Encapsulation of Potential Biocontrol Agents in an Alginate-Clay Matrix

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Trade names and company names are included for the benefit of the reader and do not imply endorsement or preferential treatment of the product.

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ABSTRACT

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A method to encapsulate microorganisms that have potential to control plant diseases was tested. Aqueous solutions containing 1% sodium alginate and 10% Pyrax® were comminuted in a blender. Solutions were amended singly with either ascospores or conidia of *Talaromyces flavus* (isolate Tf1 or Tf1-1); conidia of *Gliocladium virens* (isolate GL3), *Penicillium oxalicum*, or *Trichoderma viride* (isolate T-1-R9); or cells of *Pseudomonas cepacia* (isolate POP-S1). The alginate-Pyrax®-propagule suspension was dripped through Pasteur pipettes into a solution of either 0.25 M $CaCl_2$ or 0.1 M Ca gluconate which caused the formation of solid aggregates. The aggregates were dried overnight and stored under room conditions. Populations of encapsulated organisms were estimated after 0, 2, 4, 8, and 12 wk by dissolving the pellets in a mixture of 8.7×10^{-3} M KH_2PO_4 and 3.0×10^{-2} M Na_2HPO_4, and dilution plating on semiselective media. All fungi, but not *Pseudomonas*, were viable after pellet formation in $CaCl_2$. All organisms were viable longer after pellet formation with Ca gluconate. Initial populations ranged from 10^5 to 10^8 propagules per milliliter of alginate suspension. These populations declined during the test period: losses were 10 to 100-fold after 4 wk.

Several techniques have been employed for the delivery of biocontrol agents. For example, biocontrol organisms have been applied in liquids (12,16), in organic matter (25), as seed or seed-piece treatments (8,10,26), and in vermiculite or in clays such as Pyrax® (9,17). The concomitant addition of antagonists and germinated seedlings in Laponite® gel also has been investigated (5). Formulation and application methods are often of paramount importance in effecting biological control (19,20,22). In addition, formulation may facilitate shipping and storage of the biocontrol agent.

Reports of the incorporation of mycoherbicides into sodium alginate suggested that this method may have potential for use with biocontrol fungi (24). The resulting granular preparation is lighter than liquids, and more uniform and less bulky than most organic matter preparations.

The reaction between aqueous solutions of sodium alginate and certain metal cations such as Ca^{++} to form gels (14) has been used to formulate myco- and chemical herbicides (1,4,24). Clays, such as Pyrax®, or other materials are added as bulking agents. The process produces biodegradable pellets of relatively uniform size which are convenient for storage and are compatible with agricultural machinery.

The objectives of this study were to determine whether: biocontrol organisms would survive encapsulation in alginate, initial concentration of the organisms in alginate affects survival, spore type affects survival in alginate, and the gellant used to solidify the alginate affects survival. A preliminary report has been published (7).

MATERIALS AND METHODS

Ten grams of sodium alginate (Fischer Scientific Company, Fair Lawn, NJ) and 100 g of Pyrax® (pyrophyllite, hydrous aluminum silicate) (R. T. Vanderbuilt Co., Norwalk, CT) were added to 1-L sterile distilled water and comminuted in a blender for 1 min. One hundred and eighty milliliters of the mixture was dispensed into a sterile deep dish with a coupling at the base (Fig. 1). The mixture was amended with 20 ml of the biocontrol agent, consisting of either cells of *Pseudomonas cepacia* Burkholder (isolate POP-S1) in 1.5×10^{-2} M KH_2PO_4 + 8.2×10^{-2} M K_2HPO_4-buffered 0.1% saline (pH 6.8), or aqueous suspensions of ascospores or conidia of *Talaromyces flavus* (Klöcker) Stolk & Samson [wild-type isolate Tf1 or benomyl-resistant isolate Tf1-1 as described by Katan et al (11)], conidia of *Penicillium oxalicum* Currie and Thom, conidia of *Gliocladium virens* Miller, Giddens & Foster (isolate GL-3), or *Trichoderma viride* Pers. ex Gray [isolate T-1-R9 as described by Papavizas and Lewis (21)]. Ascospores were produced by growing *T. flavus* on potato-dextrose agar (PDA) for 4 wk at 30 C in the dark (15). *T. flavus* was grown on a molasses-corn steep medium in the light at 25 C for 1 wk to produce conidia (15). Conidia of *G. virens* were produced by growing the fungus on V-8 juice agar for 1 wk at 25 C and conidia of *P. oxalicum* and *T. viride* were produced by growing the organisms on PDA for 1 wk at 25 C. Bacteria were grown for 4 days on half-strength trypticase soy agar (Difco Laboratories, Detroit, MI) with 1.0% glucose.

