled water, using a stock of known concentration, and diluted to deliver dosages of 50, 100, 750 and 5000 PIB/μL. Formulated PIB were encapsulated using the modified basic method at similar target dosages, at rates of both 2.3 and 23.0 PIB/capsule. Capsule suspensions were passed through two layers of cheesecloth to remove any larger or fused capsules. The remaining capsules were harvested by vacuum filtration with a 20 μm Teflon® filter, rinsed with distilled water to remove any residual $CaCl_2$ solution, and then scraped from the filter with a small metal spatula and resuspended in distilled water.

Two different control treatments (distilled water and alginate capsules in distilled water) were also tested. The actual concentration of capsules in each suspension was measured with a hemacytometer as before. Because the inoculum of this virus must be ingested to infect, the suspensions (1 μL) were applied to artificial diet plugs (Duan & Otvos, 2001) with an electronic micropipette immediately after encapsulation, and the plugs were fed to newly molted fourth instar DFTM larvae, according to methods developed for use against western spruce budworm (*Choristoneura occidentalis* Freeman) (Li & Otvos, 1999). This design was intended to test the ability of the insects to consume capsules of this size, and to compare the eventual potency of formulated versus unformulated inoculum. It was not intended as a comparison of differences in feeding aversion, palatability, etc. There were 24 larvae (subsamples) treated per suspension. After inoculation, the larvae were reared individually on cubes of artificial diet in Solo® P100 Plastic Souffles (Solo Cup Company, Urbana, IL, USA) for 50 days, and mortality was evaluated daily. This experiment was replicated three times.

Multiple regression analysis was performed on the mortality data, using polynomial terms corresponding to significant ($P < 0.05$) main effects and interactions determined in analysis of variance (Statsoft Inc., 1995). Due to the variance from the target concentrations in formulated treatments, data for unformulated PIB and the two rates of formulation (2.3 PIB/capsule, 23 PIB/capsule) were analyzed separately, using actual concentrations. The distilled water controls were included as a zero dosage treatment in each analysis.

## RESULTS

### Effect of Chemical Components on Alginate Capsules

The concentration of the dispersion medium components had significant impacts on capsule size and production. Increasing lecithin concentration prevented mass fusion of capsules as the globules settled into 0.01 M $CaCl_2$, and decreased capsule diameters (Table 1). Settling did not occur in 0% lecithin, but it was complete at concentrations greater than 0.17%. In pure *n*-hexadecane, large, irregularly shaped capsules formed rapidly and completely. Adding oil allowed the slower formation of smaller, uniformly teardrop-shaped capsules, but globules were very small and did not settle into the aqueous phase in greater than 70% oil (Figure 2). The highest oil concentration also interacted with the highest and lowest alginate concentrations to prevent capsule precipitation (Table 2). The usual capsule length:width ratio of 1.4 ± 0.1 (±SE) increased to 2.2 ± 0.1 (±SE) when 1% alginate was emulsified in 90% *n*-hexadecane. No precipitation occurred in minimal solvent emulsified with buoyant (0.5%)

TABLE 1. The effect of lecithin concentration on capsule size

| Lecithin concentration (%) | Capsule diameter (μm)[a] |
|---|---|
| 0.00 | _[b] |
| 0.17 | 203.9±26.7 |
| 0.33 | 121.3±5.2 |
| 0.50 | 107.6±6.8 |

[a]Diameter values are mean±standard error.
[b]Capsules fused during formation.

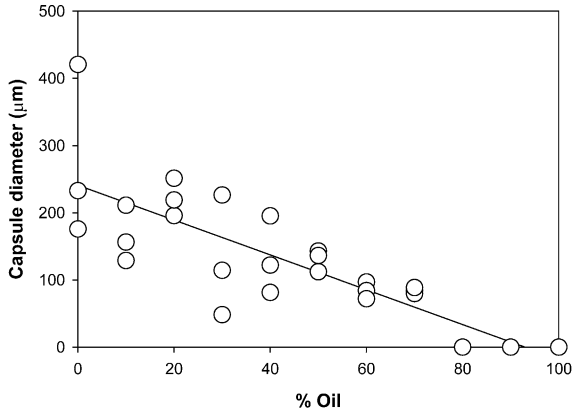


FIGURE 2. The effect of oil concentration on capsule size. The regression model ($Y = 240.9 - 2.6[x]$, $R^2 = 0.72$) is significant at $P < 0.05$.

