J. Biol. Control, 19(2): 105-113, 2005

Potential use of polyethylene glycol in the mass production of nonsynnematous and synnematous strains of *Hirsutella thompsonii* Fisher in submerged culture

P. SREERAMA KUMAR, LEENA SINGH and HABEEBA TABASSUM
Project Directorate of Biological Control (ICAR)
P. B. No. 2491, H. A. Farm Post, Bellary Road, Hebbal
Bangalore 560 024, Karnataka, India
E-mail: psreeramakumar@gmail.com

ABSTRACT: Polyethylene glycol (PEG 6000) at six different concentrations (1, 2, 3, 4, 5 and 6% w/v) in Sabouraud dextrose broth influenced both quality and quantity of the biomass of *Hirsutella thompsonii* (nonsynnematous) and *H. thompsonii* var. *synnematosa* (synnematous) in submerged culture. Whereas the number of colony-forming units (CFU), wet and dry weights of both nonsynnematous and synnematous strains increased, pellet number and size decreased with the increasing concentration of PEG in the medium. However, there was no significant effect of the chemical on conidial germination, although conidiation of biomass of both strains was slightly delayed. The intended aim of getting more of hyphal fragments and less of mycelial pellets with the chemical was achieved as reflected in the maximum CFU obtained with 6 per cent PEG in *H. thompsonii* (360.53 x 10^3/ ml) and *H. thompsonii* var. *synnematosa* (237 x 10^3/ml). Compared with the respective controls, the highest level of PEG produced 6 and 2.8 times less number of fully-formed pellets in the nonsynnematous and synnematous strains, respectively.

KEY WORDS: Biomass, fungal pathogen, *Hirsutella thompsonii*, *H. thompsonii* var. *synnematosa*, mass production, pellets, polyethylene glycol, submerged culture

INTRODUCTION

Hirsutella thompsonii Fisher (Mitosporic fungi: Hyphomycetes) is a facultative fungal parasite of the coconut mite, *Aceria guerreronis* Keifer (Acari: Eriophyidae), and several other eriophyid and tetranychid mites (McCoy, 1996; Chandler *et al.*, 2000; van der Geest *et al.*, 2000). The emergence of *A. guerreronis* as the most destructive pest of the coconuts across India and Sri Lanka resulted in drawing close attention on *H. thompsonii* as a biocontrol option for the mite (Kumar and Singh, 2000; Kumar, 2002). Consequently, mass production aspects of the fungus gained importance for product development and supply to the farmers. Though *H. thompsonii* produces aerial conidia, the infective propagules, on solid substrates (McCoy and Kanavel, 1969; Samson *et al.*, 1980), the amount of sporulation is very low in comparison with other entomopathogenic fungi. Synnemata are also produced by certain strains from the tropics

including those infective to *A. guerreronis* (Samson *et al.*, 1980; Kumar, 2002). In submerged conditions, *H. thompsonii* produces mycelial balls or pellets (McCoy *et al.*, 1972), but not blastospores unlike several of the common entomofungal species (Latgé *et al.*, 1988). Also, only a few strains of the fungus were demonstrated to be able to form true conidia in submerged culture (Lysansky and Hall, 1983; van Winkelhoff and McCoy, 1984). Because of these reasons a two-stage (liquid-solid) fermentation process was used for the first commercial product of the fungus in USA (Couch, 1982). Mycelial pellets are generally not suitable for use as inoculum of a solid substrate as they do not permit even coverage (Jenkins *et al.*, 1998). Furthermore, wherever direct mycelial application is needed it is desirable to use only a diffuse mass, which does not require blending.

Polyethylene glycol (PEG), a versatile osmoticum, has been used as an ingredient in culture media for the mass production of several fungal species that have biocontrol potential. PEG has been shown to have varied effects on biomass characteristics in addition to its influence on the shelf-life and field performance of different fungal species. In the case of certain entomofungal pathogens, PEG has been used to get more hyphal bits and increased blastospore formation (Inch and Trinci, 1987; Kleespies and Zimmermann, 1992). Studies have shown that the medium water potential can be lowered with the addition of PEG to get increased conidial density and colony-forming units (CFU) of the plant disease antagonist, *Trichoderma harzianum* Rifai (Jin *et al.*, 1991).