The alginate-propagule-Pyrax® suspension was stirred continuously while it was dripped through Pasteur pipettes with a 1-mm-diameter orifice into a solution of 0.25 M $CaCl_2$ or 0.1 M $C_{12}H_{22}CaO_{14}$ (calcium gluconate) (Figs. 1 and 2). Spores or cells were added at concentrations ranging from 10^5/ml to 10^8/ml. Concentrations recovered prior to pellet formation are listed in Table 1. The pellets were dried overnight in a laminar air flow hood.

Viable populations were determined before exposing the organism to the calcium salt, immediately after exposure to the calcium salt (pellet formation), at 24 hr (pellets first dry), and at 2, 4, 8, and 12 wk after pellet formation. Pellets were stored under room conditions in screw-capped jars. The pellets were insoluble in water but were disintegrated in a mixture of 8.7×10^{-2} M KH_2PO_4 and 3.0×10^{-2} M Na_2HPO_4 (pH 7.7) and assayed by dilution plating. Dilutions of propagules of *Trichoderma, Gliocladium*, and *Penicillium* were made on PDA amended with 0.3 g of chlortetracycline-HCl and 1 ml of Tergitol® per liter. *T. flavus* was plated on a semiselective medium recently developed for culturing

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it (15), and *Pseudomonas* was plated on a selective medium (3). Treatments of *Pseudomonas* were replicated once and repeated with five plates each at 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions. Fungal treatments were replicated four times. For each sampling time, at least two different 10-fold dilutions were performed with two plates per dilution per replicate for the fungi. Choice of dilutions for each sampling time was based on the population measured at the previous sampling time. The experiment was performed four times.

RESULTS

All organisms survived the pellet formation process when Ca gluconate was used as the gelling agent (Table 1). All fungi, but not *Pseudomonas*, were viable after pellet formation when $CaCl_2$ was used as the gelling agent. For *P. oxalicum, T. viride*, and *T. flavus*, there was no propagule viability loss from the time the liquid alginate suspensions were prepared to the time the pellets were dried. With *G. virens*, survival of conidia was 78.4 and 57.0% for $CaCl_2$ and Ca-gluconate, respectively. Cells of *Pseudomonas* were not viable after pellet formation with $CaCl_2$ and only 0.9% of the cells survived pellet formation with Ca-gluconate as the gelling agent. The maximum survival time for all organisms in alginate pellets was greater than 12 wk (Table 1). Although the pellets did not disintegrate in water, colonies developed when pellets were placed on appropriate media, even when viability was only 0.9% of the original.

The time required for the population of viable propagules to drop to half of that measured when the pellets were first dried (24 hr) (50% effective survival, ES_{50}) was calculated the same as an LD_{50} by the probit-line method (Table 1) (2). These values indicate that in most cases half of the original population of most organisms died within the first 2–3 wk after pellet formation with $CaCl_2$. *T. flavus* generally survived longer than the other organisms and ascospores of the wild-type isolate generally had an ES_{50} beyond 12 wk. Even though only a small percentage (0.9%) of the cells of *P. cepacia* survived pellet formation, the ES_{50} for the population surviving pellet formation was approximately 11.4 wk. Analysis of variance indicated that survival after 12 wk of all organisms encapsulated by using Ca gluconate as a gelling agent was significantly greater than when $CaCl_2$ was used ($P \leq 0.05$). The initial concentration of the propagules did not affect survival ($P \leq 0.05$).

