Encapsulation of *Hirsutella rhossiliensis* in hollow beads based on sulfoethylcellulose to control plant-parasitic nematodes

Anant V. Patel, Thomas Rose and Klaus-Dieter Vorlop[1]

Abstract

Plant-parasitic nematodes are important pests of a wide range of economically important plants worldwide. The endoparasitic nematophagous fungus *Hirsutella rhossiliensis* is a ubiquitous antagonist of many commercially important root-knot, cyst, and other pest nematodes. However, no commercial product containing this fungus is available because effective methods for the delivery of fungal mycelium to the soil have not yet been developed, and conidia can not be used.

The encapsulation of *H. rhossiliensis* in hollow beads should provide the following advantages:

(i) easy handling, (ii) protection from biotic and abiotic stress factors, (iii) enhanced shelf life, (iv) controlled release into the soil (controlled by environmental conditions and capsule materials) and (v) enhanced action in the soil. Amending capsules with nutrients, water-retaining substances, fillers, etc., should further enhance efficacy.

Growth experiments on petri dishes resulted in a capsule containing 15 % biomass, 15 % corn gluten and 0.5 % yeast extract. Further experiments were conducted to reduce the biomass content and capsule diameter.

During a stay at the University of California at Davis, USA (Bruce Jaffee) pathogenicity assays against *Heterodera schachtii* were conducted. The experiments indicated that high concentrations of corn gluten and yeast extract in the capsule had an adverse effect on spore transmission from fungus to nematode larvae. A reduction of nutrient content to 1.5 % corn gluten and 0.05 % yeast extract led to an increased number of infected nematode larvae of up to 50 %.

In view of the results, it may be concluded that the encapsulation of *H. rhossiliensis* in nutrient-amended hollow beads is a promising approach to the biological control of plant-parasitic nematodes.

Keywords: Hirsutella rhossiliensis, Heterodera schachtii, encapsulation, formulation, biological control

1 Introduction

The European market for biopesticides was estimated at 97.1 million US dollars in 2000 and is expected to grow by 11.7 % per year to 210.1 million US dollars in 2007 (Frost and Sullivan 2001).

While there is a wide range of microorganisms with a high biocontrol potential, the shelf life and action of cells introduced into the soil is still unsatisfactory. This is due to a lack of suitable formulation techniques (Burges 1998). The basic idea of the research presented here is to encapsulate biocontrol agents which should provide the following advantages:

- easy handling
- protection from biotic and abiotic stress factors
- enhanced shelf life
- controlled release into the soil (controlled by environmental conditions and capsule materials)
- enhanced action in the soil

The amendment of capsules with nutrients, water-retaining substances, fillers, etc., will further enhance their efficacy.

This approach was already successfully tested in our research with entomopathogenic nematodes and plant-growth promoting rhizobacteria (Patel and Vorlop 1994, Patel et al. 1995) where immobilization techniques known in classical biotechnology (Vorlop and Klein 1987) were applied. Here, this approach was to be tested for the nematophagous fungus *Hirsutella rhossiliensis* against economically important plant-parasitic nematodes.

Plant-parasitic nematodes are important pests of a wide range of plants all over the world (Oerke et al. 1994). The sugar beet nematode *Heterodera schachtii* causes an annual loss of about 90 million Euro in Europe (Müller 1999). As the hazardous chemical nematicides are being removed from the market, and the remaining control methods of integrated plant protection are not cost-effective, new control agents must be found.

The endoparasitic nematophagous fungus *Hirsutella rhossiliensis* is an ubiquitous antagonist of many commercially important root-knot, cyst, and

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other pest nematodes. It uses adhesive conidia to parasitize infective juveniles of the sugar beet nematode *Heterodera schachtii* and other *Heterodera spp.*, *Meloidogyne javanica* and other *Meloidogyne spp.*, *Globodera pallida*, etc. (Figure 1). Because the conidia lose their infectivity soon after they become detached from the mycelium (McInnis and Jaffee 1989), a liquid formulation of these conidia is impossible. It follows that the mycelium must be delivered to the field so that spores can be produced on-site in soil. Although the fungus has long been known (Minter and Brady 1980, Sturhan and Schneider 1980), no commercial product is available because methods for the delivery of mycelium to the soil have not yet been developed. Figure 2 illustrates the basic steps involved in the production of a biological control agent based on *H. rhossiliensis* to control plant-parasitic nematodes.