TABLE 2. The interactive effect of alginate and oil on capsule diameter

| Alginate (%) | Oil (%) | Capsule diameter (μm)[a] |
|---|---|---|
| 0.5 | 30 | 133±11 |
| | 50 | 132±21 |
| | 70 | 0±0 |
| 1.0 | 30 | 180±26 |
| | 50 | 157±19 |
| | 70 | 124±13 |
| 1.5 | 30 | 108±13 |
| | 50 | 163±22 |
| | 70 | 115±10 |
| 2.0 | 30 | 96±20 |
| | 50 | 133±26 |
| | 70 | 0±0 |

[a]Diameter values are mean±standard error.

or viscous (2.0%) alginate solutions. The standard 0.01 M $CaCl_2$ solution allowed minimal settling of capsules (158±19-μm diam.; ±SE) from 50% solvent, while alginate settling into concentrated (1.00 M) $CaCl_2$ was complete, forming 85±6-μm diam. (±SE) capsules.

**Encapsulation of Bioherbicide Inoculum**

*Microencapsulation of* Fusarium avenaceum *in alginate.* Encapsulated macroconidia (Figure 3A) left in the 0.01 M $CaCl_2$ solution during the experiment were viable (95–100% germination), with germ tubes emerging from the capsules (Figure 3B). Only ca. 15% of encapsulated conidia germinated at RH of 20% or higher, when [$CaCl_2$] was held to 0.01 M (Figure 4). The viability of encapsulated conidia was maintained in RH as low as 20%, although most germ tubes remained within capsules (Figure 3C); this occurred in all treatments $< 55\%$ RH (Figure 4). Germination of unformulated conidia was minimal (ca. 1%) in low ($< 85\%$) RH and it was low (5%) even at the highest (85%) RH. In lower ($< 85\%$) humidity, the residual $CaCl_2$ solution formed a viscous film around many of the capsules, potentially regulating further evaporation. When encapsulated conidia were applied to the shoots of marsh reed grass in the two-leaf stage, most conidia germinated at 85% RH, but there was very little germination in unformulated controls at the same RH. On the host

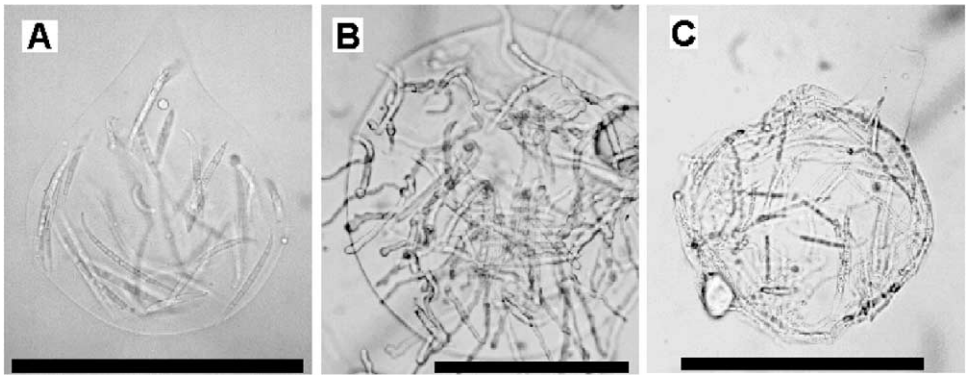


FIGURE 3. Microencapsulation of inoculum from *Fusarium avenaceum*. (A) Macroconidia of *F. avenaceum* microencapsulated in alginate. (B) Germinating hyphae extending from the encapsulated macroconidia. (C) Hyphae germinated from encapsulated *F. avenaceum* at 20% RH. The black scale bar in each panel corresponds to 100 μm.