DISCUSSION

These experiments were performed to determine the feasibility of encapsulating biocontrol agents in alginate. Even though the viability of most organisms decreased appreciably (up to 99.1%

TABLE 1. Survival in alginate pellets at room temperature of selected fungi and a bacterium used as biological control agents

Isolate and propagule	Gellant	Concentration before pellet formation (equivalent propagules/pellet)	Survival during pellet formation (%)	ES_{50}[a] (wk)	Propagule viability after 12 wk	
					Per gram of pellet ($\times 10^3$)	Per pellet
Gliocladium virens (Gl-3)						
Conidia	CC[b]	6.7×10^5[c]	89.0	0.2	0.18	12
		2.0×10^5	40.0	0	0.45	30
		3.4×10^4	78.4	0.2	1.10	73
	CG	3.4×10^4	57.0	2.2	35.00*[d]	2.3×10^3
Penicillium oxalicum						
Conidia	CC	4.3×10^6	100.0	0	0.08	5
		4.4×10^5	100.0	0	0.12	8
		9.3×10^3	100.0	1.9	0.05	3
	CG	9.3×10^3	100.0	>12.0	8.50*	5.7×10^2
Pseudomonas cepacia (POP-S1)						
Cells	CC	4.4×10^5	0	0	0	0
	CG	4.4×10^5	0.9	11.4	1.00*	67
Talaromyces flavus (Tfl)						
Ascospores	CC	2.7×10^4	100.0	>12.0	220.00	1.5×10^4
		3.3×10^3	100.0	>12.0	17.50	1.2×10^3
		1.7×10^2	100.0	6.4	5.50	3.7×10^2
	CG	1.7×10^2	100.0	>12.0	390.00*	2.6×10^4
Conidia	CC	1.5×10^6	100.0	0	0.12	8
		1.3×10^4	100.0	5.4	0	0
		1.5×10^3	100.0	0	0	0
	CG	1.5×10^3	100.0	8.6	405.00*	2.7×10^4
Talaromyces flavus (Tfl-1)						
Ascospores	CC	2.7×10^4	100.0	0.7	0	0
		2.0×10^3	100.0	4.0	0	0
		3.9×10^2	100.0	5.5	2.00	1.3×10^2
	CG	3.9×10^2	100.0	>12.0	255.00*	1.7×10^4
Conidia	CC	2.0×10^7	100.0	0	0.10	7
		2.6×10^5	100.0	9.7	0	0
		7.3×10^3	100.0	0	0.20	13
	CG	7.3×10^3	100.0	2.8	255.00*	1.7×10^4
Trichoderma viride (T-1-R9)						
Conidia	CG	6.8×10^6	100.0	2.7	0	0
		6.7×10^5	100.0	2.7	0	0
		6.6×10^4	100.0	2.5	0	0
	CG	6.6×10^4	100.0	>12.0	940.00*	6.4×10^4

[a] ES_{50} = the length of time after drying for 50% loss of the propagule viability.
[b] CC = calcium chloride; CG = calcium gluconate.
[c] Data from one experiment.
[d] Values marked by an asterisk are significantly greater ($P \leq 0.05$) compared to population values for their respective initial, air-dry concentrations.

loss) in 2–3 wk, *P. cepacia* in pellets gelled with Ca-gluconate remained viable for at least 12 wk; hence, the pellets could still function as point sources of living propagules as indicated by the production of colonies from pellets containing propagules that showed low, but measurable, viability when placed directly on culture media.

The greatest percentage losses in viability of the bacterium generally occurred during formation and drying and during the first 2–3 wk for the fungi. At the concentrations of Ca^{++} salts that we used, long-term survival was higher ($P \leqslant 0.05$) in pellets made in Ca-gluconate than in those made in $CaCl_2$. For example, the ES_{50} for *P. oxalicum* was 1.9 wk with $CaCl_2$ and more than 12 wk with Ca-gluconate. Alginate plus Ca-gluconate solidified culture media is reported to be equivalent to agar for culturing of common bacteria such as *Escherichia coli* (Migula) Castellani and Chalmers and fungi such as *Aspergillus* sp. and *Penicillium* sp. (6). Our study is the first report of the use of Ca-gluconate as a gelling agent for sodium alginate to enhance the survival of encapsulated microorganisms.

