

# SURVIVAL OF THE NEMATODE-ANTAGONISTIC FUNGUS *VERTICILLIUM LECANII* IN ALGINATE PRILLS<sup>1,2)</sup>

BY

S. L. F. MEYER<sup>3)</sup> & R. J. MEYER<sup>4)</sup>

<sup>3)</sup> USDA, ARS, Nematology Laboratory, Bldg. 011A, Rm. 153, BARC-West, 10300 Baltimore Avenue, Beltsville, Maryland 20705-2350, U.S.A.; <sup>4)</sup> Department of Botany, University of Maryland, College Park, MD 20742-5815, U.S.A.

Strains of the fungus *Verticillium lecanii* have been incorporated into alginate prills and studied as potential management agents for plant-parasitic nematodes. Since long-term storage is essential in material intended for field applications, the viabilities of two *V. lecanii* strains in prill formulations were recorded after storage in the freezer (-15°C), refrigerator (4°C), ambient room conditions (ca. 25°C), and glasshouse (15-43°C). In a wheat bran formulation, both strains generally grew from 90%-100% of prills stored in the refrigerator for up to 43-45 months (although one batch of the mutant strain lost viability by 12 months). Viability at -15°C did not always remain as high as when prills were stored at 4°C. Viability of both strains was 0% within 2 years at ambient room conditions and within 7 months in the glasshouse. Similar results were obtained with the mutant strain in a pyrophyllite formulation. One batch of wheat bran prills made with the mutant strain plus soybean cyst nematode sex pheromone (vanillic acid) was also tested. Fungus viability was 0% after 25 months (freezer, refrigerator), 4 months (ambient room conditions), and 1 month (glasshouse). Fungus in prills made with the pheromone analogue syringic acid had a similar longevity to fungus in prills without added pheromone.

*Keywords:* biocontrol, biological control, alginate prills, formulation, delivery, viability, *Verticillium lecanii*.

Efficacious formulations are essential for successful application of microbial pest control agents. Consequently, development of formulations is a high priority in biological control research. Alginate formulations (Fravel *et al.*, 1985) have been used for delivery of biocontrol fungi in a number of studies, including investigations on management agents for plant-parasitic nematodes (Cabanillas *et al.*, 1989; Kim & Riggs, 1992; Meyer, 1994; Meyer & Huettel, 1993; Schuster & Sikora, 1992a, 1992b). These formulations can be made with a food base for the fungus or with an inert carrier. In addition, compounds such as fertilizers and chemical pesticides can be added as needed to enhance control, promote plant growth, or reduce competitive effects of other microorganisms (Fravel & Lewis, 1992).

---

<sup>1)</sup> Mention of a trademark or proprietary product does not constitute a guarantee, warranty, or endorsement by the United States Department of Agriculture and does not imply approval to the exclusion of other similar products.

<sup>2)</sup> Prills are granules manufactured in a specific way; they are homogeneous in character and have a smooth surface.

No matter how effective the formulation proves to be, however, the product can only be used if it gives the organism a shelf life that makes its production, distribution and sale as a biocontrol agent both economically and practically feasible. An important component of shelf life is the viability of the biocontrol organism in the formulation. For this reason, the viabilities of various fungi in alginate formulations have been investigated (Fravel *et al.*, 1985; Jackson *et al.*, 1991; Lewis & Papavizas, 1985; Papavizas *et al.*, 1987). Such studies have been conducted on only two biocontrol fungi for nematodes: *Paecilomyces lilacinus* and the unidentified fungus designated ARF18 (Cabanillas *et al.*, 1989; Kim & Riggs, 1992). *Verticillium lecanii* (A. Zimmermann) Viégas, which has been sold for insect control (Hussey, 1984), has also shown promise as a management agent for plant-parasitic nematodes, as indicated by laboratory, glasshouse and field studies (Gintis *et al.*, 1983; Hänssler, 1990; Hänssler & Hermanns, 1981; Meyer *et al.*, 1990; Meyer & Huettel, 1993). For some biocontrol studies, a wild type strain of this fungus and a UV-induced mutant with increased tolerance to the fungicide benomyl (Meyer, 1992) were incorporated into alginate prills (Meyer, 1994; Meyer & Huettel, 1993). The objective of the study reported here was to determine survival of the two *V. lecanii* strains in the intact alginate prills during storage under conditions that might be used by a grower or supplier. Measured variables included temperature, addition of vanillic acid or syringic acid to the prills as potential nematode management agents, and inclusion of pyrax vs. wheat bran as carriers in the formulation.