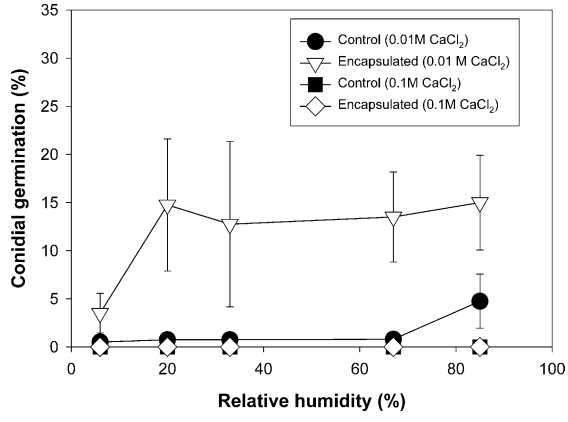


FIGURE 4. The effects of RH and $CaCl_2$ concentration on the germination of unformulated or microencapsulated macroconidia of *Fusarium avenaceum*. Vertical bars = SE.

plants, encapsulated conidia caused 23 ± 3% leaf area damage (mean ± SE) after 1 week, versus 4 ± 3% for unformulated controls.

*Microencapsulation of* B. sorokiniana *in gelatin and agar.* Although the lipophilic conidia of *B. sorokiniana* migrated to the oily dispersion medium when gelatin was used as the sole encapsulating material in preliminary trials, combining agar and gelatin allowed most of the conidia to remain in the aqueous phase in this experiment. Uncovered seedlings experienced moderately low (30–50%) RH during the night following incubation. Even in this low humidity, enough water remained in the lower portion of some of the inoculated plants to permit lesion formation. For unencapsulated conidia, lesions tended to form at points where inoculum was redistributed and concentrated by water droplets. The incidence of lesions in the other treatments was relatively minor; a few lesions (1–2/plant) appeared in controls. In the dew treatments, unformulated conidia caused minor damage (7.9 lesions/plant), but that level of damage was nearly trebled (21.3 lesions/plant) by microencapsulated conidia (Table 3). When all of the plants were misted, inoculum revived and proceeded to cause lesions, irrespective of encapsulation.

**Encapsulation of Bioinsecticide Inoculum**

Recycled dispersion media used for the insect assay produced 93.4 ± 27.5% (± SE) of the targeted PIB concentrations. Mortality of DFTM larvae was low in the check treatments: 9.2 ± 0.1% (14 days) and 13.8 ± 2.6% (21 days) for distilled water, or 17 ± 1.7% (14 days) and 6.7 ± 3.1% (21 days) for insects treated with alginate capsules lacking inoculum (± SE). Capsules did not appear to affect the insects; a response surface showing the interaction between time and the dosage of control capsules is not included here, because the $\beta$ value for capsule dosage was not significant after multiple regression analysis ($P > 0.05$) and most of the values for these controls were near zero. A response surface graph for the distilled water treatments also is not shown here, because there was no dosage factor applicable to water. Most of the larval mortality that did occur in the controls (including the distilled water control) appeared to result from improper molting that is normally associated with artificial diet. Mortality of larvae fed with unformulated PIB increased with time and dosage, and interaction between these main effects (Table 4; Figure 5). The viability of *Op*MNPV was preserved when PIB were formulated, where time, dosage, and interaction between the two were also significant (Table 4). At the 2.3 PIB/capsule rate, efficacy of the virus (larval mortality) was enhanced at lower dosages (Figure 6) compared to mortality caused by unformulated PIB. However, this enhancement was not as great at the 23 PIB/capsule rate, and the onset of mortality was also delayed (Figure 7).