Figure 1:
Left: Spore of *Hirsutella rhossiliensis* adhering to the head of a juvenile *Heterodera schachtii*. Right: Early sporulation of *Hirsutella rhossiliensis* from a parasitized juvenile of *Heterodera schachtii*. The spore that initiated the infection is on the surface of the cuticle, to the right of the stylet. The body cavity is filled with hyphae. The first phialide and spore produced are to the right of the nematode (photos courtesy of B. Jaffee).

Earlier, a novel type of hollow bead based on sulfoethycellulose was developed (Patel 1998) and optimised (Rose et al. 2000). It was reported that *H. rhossiliensis* can be encapsulated in this type of hollow bead and that the addition of corn gluten and yeast extract enhanced growth of mycelium out of the capsules (Patel et al. 1996). Thus, this capsule system may offer an alternative to the conventional calcium alginate beads used to deliver microbes to the soil (Cassidy et al. 1996, Burges 1998). In a first pathogenicity assay, a high reduction of nematode invasion into roots of sugar beet plantlets was found. However, the transmission of spores to nematodes was not measured.

Here, we present data on the encapsulation of *H. rhossiliensis* mycelium in hollow beads based on sulfoethylcellulose together with nutrients (corn gluten and yeast extract). Then, the influence of nutrients (corn gluten, yeast extract) on the growth of fungus from capsules (colony diameter, mycelium density, sporulation) was monitored using interference contrast microscopy. Finally, the resulting formulations were tested in pathogenicity assays against *Heterodera schachtii* at the UC Davis (Prof. Bruce Jaffee) where the transmission of spores from mycelium to infective juveniles was determined in heated and unheated field soils.

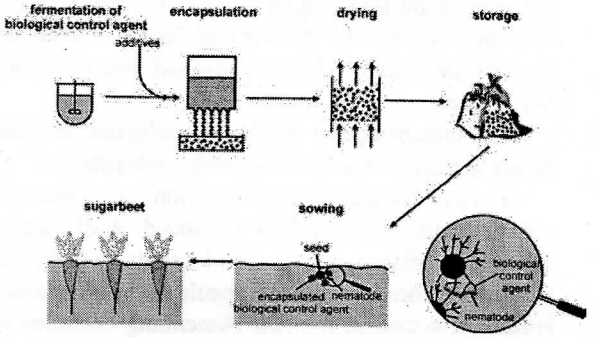

Figure 2:
Encapsulation of *Hirsutella rhossiliensis* to control *Heterodera schachtii*

2 Experimental

First, fungus was raised in a liquid shake culture (Lackey et al. 1993) and the mycelium encapsulated in hollow beads together with nutrients, resulting in a hollow bead which contained 15 % (w/w) wet biomass, 15 % (w/w) corn gluten and 0.5 % (w/w) yeast extract. Figure 3 illustrates the encapsulation of fungal mycelium in hollow beads according to Patel et al. (1996). The capsule formation is based on the reaction of the polyanion sulfoethylcelluose with a corresponding polycation such as polydiallyldimethylammoniumchloride (PDADMAC) or chitosan resulting in a semi-permeable symplex gel membrane.

Conventional calcium alginate beads were prepared according to Lackey et al. (1993) and contained no nutrients, but about 25 % (w/w) biomass.

In vitality assays, radial growth of fungus out of the capsules on water agar and soil was measured. Finally, the formulations were tested against infective juveniles of *Heterodera schachtii* (sugar beet nematode) in pathogenicity assays as described in Lackey et al. (1993): Briefly, capsules were transferred into snap cap bottles with heated or non-heated field soil and incubated for 14 d at 20°C. Then, 500-800 infective juveniles (IJ) of *H. schachtii* were added. After

another 2 d incubation, IJ were extracted and the number of IJ with one or more spores adhering to the cuticle (Figure 1, left) or with no spores adhering were counted.