#### MATERIALS AND METHODS

##### *Production of prills*

Two strains of *V. lecanii* were tested for longevity in storage: Mutant strain M2S1 (Meyer, 1992; deposited at the Agricultural Research Service Culture Collection, NRRL no. 18726) and wild type strain (American Type Culture Collection no. 58909). Prill production began by the inoculation of each strain onto potato dextrose agar (PDA) in Petri dishes. After incubation for one week at *ca.* 25°C, the fungus colonies were put into a Waring blender, homogenized in potato dextrose broth (PDB, DIFCO), and poured into Erlenmeyer flasks. Each 1 litre Erlenmeyer flask received the equivalent of one entire Petri dish colony in broth suspension, and the broth volume per flask was adjusted to 250 ml with additional PDB. The flasks were rotated (*ca.* 200-240 rpm) on orbital shakers for 2 days at room temperature (*ca.* 25°C). The fungus was collected from the broth by centrifugation at 13,000 *g* (9000 rpm for 10 min) in a Sorvall® GSA rotor. Pellets of approximately 22-44 g (wet weight) were harvested from each Erlenmeyer flask. Dry weight to wet weight ratios ranged from 0.055-0.12 for the mutant strain and 0.058-0.085 for the wild type strain. The mycelia and conidia were homogenized in water with a VirTis "45" mechanical homogenizer so that the mycelia could pass through the prill-

making apparatus. Alginate prills (spherical pellets made from an alginate solution; technique from Fravel *et al.*, 1985) were produced by dripping a mixture (100 g wet weight fungus, 5 g wheat bran ground to a particle size of less than 1 mm, and 15 g alginate, all per litre solution) through 1-2 mm diam apertures into 0.25 M aqueous calcium chloride. The prills were soaked in the solution for 20-30 min, rinsed with water, and dried overnight at room temperature with moving air provided by tabletop fans. Dry weight of prills produced per litre of bran-alginate-fungus solution ranged from 26-33 g.

One batch of prills made with the mutant strain did not contain the bran food source. Instead, 5 g of pyrophyllite (hydrous aluminum silicate, sold as Pyrax®, R. T. Vanderbilt Company, Inc., Norwalk, Connecticut) were added per litre alginate solution.

Two additional types of prills were made with the mutant strain. One was formulated with a soybean cyst nematode sex pheromone, vanillic acid (0.67 g in 7.5-8.3 ml ethanol per litre alginate solution) and the other with the pheromone analogue syringic acid (0.13 g in 6.7 ml ethanol per litre). One batch of each was tested to determine viability.

#### *Storage of prills and tests for viability*

Prills were put in unscaled plastic or glass screw-cap bottles and stored under four different conditions: a) freezer (-15°C); b) refrigerator (4°C); c) ambient room conditions (heated and air conditioned laboratory, *ca.* 25°C); and d) glasshouse (temperatures ranged from 15-43°C). Prill viability could have been evaluated by testing whole prills, which gives a measure of viable point sources, or by testing ground-up prills, which gives an estimation of viable "colony forming units." Because prills used for biocontrol studies are applied in the field as whole prills and not ground up before application, each prill is a point source for the fungus in the soil, so it is important to establish the number of viable point sources. Therefore, viability was determined by plating the prills onto agar and counting the number of prills from which fungus grew. For each storage condition/age combination, thirty prills from each tested batch were plated onto PDA in 3 Petri dishes (10 prills per dish) and placed at room temperature (*ca.* 25°C). Viability was first observed after 2 days; the colonies were then allowed to grow long enough for identification to be verified. Even at the earliest stage of visible growth, the *Verticillium* morphology on the prills was so distinctive that there were no instances where it was later found that a fungus contaminant growing from a prill without viable *Verticillium* had been mistakenly counted as *Verticillium*. Intervals between viability tests were a minimum of 1 month.