Survival varied with spore type, isolate, and species (Table 1). Ascospores of *T. flavus* survived longer than conidia. This may be due to the spore morphology and/or physiology. Ascospores are long-term, overwintering structures, while conidia are short-term and more ephemeral. Ascospores of *T. flavus* also are known to survive better in the field and to be more compatible with fungicides than conidia (8). Ascospores and conidia of the Tf1 isolate of *T. flavus* survived longer than those of isolate Tf1-1 in alginate granules. Ascospores of *T. flavus* have previously been reported to survive longer than conidia in field soil or dried in Pyrax® alone (9). Differences also were observed between different species of the same genus. Conidia of *P. oxalicum* did not survive as well as those of *P. dangardeii* (the anamorph of *T. flavus*).

Although the initial concentration of the propagules did not affect survival ($P \leqslant 0.05$), for some organisms there was apparently an optimum concentration. For example, ascospores of Tf1 survived better at initial concentrations of 10^7/ml and 10^8/ml than at 10^6/ml. In contrast ascospores of Tf1-1 and conidia of *P. oxalicum* survived better at 10^5/ml than at 10^7/ml.

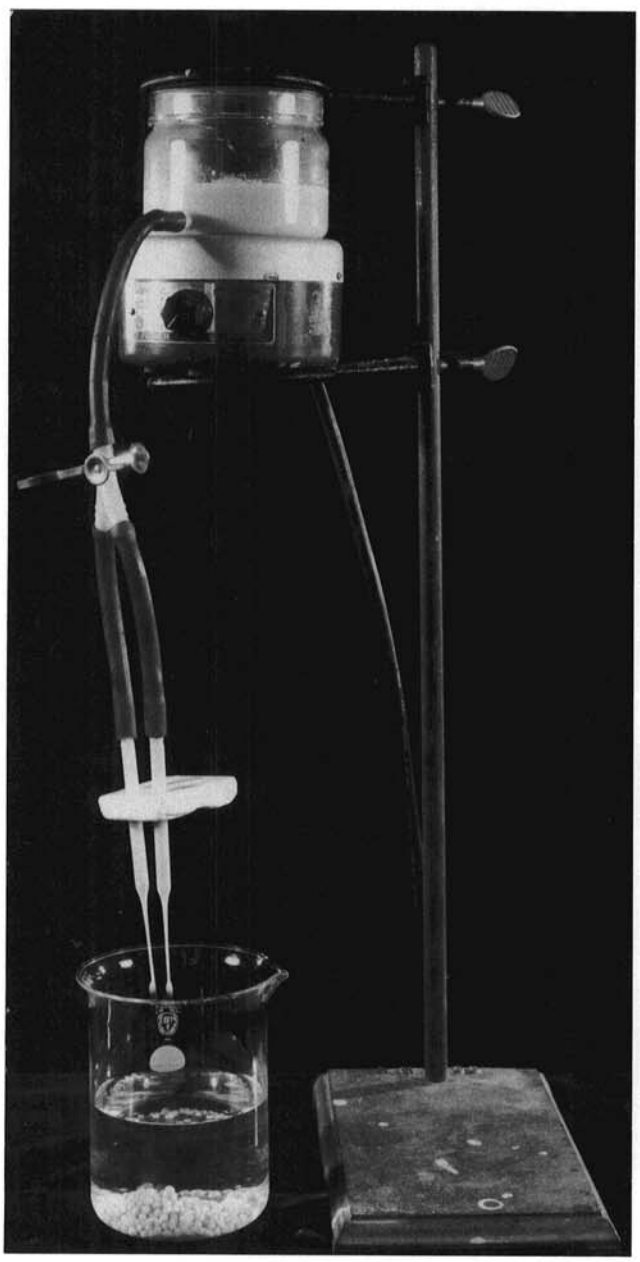

Fig. 1. Apparatus used to form alginate-clay pellets. An aqueous suspension of biocontrol organism, sodium alginate, and a bulking agent (Pyrax®) is dripped into a solution of either calcium chloride or calcium gluconate. The calcium displaces the sodium, causing formation of a solidified shell around the drop.