Studies were, therefore, undertaken to find out the effect of PEG on the biomass of nonsynnematous and synnematous strains of *H. thompsonii* obtained through submerged culturing. The main goal of the study was to get more of hyphal fragments and less of mycelial pellets.

MATERIALS AND METHODS

Details about test fungi

The nonsynnematous *H. thompsonii* strain MF(Ag)5, and the synnematous strain MF(Ag)27, identified as *H. thompsonii* var. *synnematosa* Samson, McCoy & O'Donnell, both derived from *A. guerreronis* in India were used in all experiments. Both strains were routinely grown on homemade potato dextrose agar (PDA) in test tubes at 25 °C under a 12-h photoperiod.

Experimental methods

The effect of PEG 6000 (HiMedia, Mumbai, India) on the biomass of *H. thompsonii* and *H. thompsonii* var. *synnematosa* was assessed by growing the fungi in Sabouraud dextrose broth (SDB) (HiMedia) in submerged culture.

Six different concentrations, *viz.* 1, 2, 3, 4, 5 and 6 per cent (w/v) of PEG were prepared separately and dispensed into 500-ml Erlenmeyer flasks at the rate of 200 ml of the amended medium per flask. The pH of the medium so prepared was adjusted to 6.5 with either N/10 HCl or NaOH before plugging the flasks with cotton and sterilizing for 15 minutes at 121 °C and 1.05 kg/cm^2 pressure in an autoclave. Conidial suspension (5.4 x 10^5 conidia/ml of sterile deionized water) prepared from an actively growing 1-month-old PDA (homemade) culture was used as inoculum (2 ml/ flask).

The inoculated flasks were loaded onto a table-top laboratory orbital shaker ('Orbitek-L', Scigenics Biotech (Pvt.) Ltd., India) used for shake (25-mm throw) culture and was run for 7 days at room temperature (28 ± 2 °C during daytime) and light conditions at an agitation rate of 150 rpm. After every 24 h, the shaker speed was increased to 300 rpm for a minute to dislodge the mycelium sticking to the surface of the flask so as to prevent conidia formation and reinoculation of the medium. Due to limited shaker space, the experiment for each fungus was conducted in three different sets consisting of two consecutive concentrations at a time with a suitable control, which was SDB without PEG. Each concentration and control had three replicates. For statistical analysis, data from controls in each set were pooled.

At the end of the incubation period, the number of CFU, pellet number, pellet size, wet and dry weights, and conidiation of biomass were

determined. CFU was determined by plating 1 ml sample of the liquid culture at 10^3 dilution on homemade PDA with five replicates for each flask. Plates were incubated at 25 °C and the number of colonies was counted at the end of 72 h and expressed as number per ml. For estimating the pellet size, 10 fully-formed (spherical and compact/ loose) pellets per replicate were measured, and for pellet number the numbers of such pellets per 10 ml were counted.

For studying the wet and dry weights of the fungus 100 ml of culture was taken separately and the biomass was separated from the spent medium by filtration soon after the incubation period and weighed. The same was dried at 60 °C for 24 h for obtaining the dry weight.

For checking the conidiation of the biomass, nine pellets (compact or loose) from all replicates were kept in Petri-dishes lined with filter paper, at the rate of three pellets per dish and incubated as done for CFU estimation to allow conidiogenesis.

Before setting up the main experiment on the effect of PEG on the biomass characteristics of *H. thompsonii* and *H. thompsonii* var. *synnematosa*, its effect on spore germination of the two strains was assessed. Spore suspensions (5 x 10^5 conidia/ ml) of both fungi were prepared in sterile deionized water and the same was used to prepare the six test concentrations of PEG. Ten ml quantities each in a 30-ml glass vial served as replicates. For each concentration and control (no PEG), three replicate vials were maintained. The contents of each vial were shaken well and incubated for 2 h under room conditions. A haemocytometer was used to count germinated and non-germinated spores from each suspension in 1 mm^2- grid and worked out the percentage of germination. Conidia that showed initiation of germtube were considered to have germinated.