Results are reported as the percentage of prills with viable fungus in the months succeeding prill production. Means and standard errors were calculated for each storage condition/age combination where more than one prill

batch was tested. Repetitions were conducted for many of the storage condition/age combinations with different prill batches. The number of prill batches tested for each storage condition/age combination varied because some prill batches had fewer prills and were used up earlier than others or were not tested each month.

Eighteen batches of the mutant strain in wheat bran prills made in the years 1990-1993 were tested for viability. One (30 prills) to 11 (330 prills) prill batches were tested for each storage condition/age combination. Graph points on Fig. 1 that are represented by a single prill batch: freezer, 10 and 14 months; glasshouse, 10 months; and most storage times of 16 months or longer.

Data on viability of the wild type strain were collected from eight batches of prills made in the years 1990-1992. Each graph point from 1-16 months represents results from 1-5 batches of prills. All results from 18 months and later are from single prill batches.

The results for the M2S1-Pyrax® prills came from one prill batch made in 1991 (30 prills per graph point). The information on fungus/vanillic acid and fungus/syringic acid prills also represents one batch of each type of prill made in 1991.

## RESULTS

### *Mutant strain in wheat bran prills*

Strain M2S1 in wheat bran prills survived better in the refrigerator than under other storage conditions (Fig. 1). Eleven prill batches were stored in the refrigerator, with 1 to 8 batches tested for each time period. Overall, viability was generally 90-100% in refrigerator storage, even after 45 months (Fig. 1B). Only one batch of M2S1 prills stored in the refrigerator dropped to 0% fungus viability (recorded after 12 months storage). The other five batches tested after 12 months refrigerator storage all had 100% fungus viability. The batch with 0% viability was plated out only once more (after 22 months); it was the sole batch tested at that time (Fig. 1B). The batch was not stored under other conditions.

Average viability of M2S1 stored in the freezer (6 prill batches total, 1 to 6 batches sampled per month) was between 95 and 100%, with the exception of two batches (Fig. 1A). One batch had 93% fungus viability after 12 months, 80% after 15 months, and 0% after 29 and 40 months (the next dates on which it was sampled). This same batch retained 97% viability in the refrigerator when tested after 29 and 40 months storage. The other freezer batch that dropped to 0% fungus viability (recorded after 32 and 43 months storage) had 100% fungus viability after 43 months storage in the refrigerator.

At ambient room conditions (15 batches total, 1 to 11 tested per month), viability of M2S1 was 95-100% after one month (Fig. 1B). The viability decreased after that time, reaching 0% in all batches tested after 24 months of