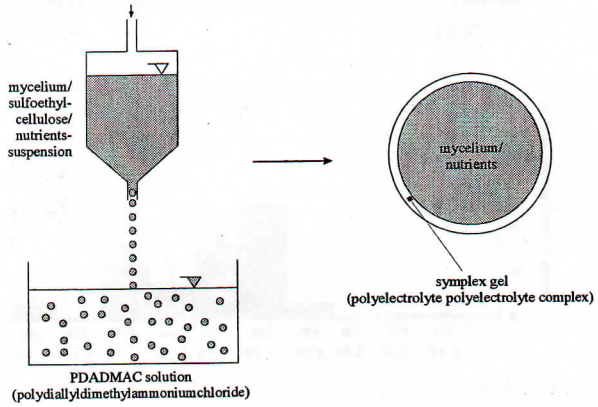

Figure 3:
Encapsulation *of H. rhossiliensis* mycelium in hollow beads based on sulfoethylcellulose

3 Results

Figure 4 shows the influence of biomass content on the growth of mycelium out of the capsules placed on water agar. It is very important to note that due to the presence of nutrients in the capsule, a reduction of biomass content from 15 % down to only 0.1 % does not reduce the growth. It illustrates one of the main advantages of such a formulation type: costly biomass can be saved. The capsule acts as a "microfermenter" where even small amounts of biomass (maybe even fermentation liquid) can multiply. The capsule also acts as a depot, because the filamentous fungus can grow out of the capsule while still using the nutrients. This gives a weak saprophyte a competitive advantage over antagonists in soil.

Figure 5 shows the influence of capsule diameter on radial growth of mycelium out of hollow beads (placed on soil at 14 % (w/w) soil moisture). Naturally, capsules with a higher diameter form bigger colonies. If the number of colonies is considered, one has to look at the "mycelium yield" per 1 ml capsule volume: Out of 1 ml encapsulation liquid, 566 capsules with 1.5 mm diameter can theoretically be formed (if shrinkage of capsules and other effects are not considered). In the other cases, with higher diameters, only 122, 71 or 30 capsules can be formed. Figure 6 shows the "mycelium volume" which is produced per ml encapsulation liquid. The same results are obtained if the surface is considered instead of the volume (data not shown). In other words: the smaller the capsules, the more mycelium will be obtained. Also, more capsules ("colony forming units") may give a better distribution of the biocontrol agent in soil. These results indicate the importance of producing small capsules, probably less than 1 mm diameter, which, to the knowledge of the authors, has not been discussed in detail in the biocontrol literature. The lowest capsule size possible will be dictated by the technological and biological limits and still needs to be investigated.

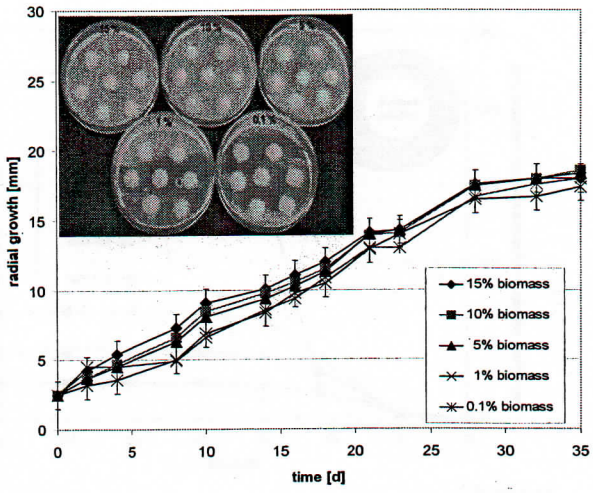

Figure 4:
Influence of biomass content on radial growth of fungal mycelium out of hollow beads containing 15 % corn gluten and 0.5 % yeast extract and placed on water agar, standard deviation for n = 10 capsules