RESULTS AND DISCUSSION

Our studies on the effect of PEG on both non-synnematous and synnematous strains of *H. thompsonii* in submerged culture showed that the chemical has several beneficial effects on the biomass characteristics. PEG had significant effects on various parameters as seen on comparing different concentrations of the chemical with control. Both strains produced mycelia and hyphal fragments but no conidia in submerged culture. This phenomenon was foreseen as so far only one pathotype of *H. thompsonii* var. *synnematosa* from Ivory Coast (HtIC) has been demonstrated to be able to produce conidia in submerged culture, although in semi-solid fermentation moderate sporulation of all pathotypes occurs (van Winkelhoff and McCoy, 1984).

In the case of *H. thompsonii* (Fig. 1), PEG brought about an increase in the number of CFU, which was the highest at 6 per cent concentration and the lowest in unamended medium. Even at 1 per cent level, PEG was able to produce a significantly higher number of CFU compared with the control (Fig. 3). The colonies from the higher concentrations of PEG were smaller than those from control, indicating the fragmentation of the mycelia.

The increase in CFU by PEG amendment was due to the decrease in the pellet number and size by the chemical at all the six test concentrations (Table 1). Both the number and size of fully-formed pellets were the maximum in control (Fig. 4) suggesting that there was no breaking of the pellets and that they were able to maintain their number and size whereas in the other treatments there was either no normal pellet formation or the pellets got broken into 'baby' pellets, because of the effect of PEG. At 6 per cent, PEG brought about more or less homogeneous biomass of the fungus.

Wet and dry weights of the biomass considerably increased by the addition of PEG in the medium, though the first two lower concentrations had no effect on these parameters (Table 1). Marked increase in the biomass weight was detected only from 3 per cent level onwards.

There seems to be no direct influence of PEG on the conidial germination, because there was rapid germination of conidia treated with the chemical (Table 1). There was no significant difference between different treatments, the per cent germination ranged from 75 to 82 in 2 h. In contrast,