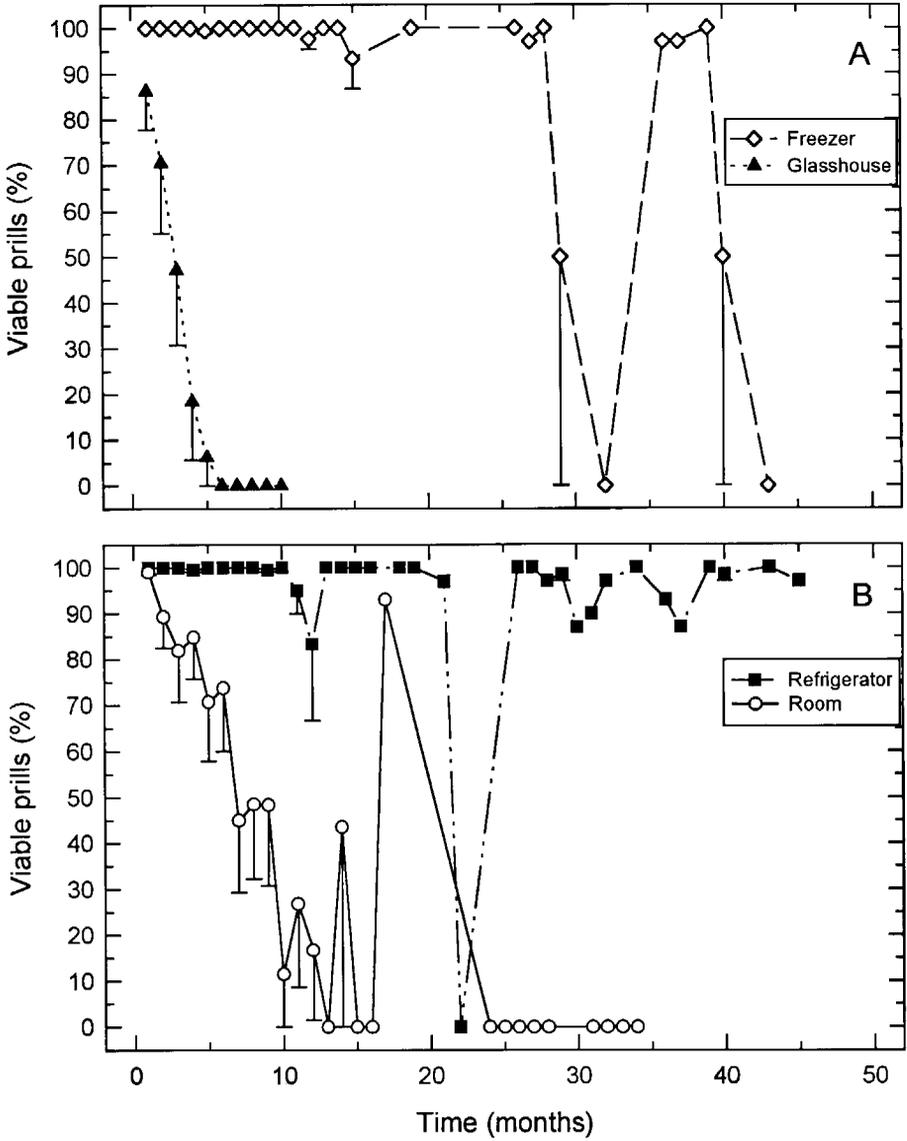


Fig. 1. Viability after storage of *Verticillium lecanii* mutant strain M2S1 in a wheat bran-alginate prill formulation. A. In freezer and glasshouse. B. In refrigerator and at ambient room conditions. Vertical bars represent standard error.

storage. The sole batch used for the 17 month test had an unusually high fungus viability for much of the experiment; 97% viability after 11 months, 87% after 14 months, and 93% after 17 months (Fig. 1B). However, that batch also dropped to 0% fungus viability during the course of the experiment. Glasshouse storage (7 prill batches, 1 to 7 batches tested per month) resulted in an even faster decline in viability, with 0% of the prills containing viable fungus after 6 months (Fig. 1A).

#### *Mutant strain in Pyrax® prills*

In the single batch of prills containing M2S1 and Pyrax® carrier, results were similar to those obtained with alginate-bran prills (Fig. 2). Fungus viability in the refrigerator remained 100% after 40 months, but dropped slightly to 90% in the freezer at that time. Viability at ambient room conditions dropped from 100% to 93% after 4 months, and reached 0% after 12 months. Viability in the glasshouse declined to 80% after 2 months and 0% after 4 months.