TABLE 3. Effect of agar/gelatin microencapsulation on incidence of lesions caused by conidia of *Bipolaris sorokiniana* on seedlings of green foxtail (*Setaria viridis*)

| Conidial dosage ($10^5$/mL) | Microcapsule dosage ($10^4$/mL) | Dew period (h) | Damage 2 days post-inoculation (lesions/plant)[z] | Damage 6 days post-inoculation (lesions/plant)[z] |
|---|---|---|---|---|
| 0 | 0 | 0 | 1.2 A | 0.7 A |
| 0 | 1 | 0 | 0.0 A | 0.7 A |
| 0 | 0 | 18 | 0.9 A | 0.7 A |
| 0 | 1 | 18 | 0.0 A | 2.4 A |
| 1 | 0 | 0 | 3.5 A | 14.2 B |
| 1 | 1 | 0 | 0.8 A | 11.0 B |
| 1 | 0 | 18 | 7.9 B | 23.9 C |
| 1 | 1 | 18 | 21.3 C | 29.5 C |

[z]Means in the same column followed by the same letter are not significantly different according to the Newman–Keuls test ($P = 0.05$).

TABLE 4. Analysis of variance of Dougals-fir tussock moth mortality in the *Op* MNPV trials. Each trial represents a separate analysis, with distilled water controls serving as the zero dosage treatment

| Trial | Factor | df | *F* | *P* level |
|---|---|---|---|---|
| Unformulated PIB | Dosage | 4 | 1358.1 | 0.01 |
| | Time | 49 | 179.5 | 0.01 |
| | Dosage × Time | 196 | 7.7 | 0.01 |
| 2.3 PIB/capsule | Dosage | 12 | 191.4 | < 0.001 |
| | Time | 49 | 146.9 | < 0.001 |
| | Dosage × Time | 588 | 1.9 | < 0.001 |
| 23 PIB/Capsule | Dosage | 12 | 166.9 | < 0.001 |
| | Time | 49 | 149.9 | < 0.001 |
| | Dosage × Time | 588 | 1.4 | 0.01 |

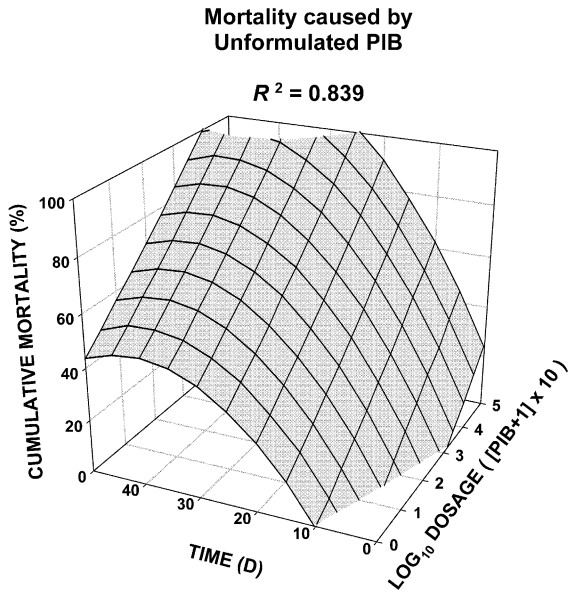


FIGURE 5. The effect of unformulated *Op* MNPV PIB (polyhedral inclusion bodies) dosage versus time on cumulative mortality of Douglas-fir tussock moth larvae. For the response surface, cumulative mortality is $37.3+10.4(\text{dose})+4.16(\text{time})+0.361(\text{dose}^2)+0.043(\text{dose}(\text{time}))-0.051(\text{time}^2)$, where dosage is $\log_{10}[(\text{PIB}+1)\times 10]$. The indicated dosage transformation was used to allow for display of all dosages on a log scale. Concentrations are actual dosages rather than targeted dosages.

## DISCUSSION

Encapsulation via phase separation was rapid, used little material, and was nontoxic to the two fungi and the entomopathogenic virus that were tested. In addition, the technique proved to be a facile method for producing polymer capsules of uniform size, unencumbered by hydrophobic materials. Extraction into an aqueous phase avoided oily inoculum, which can be difficult to concentrate or apply. For field applications, it will be necessary to understand how this technique will perform at larger scales; in vats or large mixing chambers, for example. Purification or reconstitution of recycled dispersion media may be necessary at such scales to avoid the kind of variable production efficiency noted in the virus experiment.