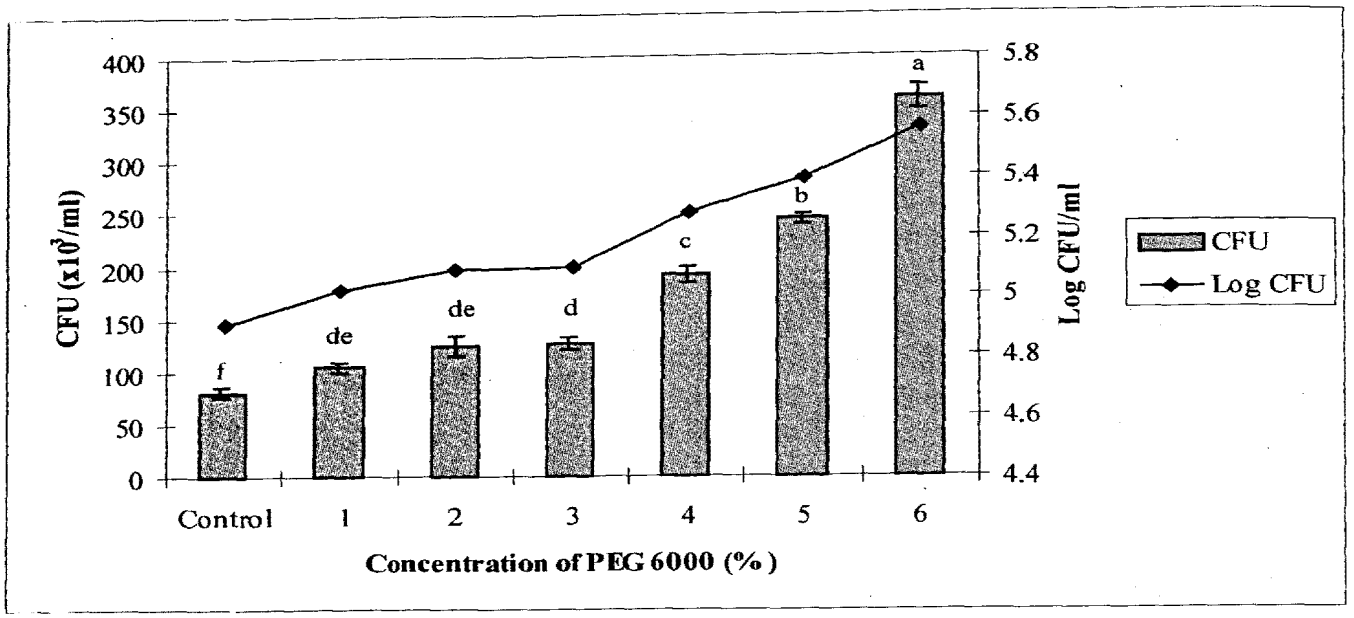

Fig. 1. Effect of PEG 6000 on the CFU of *H. thompsonii* (mean ± SEM)
Different letters denote significant differences between concentrations.

Table 1. Effect of PEG 6000 on the biomass and conidial germination of *H. thompsonii*

Concentration of PEG 6000 (%)	Pellet number / 10 ml[a]	Pellet size (mm)[b]	Wet weight (g/100 ml)	Dry weight (g/100 ml)	Conidial germination (%) after 2 h[c]
1	5.83 ± 0.45*	4.00 ± 0.44	6.30 ± 0.46	0.23 ± 0.07	82.0 ± 2.31 (65.0)
2	1.75 ± 0.43	3.00 ± 0.00	7.27 ± 0.28	0.33 ± 0.07	79.3 ± 2.40 (63.0)
3	1.46 ± 0.07	3.00 ± 0.00	13.00 ± 0.81	1.07 ± 0.15	78.0 ± 2.00 (62.1)
4	1.36 ± 0.98	3.00 ± 0.00	15.63 ± 0.52	1.77 ± 0.28	76.7 ± 2.40 (61.2)
5	1.11 ± 0.06	2.00 ± 0.00	16.17 ± 0.26	2.83 ± 0.03	76.7 ± 2.40 (61.2)
6	0.97 ± 0.09	1.75 ± 0.14	16.73 ± 0.59	2.90 ± 0.06	74.7 ± 2.90 (59.9)
Control	5.83 ± 0.35	5.02 ± 0.06	8.56 ± 0.60	0.22 ± 0.06	80.7 ± 1.77 (64.0)
SEM ±	0.27	0.18	0.56	0.14	1.64
CD (*P*=0.01)	1.19	0.78	2.41	0.59	NS

[a] Pellet number indicates the number of fully-formed pellets.
[b] Pellet size indicates the diameter of fully-formed pellets.
[c] Figures in parentheses are arcsine-transformed values.
* Standard error of mean (SEM).

Jackson *et al.* (1991) reported that on PDA hyphal extension rates and conidial germination of *Trichoderma* spp. declined with decreasing water potential adjusted with PEG over the range -0.7 to -14.0 MPa.

Conidiation occurred within 24 h in the pellets obtained from control treatment whereas it took about 30 h for the conidiation of pellets from the PEG-containing flasks. The time to conidiate was found to be dependent on the PEG level in the medium, indicating that the chemical could cause little delay in conidiation.

The colour of the pellets in control was creamish brown and they were mostly of the same size. Slight colour differences were observed among the pellets or biomass obtained from different PEG treatments. For example, the colour of pellets varied from brownish cream to dark grayish green. Pellets obtained from 4 and 5 per cent PEG were dark brown from the beginning which might be due to the high stress conditions the fungus was facing from the time of their formation.

As shown in Fig. 2 and Table 2, the CFU, wet and dry weights of *H. thompsonii* var. *synnematosa* increased with increase in PEG concentration in the medium, but this boost was not as consistent as seen in the nonsynnematous strain.

A comparison of CFU when *H. thompsonii* var. *synnematosa* was grown with PEG showed that the highest concentration (6 %) of the chemical produced the maximum numbers, while the two lowest concentrations (1 & 2 %) were on a par with control (Fig. 2).

The pellet number and size also did not show a regular pattern in the case of *H. thompsonii* var. *synnematosa* (Table 2). Although, in general, 6 per cent PEG was the best in terms of the number of CFU, and wet and dry weights of the fungus. The least number of fully-formed pellets and the smallest size were also seen at the highest level of PEG. Unlike in the case of *H. thompsonii*, the pellets were loose.