#### *Wild type strain in wheat bran prills*

The wild type strain in wheat bran prills also gave similar results to the mutant strain (Fig. 3). Viability in the refrigerator (5 prill batches, 1 to 3 batches sampled per time period) was generally 90-100%. Interestingly, 87% fungus viability was recorded from a single batch after 9 months, but from then on was 93-100% for that batch (with 100% viability recorded after 43 months storage).

Viability of the wild type strain in the freezer was recorded from two prill batches, with one or both sampled at each time period. One batch exhibited 100% fungus viability throughout the experiment (37 months total) in both the freezer and the refrigerator. The other batch, which had 87-100% fungus viability when stored in the refrigerator, dropped substantially in fungus viability in the freezer; 60% viability after 32 months freezer storage and 70% when measured again after 43 months (compared with 100% in the refrigerator after 43 months).

Viability of the wild type strain at ambient room conditions (recorded from a total of 7 prill batches, 1 to 5 tested per month) did not drop below 90% in any batches until 6 months of storage, but was less than 10% after 13 months, and remained 0% after 24 months. Viability in the glasshouse, recorded from one to three prill batches per month, was 0% by 7 months.

#### *Mutant strain in wheat bran prills formulated with vanillic acid or syringic acid*

The vanillic acid formulation (1 prill batch tested) had the shortest fungus viability, decreasing to less than 50% after 4 months in the refrigerator and 8 months in the freezer, and reaching 0% by 25 months in both the refrigerator

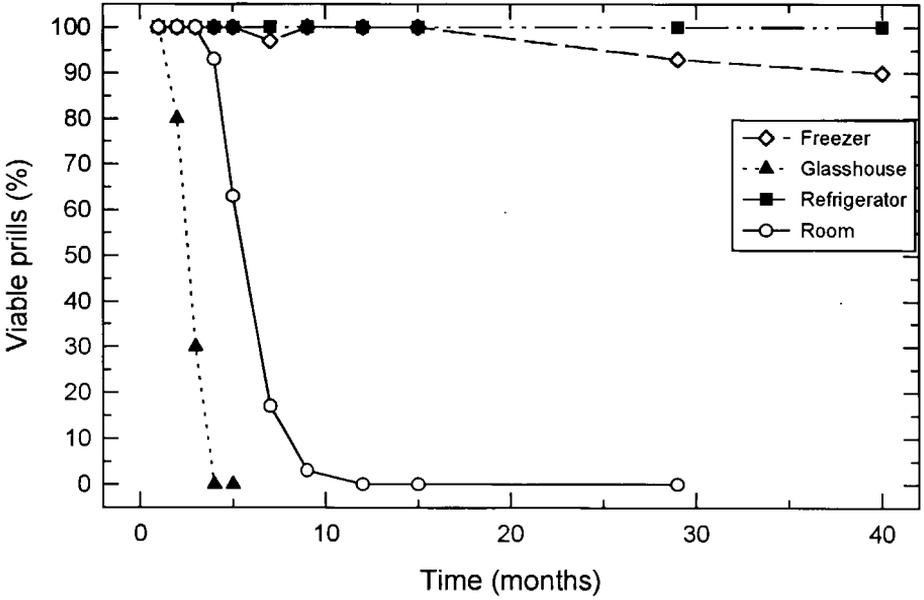


Fig. 2. Viability after storage of *Verticillium lecanii* mutant strain M2S1 in a Pyrax®-alginate prill formulation.