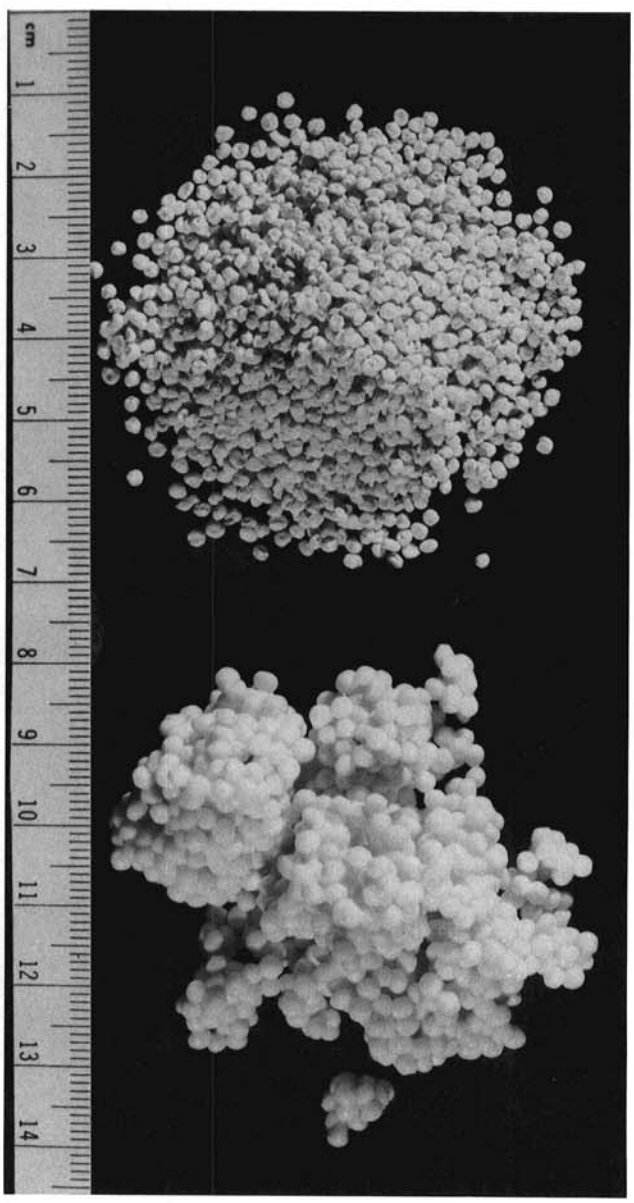

Fig. 2. Alginate-clay pellets immediately after formation (bottom) and after being dried (top).

Although survival for 12 wk at room temperature should be sufficient time to make biocontrol formulations feasible for use, survival of *T. flavus* in alginate was less than in Pyrax® alone at 25 C (9). Improvement of formulations and delivery systems for biocontrol agents should facilitate the introduction of these organisms as well as make them commercially more feasible by making them compatible with current production practices. The alginate formulations have several advantages. The process is inexpensive and very versatile in size and composition. The pellets have been formulated for a variety of agricultural compounds including mycoherbicides (24) and herbicides (1). Sodium alginate also has been used in the formulation of insect juvenile hormones (23) and slow-release fertilizers (18). Proliferation of the biocontrol agent may be enhanced by substitution of food bases such as bran for the clay (13). Sodium alginate and $CaCl_2$ are commonly used as food additives and are considered to be nontoxic to nontarget organisms. This technique may also be useful for uniformly infesting test areas with propagules of plant pathogens.

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