In the unamended medium there were just 2.78 fully-formed pellets compared with almost double that number per 10 ml of the medium in respect of *H. thompsonii* (Tables 1 & 2). Unlike in the case of *H. thompsonii*, the synnematous strain, in general, showed a weak pelleting capacity. The addition of PEG aided in further homogenization of the biomass. Previously, van Winkelhoff and McCoy (1984) observed that the mycelium of *H. thompsonii* var. *synnematosa* was more fragmented with a fewer spherical colonies in the liquid culture medium containing Tween 80 (0.2%), and stated that the surfactant's effect was more physical than nutritional.

PEG did not significantly affect germination of conidia in comparison with control; it ranged from about 77 to 92 % (Table 2).

As in the case of the nonsynnematous strain, it took approximately 24 h for the biomass of *H. thompsonii* var. *synnematosa* from control to initiate conidiation, whereas a 30-h period was required for the biomass from PEG treatments, indicating some delaying effect of PEG on the conidiogenesis of the fungus. Unlike in the case of the other strain, no very specific colour change in the biomass was seen in synnematous strain, the fungus was uniformly creamish brown in all the replicates.

Addition of certain polymers in growth media is one of the various techniques through which mycelial pellet formation can be decreased by encouraging diffuse mycelial growth or formation of tiny hyphal fragments or blastospores. Inch and Trinci (1987) and Humphreys *et al.* (1989) reported that the addition of PEG 200 suppressed the formation of pellets in liquid cultures of certain entomopathogenic fungi having commercial value. Similarly, Kleespies and Zimmermann (1992) have also obtained increased blastospore production and reduced pellet formation of *Metarhizium anisopliae* (Metschn.) Sorokin using PEG 200, Tween 80 and high or low pH. Geetha and Balaraman (2001) reported that PEG (2 %) favoured both higher biomass and blastospores in the case of *Beauveria bassiana* (Bals.-Criv.) Vuill.

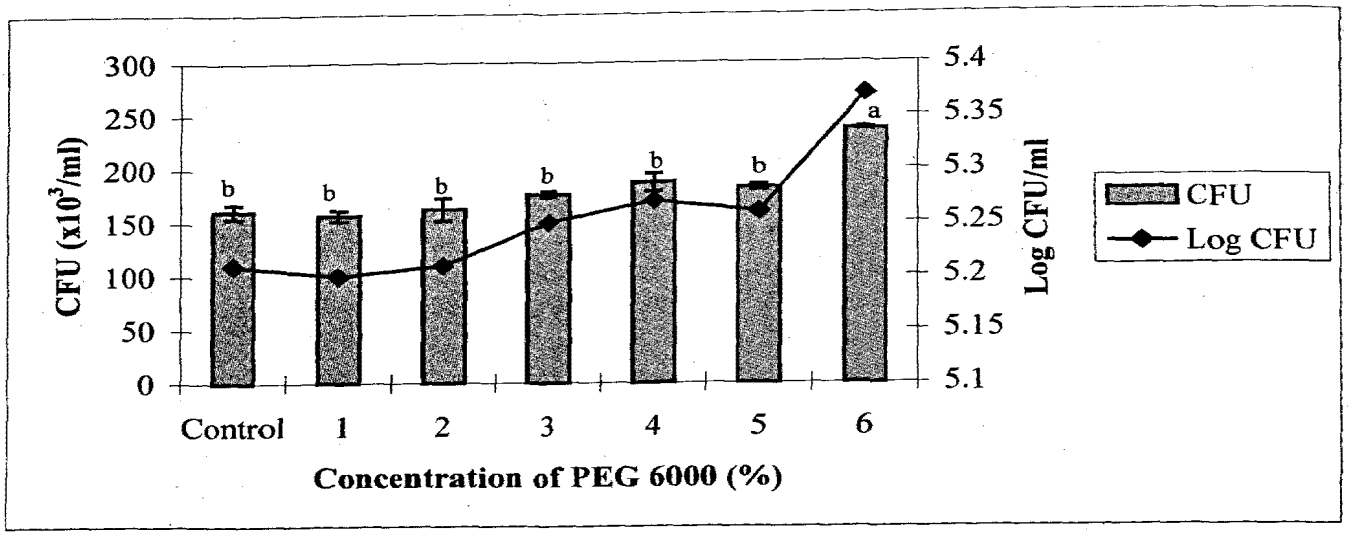

Fig. 2. Effect of PEG 6000 on the CFU of *H. thompsonii* var. *synnematosa* (mean ± SEM). Different letters denote significant differences between concentrations.