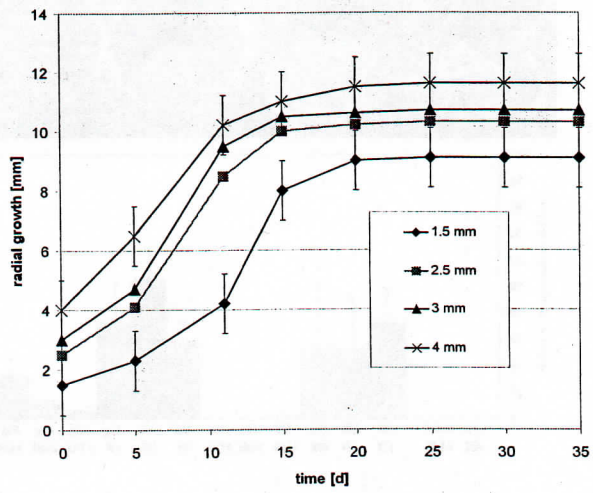

Figure 5:
Influence of capsule diameter on radial growth of fungal mycelium out of hollow beads containing 15 % corn gluten and 0.5 % yeast extract and placed on soil at 14 % (w/w) moisture, standard deviation for n = 10 capsules

The hollow beads were then tested in pathogenicity assays where the transmission of spores from mycelium to infective juveniles of *H. schachtii* was measured. In the first experiments, it was observed

that the fungus grew very well from hollow beads containing 15 % corn gluten, 0.5 % yeast extract and 15 % biomass, not only on water agar, but also in heated and unheated soil compared to alginate beads (Figure 7A,B). Surprisingly, the infection of nematode larvae was very low, despite excellent mycelial growth for hollow beads containing 15 % biomass, 15 % corn gluten and 0.5 % yeast extract (Figure 7C).

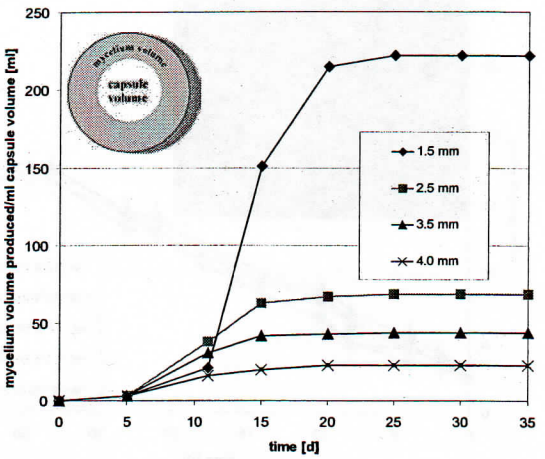

Figure 6:
Mycelium volume produced per ml capsule volume calculated for hollow beads with 1.5-4 mm diameter (explanation see text)

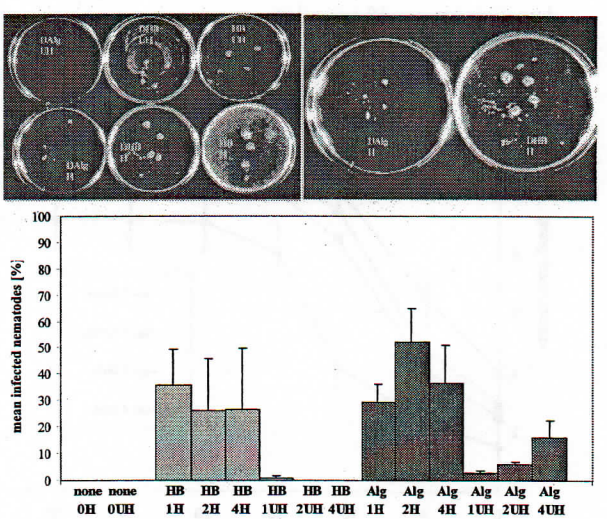

Figure 7:
Hollow beads and alginate beads extracted from soil after 16 d (A: top left, B: top right) and infection of nematode larvae in a bioassay with *H. rhossiliensis* encapsulated in hollow beads and alginate beads (C: bottom); HB: hollow bead; 1, 2, 4: number of capsules added to bioassay, H: heated soil; UH: unheated soil; Alg: alginate beads, DAlg: dried alginate beads (standard error for n=3-6)

In a "pulse experiment" it was to be investigated if the 16 d long bioassay can be accelerated by raising the temperature to 25°C. It was observed that there was a variation in the infection over time (Figure 8): There was a distinct increase in spore transmission after 5 d, which subsequently decreased. Extraction efficiency was around 50 % in all experiments (data not shown).