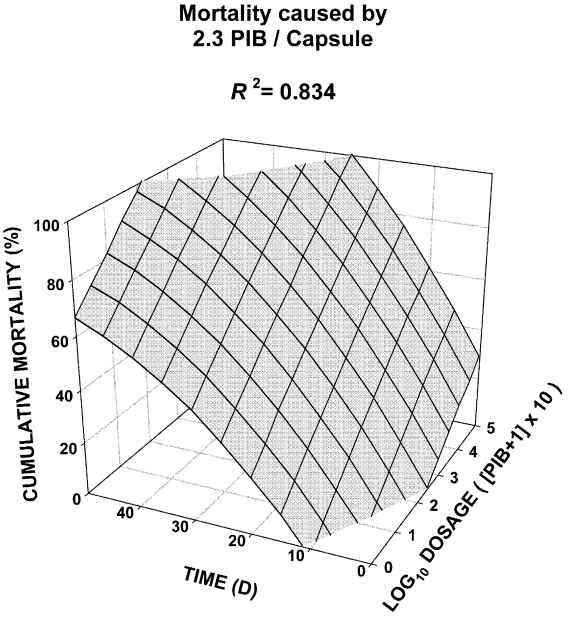


FIGURE 6. The effect of formulated *Op* MNPV PIB (polyhedral inclusion bodies) dosage versus time on cumulative mortality of Douglas-fir tussock moth larvae. PIB were formulated using an average of 2.3 PIB/capsule. For the response surface, cumulative mortality is $33.3+13.9(\text{dose})+3.24(\text{time})-0.285(\text{dose}^2)+0.045(\text{dose}(\text{time}))-0.025(\text{time}^2)$, where dosage is $\log_{10}[(\text{PIB}+1)\times 10]$. The indicated dosage transformation was used to allow for display of all dosages on a log scale. Concentrations are actual dosages rather than targeted dosages.

It should be noted that the agar/gelatin method has some limitations, because the addition of material to the dispersion medium results in cooling and emulsification must be achieved rapidly, before gelation occurs. Materials that gel at lower temperatures or techniques that control emulsion temperatures may be necessary to successfully scale-up such methods.

Agar, gelatin, or alginate microcapsules provide some protection from low RH, but they do not provide a panacea for overcoming the problems posed by lack of free moisture. Inoculum subjected to low RH levels will eventually desiccate if it is encapsulated in agar and gelatin, or it will fail to grow outside the capsule if it is encapsulated in alginate and applied with a $CaCl_2$ solution. But at higher RH levels, microcapsules do appear to provide a moderately improved infection court for phytopathogens by concentrating inoculum into a microscopic bead with humectant properties, and possibly by providing a point for droplets of dew to condense and persist.

If phytopathogens are to be exposed to constant atmospheric moistures below ca. 50% RH, then different polymers or formulation strategies may be necessary to support full infectivity of inoculum. Removal of alginate-encapsulated conidia from the $CaCl_2$ solution may be an important consideration, because drying and concentration of aqueous $CaCl_2$ on leaf surfaces reduced conidial germination. However, there was some germination even at the lowest RH levels, and this might be beneficial where performance in arid climates is necessary. Some conidia apparently equilibrated with the salt solution or tolerated it in some other way. Oils and other substances could also be used to coat the capsules and improve activity in arid conditions; this could be achieved, for example, by allowing agar/gelatin capsules to remain in the oil phase. Leaving fungal spores in the dispersion medium is still probably not the best option, since inoculum in this form would have to be applied immediately unless germination can be delayed. Desiccated capsules were capable of