Table 2. Effect of PEG 6000 on the biomass and conidial germination of *H. thompsonii* var. *synnematosa*

Concentration of PEG 6000 (%)	Pellet number/ 10 ml[a]	Pellet size (mm)[b]	Wet weight (g/100 ml)	Dry weight (g/100 ml)	Conidial germination (%) after 2 h[c]
1	2.14 ± 0.07*	3.42 ± 0.17	9.09 ± 0.26	0.38 ± 0.03	88.0 ± 4.16 (70.2)
2	2.05 ± 0.33	2.98 ± 0.08	9.88 ± 0.60	0.38 ± 0.07	88.0 ± 5.29 (70.7)
3	2.26 ± 0.28	2.62 ± 0.03	9.35 ± 0.49	0.40 ± 0.03	92.0 ± 4.16 (74.7)
4	2.04 ± 0.30	2.73 ± 0.04	11.02 ± 1.00	0.48 ± 0.03	80.0 ± 1.15 (63.5)
5	1.13 ± 0.18	2.25 ± 0.05	11.39 ± 0.33	0.56 ± 0.05	80.0 ± 5.03 (63.9)
6	0.99 ± 0.13	2.15 ± 0.08	11.58 ± 1.07	0.64 ± 0.09	78.7 ± 10.42 (64.2)
Control	2.78 ± 0.29	3.52 ± 0.24	9.59 ± 0.19	0.38 ± 0.04	76.7 ± 6.56 (61.5)
SEM ±	0.23	0.11	0.69	0.05	5.02
CD (*P*=0.01)	1.01	0.48	NS	NS	NS

[a] Pellet number indicates the number of fully-formed pellets.

[b] Pellet size indicates the diameter of fully-formed pellets.

[c] Figures in parentheses are arcsine-transformed values.

* Standard error of mean (SEM).