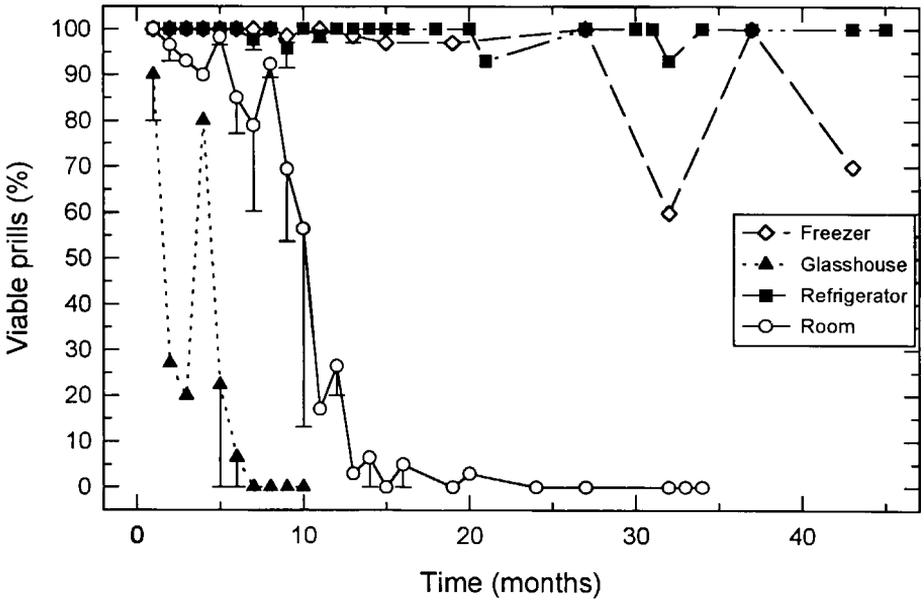


Fig. 3. Viability after storage of *Verticillium lecanii* wild type strain ATCC 58909 in a wheat bran-alginate prill formulation. Vertical bars represent standard error.

and freezer. Fungus viability was 0% after 4 months at ambient room conditions and 1 month in the glasshouse. Although these results are based on only one prill batch, they corroborate unpublished observations made when vanillic acid/M2S1 prills were tested for fungus viability before and after biocontrol experiments. The formulation with the pheromone analogue syringic acid had a similar viability to the M2S1 preparation not amended with vanillic acid. Fungus viability of the syringic acid batch was 90-100% after 25 months in the refrigerator and freezer (last date recorded), 0% after 2 years at ambient room conditions, and 0% after 4 months in the glasshouse.

#### DISCUSSION

The two strains of *V. lecanii* survived long-term storage in alginate prills when refrigerated, but did not remain viable for such long periods at ambient room or glasshouse conditions. This corroborated previous studies indicating that refrigeration tends to prolong viability of fungi in this type of formulation (Papavizas *et al.*, 1987). However, freezer storage of *V. lecanii* also resulted in decreased viability of some prill batches compared with refrigerator storage. Even though this effect of freezing temperatures was not recorded from all prill batches, the occurrence of this phenomenon indicated that refrigeration is the best method for maintaining high viability of *V. lecanii* in alginate formulations. If refrigeration cannot be provided, other formulations may be necessary to prolong viability.

As with *V. lecanii*, viabilities of other biocontrol fungi in alginate formulations have generally been good. However, most longevity studies covered periods of months rather than years. Four fungi incorporated into Pyrax®-alginate prills survived longer than 12 weeks at room temperature (Fravel *et al.*, 1985). *Beauveria bassiana* (Balsamo) Vuillemin, incorporated into alginate prills for control of cereal aphids, sporulated from all prills after 5 months storage at room temperature (Knudsen *et al.*, 1990). *Paecilomyces lilacinus* (Thom) Samson was formulated in alginate pellets with Pyrax® filler for application to soil as a control agent for *Meloidogyne incognita* (Kofoid & White) Chitwood on tomato. At 25°C, viability of this fungus dropped from *ca.* 7.5 ( $\log_{10}$  colony forming units per g carrier) to *ca.* 6.5 after 7 days storage (Cabanillas *et al.*, 1989). The last test date was after 56 days storage and viability was still similar to that measured at 7 days. The nematode-antagonistic fungus labelled ARF18 survived at least 3 months storage in a sodium alginate formulation (Kim & Riggs, 1992). In some cases, longer viability might have been recorded if the experiments had been prolonged. *Talaromyces flavus* (Klöcker) Stolk & Samson, when formulated into alginate prills, usually showed decreases in viability after 4-6 weeks storage. A slight increase in colony forming units was usually measured after 6-12 weeks (possibly caused by ascospores coming out of dormancy), after which populations then declined (Fravel, Lewis & Chittams, personal communication). However, a long term study found that the remaining propagules had a viability of

at least 7 years in Pyrax®-alginate prills stored at room temperature (Fravel, personal communication).