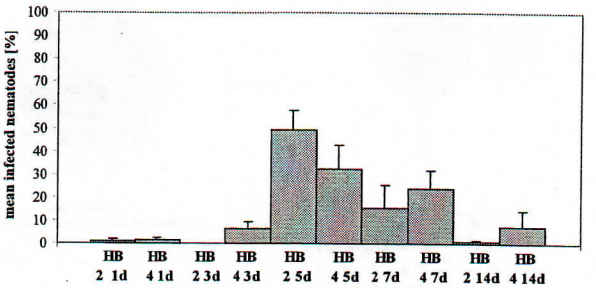

Figure 8:
Infection of nematode larvae in a bioassay after 1, 3, 5, 7 and 14 days of incubation of the hollow beads in heated soil at 25°C, HB: hollow beads, 2,4: number of capsules added to bioassay (standard error for n = 6)

In the original bioassay, the soil was tamped after addition of capsules (Lackey et al. 1993). Because of the danger of breaking moist hollow beads in the process, in the first experiments the soil was not tamped. However, it is known that porosity of the soil can have an influence on spore transmission (Tedford et al. 1992), so, in another experiment, the influence of tamping on spore transmission was investigated using dried (and thus more stable) hollow beads. No effect of tamping was observed (Figure 9).

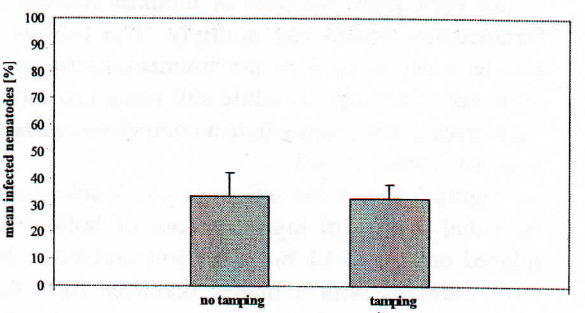

Figure 9:
Infection of nematode larvae in heated soil which was either tamped or not tamped after addition of 1 dried hollow bead (standard error for n = 6)

Finally, it was tested whether the nutrient content of the capsules has an influence on the infection of nematode larvae. It was observed that with hollow beads containing only 1/10 of the nutrients used in the experiments before (1.5 % corn gluten, 0.05 % yeast extract), the infection was significantly higher (Figure 10, Figure 11), while growth of mycelium from capsules was still good (Figure 12, Figure 13).

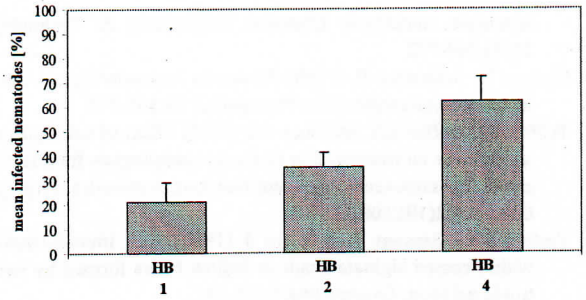

Figure 10:
Infection of nematode larvae in bioassays using 1, 2 or 4 moist hollow beads with reduced nutrient content in heated soil (standard error for n = 6)

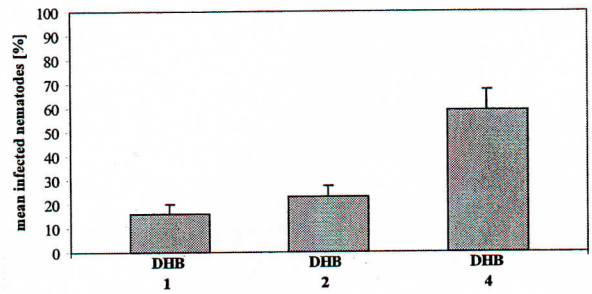

Figure 11:
Infection of nematode larvae in bioassays using 1, 2 or 4 dried hollow beads with reduced nutrient content in heated soil (standard error for n = 6)