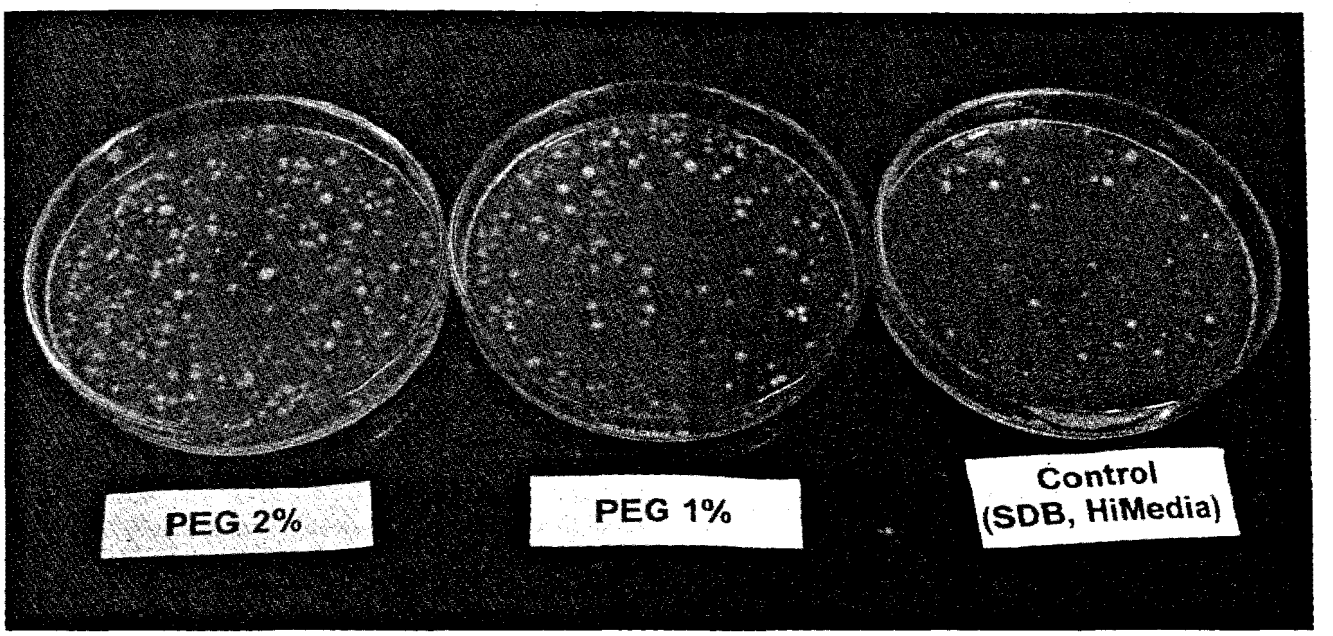

Fig. 3. *H. thompsonii* colonies obtained from control and PEG (1 & 2%)- amended medium

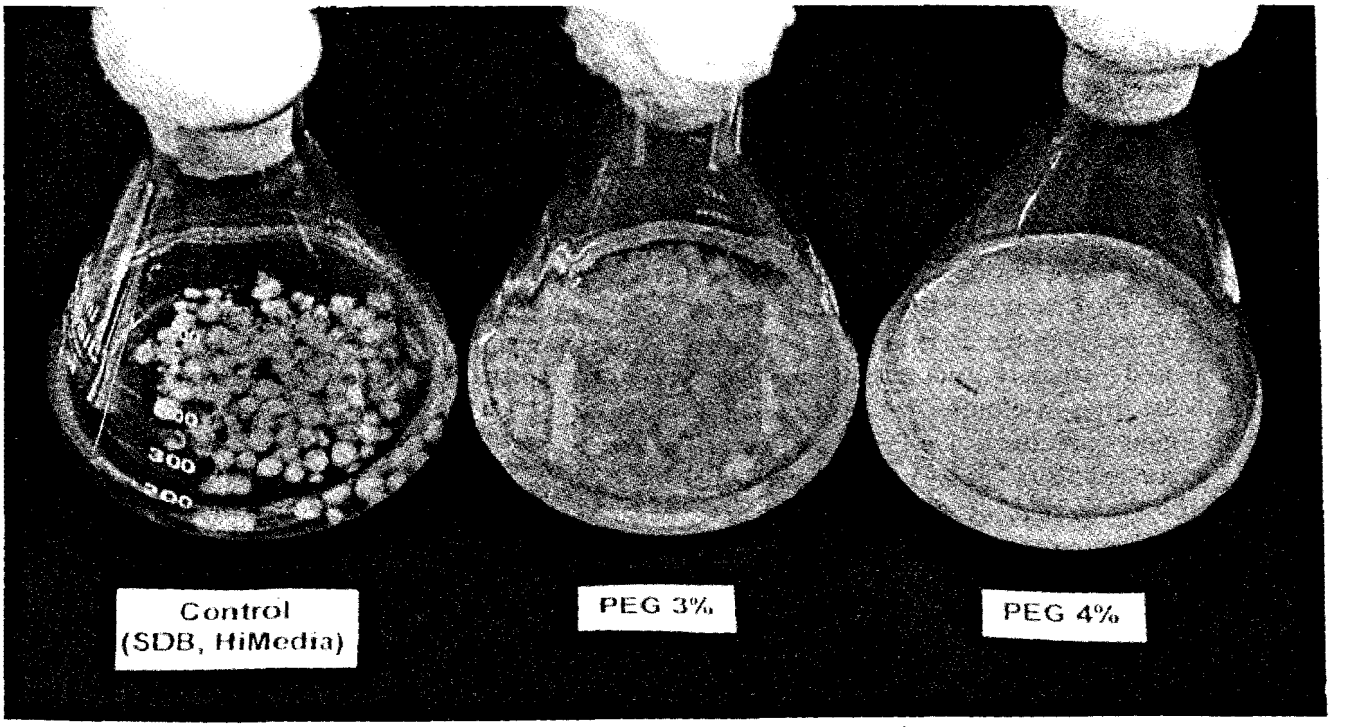

Fig. 4. Biomass of *H. thompsonii* in control and PEG (3 & 4%)- amended medium

It has been demonstrated through the research presented here that PEG is useful in limiting pellet formation and that a diffuse biomass of both nonsynnematous and synnematous strains could be achieved with the chemical.