Viability can be affected by addition of other compounds, as demonstrated by the *V. lecanii* formulation with vanillic acid. Longevity is also influenced by the carrier used in the prills. *Talaromyces flavus* was formulated into alginate prills made with pyrophyllite clay, corn cobs, milled chitin, neem cake, fish meal, soy fibre, wheat bran, or peanut hulls (Fravel, Lewis and Chittams, personal communication). Viability (measured for 18 weeks at 5°C and at 22-24°C) was greatest in prills made with soy fibre, corn cobs, and peanut hulls. In the current study on viability of *V. lecanii*, prills formulated with pyrophyllite did not give substantially different results from prills made with wheat bran.

Thanks are extended to Robin Huettel, Paula Crowley and Robert Reise for assistance in the laboratory; to Deborah Fravel, Martha Hollenbeck, and Jack Lewis for demonstration of prill-making techniques; and to Lorin Krusberg, Department of Botany, University of Maryland, for arrangement of a cooperative agreement that facilitated this research.

#### RÉSUMÉ

##### *Survie du champignon antagoniste des nématodes Verticillium lecanii dans des granules d'alginate*

Des souches du champignon *Verticillium lecanii* ont été incorporées dans des granules d'alginate de formulation particulière ("prills") et leurs potentialités en tant qu'agent de contrôle des nématodes phytoparasites ont été étudiées. Le stockage de longue durée étant une contrainte pour les matériaux utilisés au champ, la viabilité de deux souches de *V. lecanii* formulées en granules a été évaluée après stockage au congélateur (-15°C), au réfrigérateur (4°C), en conditions ambiantes (environ 25°C) et en serre (15-43°C). Sur un milieu à base de son de blé, les deux souches se développent à partir de 90 à 100% des granules stockés au réfrigérateur pendant une période allant jusqu'à 43-45 mois (quoique l'un des lots de la souche mutante ait perdu toute viabilité après 12 mois). La viabilité à -15°C ne demeure pas toujours aussi élevée qu'à 4°C. La viabilité des deux souches est de 0% après deux ans à température ambiante, et après 7 mois en serre. Des résultats similaires sont obtenus avec une souche mutante dans une formulation à base de pyrophyllite. Un lot de granules à base de son de blé contenant la souche mutante additionnée de la phéromone sexuelle du nématode à kyste du soja (acide vanillique) a été également testé. La viabilité du champignon est de 0% après 25 mois (congélateur, réfrigérateur), 4 mois (température ambiante) et 1 mois (serre). Les champignons présents dans des granules contenant de l'acide syringique (analogue de la phéromone) ont une longévité équivalente à celle des champignons contenus dans des granules sans phéromone.

#### REFERENCES

- CABANILLAS, E., BARKER, K. R. & NELSON, L. A. (1989). Survival of *Paecilomyces lilacinus* in selected carriers and related effects on *Meloidogyne incognita* on tomato. *Journal of Nematology* **21**, 121-130.
- FRAVEL, D. R. & LEWIS, J. A. (1992). Production, formulation and delivery of beneficial microbes for biocontrol of plant pathogens. In: *Pesticide Formulations and Application Systems*, 11th volume, **1112**, pp. 173-179. Eds L. E. Bode & D. G. Chasin. Philadelphia, USA: American Society for Testing and Materials.
- FRAVEL, D. R., MAROIS, J. J., LUMSDEN, R. D. & CONNICK, W. J. Jr. (1985). Encapsulation of potential biocontrol agents in an alginate-clay matrix. *Phytopathology* **75**, 774-777.