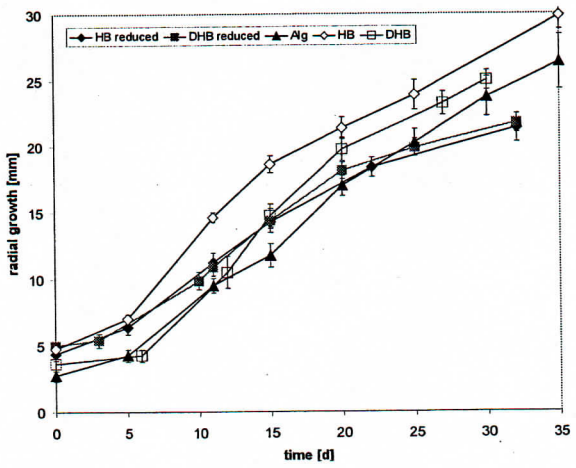

Figure 12:
Radial growth of *H. rhossiliensis* from alginate beads and from hollow beads with and without reduced nutrient content on water agar: HB: hollow beads, DHB: dried hollow beads, HB reduced: reduced nutrient content, DHB reduced: reduced nutrient content, Alg: alginate beads without nutrients (standard deviation for n = 10 capsules)

4 Discussion

We reported here that *H. rhossiliensis* can be successfully established in soil by encapsulation in a novel type of hollow bead amended with nutrients as an alternative to conventional alginate beads. The addition of nutrients to the capsule in order to give the fungus, which is reported to be a weak saprophyte (Jaffee and Zehr 1985), a competitive edge in soil is still considered a sound idea. However, care has to be taken that nutrients do not leak out of the capsule. This problem will be investigated in future research by changing membrane permeability, coating the hollow beads or introducing high molecular weight nutrients into the capsule.

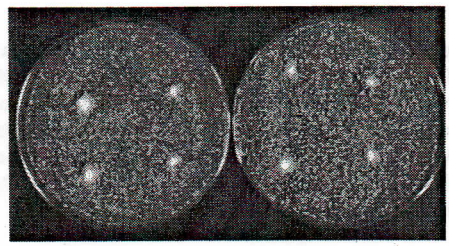

Figure 13:
Growth of *H. rhossiliensis* from hollow beads with reduced nutrient content on heated and unheated soil after 25 d

Then, in addition to the growth of mycelium, it should be investigated how the sporulation is affected by the nutrients (i.e., by the C:N ratio). Maybe (although it is considered unlikely) the poor infection in spite of excellent mycelium growth in soil can be explained with a lack of sporulation or the formation of less infectious (sticky) spores.

It also seems advisable to combine the spore transmission bioassay with a pathogenicity bioassay of *H. schachtii* where the invasion of sugar beet plantlets is investigated. This will be especially valuable in cases where the invasion of sugar beets by nematodes is very low. In these cases it should be checked if the protection of the plant is actually due to fungal activity or due to other effects such as a direct or indirect repellent effect of capsule ingredients, activity of other nematode antagonists, etc. These investigations are being carried out in the lab of J. Müller at the BBA Münster and will be published elsewhere. More systematic investigations regarding the interaction of formulation additives/fungus/nematode/host plant/rhizosphere are needed in order to develop a reliable biocontrol agent.

Finally, a focus of future research will be the capsule application. This may be done by incorporation of capsules into sugar beet pills, which may result in a release of the biological control agents only in the rhizosphere and thus in a reduction of dosage. Alternatively, dried capsules may be delivered separately as granules or powder during seed sowing or combined with an underground fertilization process as

used in maize. All formulations need to be tested in more experiments in greenhouses and in the field. More results regarding the cultivation, encapsulation and drying of *H. rhossiliensis* will be published elsewhere.

In view of the results, it may be concluded that the encapsulation of *H. rhossiliensis* in nutrient-amended hollow beads is a promising approach to the biological control of plant-parasitic nematodes.

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