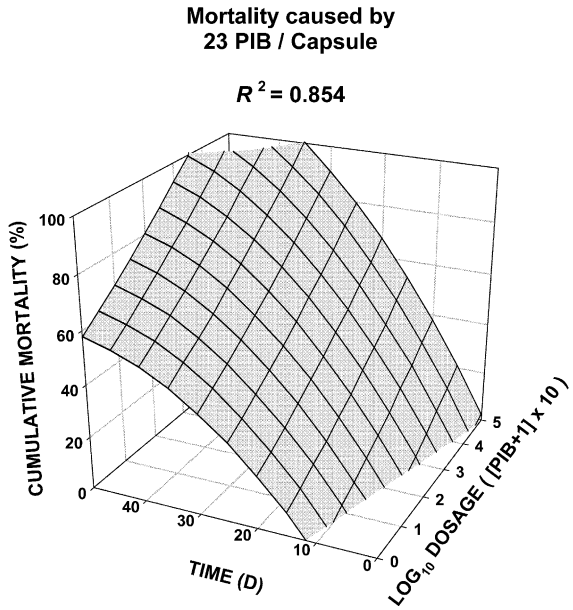


FIGURE 7. The effect of formulated *Op* MNPV PIB (polyhedral inclusion bodies) dosage versus time on cumulative mortality of Douglas-fir tussock moth larvae. PIB were formulated using an average of 23 PIB/capsule. For the response surface, cumulative mortality is $-34.4+5.33(\text{dose})+3.29(\text{time})+0.404(\text{dose}^2)+0.104(\text{dose}(\text{time}))-0.029(\text{time}^2)$, where dosage is $\log_{10}[(\text{PIB}+1)\times 10]$. The indicated dosage transformation was used to allow for display of all dosages on a log scale. Concentrations are actual dosages rather than targeted dosages.

supporting infection when humidity increased again in the *Bipolaris* study, suggesting that desiccation may provide a means for storage of microencapsulated inoculum for later use. Shelf life and potency of inoculum would still need to be established, however. Understanding the balance between humectant properties and properties necessary for desiccation and storage will be important in future development of microencapsulation methods. If the proper balance between these requirements can be achieved, then microencapsulation could be particularly useful in protecting spores, hyphae, or other types of cells where durable propagules are difficult to obtain.

In the green foxtail study, inoculum of *B. sorokiniana* apparently benefited from concentration into focused infection courts (capsules). Although the number of lesions formed by encapsulated conidia was roughly equivalent to the number of lesions formed by unformulated conidia, this occurred despite the 10-fold reduction in coverage for conidia concentrated within the capsules. Efficacy might be improved by enclosing fewer conidia within the capsules. For any particular agent, it will be critical to understand the relationship between lesion density and eventual leaf mortality before the benefits of microencapsulation can be accurately weighed.

For entomopathogens, encapsulation of *Op* MNPV reduced the viral dosage required to cause acceptable mortality of DFTM larvae at the lower PIB/capsule rate. Although the reasons for attenuation of this effect at higher PIB/capsule rates are unclear, it should be noted that a greater number of capsules ($10\times$) were delivered at the 2.3 PIB/capsule rate to achieve a dosage per insect equivalent to the 23 PIB/capsule rate, and that this may have affected mortality. Again, a better understanding of the relationship between inoculum coverage and mortality will be critical in evaluating the benefits of microencapsulation. Rapid delivery of acute viral dosages to insects could improve the performance and economic

viability of viral agents, and possibly enhance the delivery of other needed improvements such as UV protection or phagostimulants. This strategy could be particularly effective in cases where larvae avoid feeding on concentrations of pathogenic virions or bacteria, because the microcapsules could be used to mask the presence of the agent while remaining small enough to be ingested. Semiochemicals capable of attracting the insects could also be incorporated into such a strategy. Microencapsulation could also have additional benefit if the capsules are shown to protect pathogens from UV light or if they adhere to foliage.

It should be possible to develop economical microencapsulation methods capable of modulating microbial behavior or properties at a cellular scale. Compounds that promote a long, stable shelf life (Zhu, 1996), UV-blocking compounds (Prasad, 1995; Li & Otvos, 1999), adhesives (Shasha & McGuire, 1998), nutrients (Green *et al*., 1998), pheromones, phagostimulants, flavors, and pigments are just a few of the materials that might be incorporated as persistent components of microscopic formulations. Moreover, the general approach used in this study could be applied to materials other than alginate, gelatin, or agar. Various polymeric materials or mixtures of polymeric materials can also be utilized providing that the process used to produce them is not toxic to the inoculum. These other materials could have special properties, such as modified composition with respect to time (Ramstack *et al*., 1997; Rickey *et al*., 1998) temperature, or UV light. In future research, these additional components could be added to inoculum in a persistent, sprayable format, with the specific composition depending on the special requirements of the agent in question.

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