- GINTIS, B. O., MORGAN-JONES, G. & RODRÍGUEZ-KÁBANA, R. (1983). Fungi associated with several developmental stages of *Heterodera glycines* from an Alabama soybean field soil. *Nematropica* **13**, 181-200.
- HÄNSSLER, G. (1990). Parasitism of *Verticillium lecanii* on cysts of *Heterodera schachtii*. *Journal of Plant Diseases and Protection* **97**, 194-201.
- HÄNSSLER, G. & HERMANN, M. (1981). *Verticillium lecanii* as a parasite on cysts of *Heterodera schachtii*. *Journal of Plant Diseases and Protection* **88**, 678-681.
- HUSSEY, N. W. (1984). Biological control in integrated pest control programs in Europe. In: *The Role of Biological Control in Pest Management*, pp. 128-136. Eds G. Allen & A. Rada. Ottawa, Canada: University of Ottawa Press.
- JACKSON, A. M., WHIPPS, J. M. & LYNCH, J. M. (1991). Production, delivery systems, and survival in soil of four fungi with disease biocontrol potential. *Enzyme and Microbial Technology* **13**, 636-642.
- KIM, D. G. & RIGGS, R. D. (1992). Biological Control. In: *Biology and Management of the Soybean Cyst Nematode*, pp. 133-142. Eds R. D. Riggs & J. A. Wrather. St. Paul, USA: APS Press.
- KNUDSEN, G. R., JOHNSON, J. B. & ESCHEN, D. J. (1990). Alginate pellet formulation of a *Beauveria bassiana* (Fungi: Hyphomycetes) isolate pathogenic to cereal aphids. *Journal of Economic Entomology* **83**, 2225-2228.
- LEWIS, J. A. & PAPAIVIZAS, G. C. (1985). Characteristics of alginate pellets formulated with *Trichoderma* and *Gliocladium* and their effect on the proliferation of the fungi in soil. *Plant Pathology* **34**, 571-577.
- MEYER, S. L. F. (1992). Induction of increased benomyl tolerance in *Verticillium lecanii*, a fungus antagonistic to plant-parasitic nematodes. *Journal of the Helminthological Society of Washington* **59**, 237-239.
- MEYER, S. L. F. (1994). Effects of a wild type strain and a mutant strain of the fungus *Verticillium lecanii* on *Meloidogyne incognita* populations in greenhouse studies. *Fundamental and Applied Nematology* **17**, 563-567.
- MEYER, S. L. F. & HUETTEL, R. N. (1993). Fungi and fungus/bioregulator combinations for control of plant-parasitic nematodes. In: *Pest Management: Biologically Based Technologies*, pp. 214-221. Eds R. D. Lumsden & J. L. Vaughn. Washington, DC, USA: American Chemical Society.
- MEYER, S. L. F., HUETTEL, R. N. & SAYRE, R. M. (1990). Isolation of fungi from *Heterodera glycines* and in vitro bioassays for their antagonism to eggs. *Journal of Nematology* **22**, 532-537.
- PAPAIVIZAS, G. C., FRAVEL, D. R. & LEWIS, J. A. (1987). Proliferation of *Talaromyces flavus* in soil and survival in alginate pellets. *Phytopathology* **77**, 131-136.
- SCHUSTER, R.-P. & SIKORA, R. A. (1992a). Persistence and growth of an egg pathogenic fungus applied in alginate granules to field soil and its pathogenicity toward *Globodera pallida*. *Fundamental and Applied Nematology* **15**, 449-455.
- SCHUSTER, R.-P. & SIKORA, R. A. (1992b). Influence of different formulations of fungal egg pathogens in alginate granules on biological control of *Globodera pallida*. *Fundamental and Applied Nematology* **15**, 257-263.