The need for blending of the biomass before formulating the product during the commercial production of a mycoacaricide based on *H. thompsonii*, could be eliminated in liquid with the use of PEG. The diffuse biomass obtained could be used for direct application on plant surfaces or for inoculating solid substrates for getting aerial conidia. Alternatively, it can be utilized for developing better formulations.

Harman *et al.* (1991) reported that the production, desiccation tolerance and biocontrol efficacy of conidia of a strain of the plant disease antagonist, *T. harzianum* were enhanced after growth in the media of low water potential. Further work should investigate if the biomass of *H. thompsonii* produced with PEG could have better desiccation tolerance, shelf-life and bioefficacy. Also, the possibility of inducing microcycle conidiation (Latgé *et al.*, 1988) in different strains of *H. thompsonii* available in India through the use of PEG in combination with other chemicals should be specifically studied.

ACKNOWLEDGEMENTS

This research was done in the Production System Research Project of the Coastal Agro-ecosystem (Project Number: P-16) under the World Bank-sponsored National Agricultural Technology Project of the Indian Council of Agricultural Research. Thanks are due to the Project Director, Project Directorate of Biological Control, Bangalore, for the facilities provided.

REFERENCES

Chandler, D., Davidson, G., Pell, J. K., Ball, B. V., Shaw, K. and Sunderland, K. D. 2000. Fungal biocontrol of Acari. *Biocontrol Science and Technology*, **10**: 357-384.

Couch, T. L. 1982. Production of hyphomycetes, pp. 178-180. In: *Invertebrate pathology and microbial control: Proceedings of the Third international colloquium on invertebrate pathology, Fifteenth annual meeting of the Society for Invertebrate Pathology*, 6-10 September 1982, University of Sussex, Brighton, UK.

Geetha, I. and Balaraman, K. 2001. Biomass and blastospore production in *Beauveria bassiana* (Bals.) Vuill. as influenced by media components. *Journal of Biological Control*, **15**: 93-96.

Harman, G. E., Jin, X., Stasz, T. E., Peruzzotti, G., Leopold, A. C. and Taylor, A. G. 1991. Production of conidial biomass of *Trichoderma harzianum* for biological control. *Biological Control*, **1**: 23-28.

Humphreys, A. M., Matewele, P. and Trinci, A. P. J. 1989. Effects of water activity on morphology, growth and blastospore production of *Metarhizium anisopliae*, *Beauveria bassiana* and *Paecilomyces farinosus* in batch and fed-batch culture. *Mycological Research*, **92**: 257-264.

Inch, J. M. M. and Trinci, A. P. J. 1987. Effects of water activity on growth and sporulation of *Paecilomyces farinosus* in liquid and solid media. *Journal of General Microbiology*, **133**: 247-252.

Jackson, A. M., Whipps, J. M. and Lynch, J. M. 1991. Effects of temperature, pH and water potential on growth of four fungi with disease biocontrol potential. *World Journal of Microbiology and Biotechnology*, **7**: 494-501.

Jenkins, N. E., Heviefo, G., Langewald, J., Cherry, A. J. and Lomer, C. J. 1998. Development of mass production technology for aerial conidia for use as mycopesticides. *Biocontrol News and Information*, **19**: 21N-31N.

Jin, X., Harman, G. E. and Taylor, A. G. 1991. Conidial biomass and desiccation tolerance of *Trichoderma harzianum* produced at different medium water potentials. *Biological Control*, **3**: 237-243.

Kleespies, R. G. and Zimmermann, G. 1992. Production of blastospores by three strains of *Metarhizium anisopliae* (Metch.) Sorok. in submerged culture. *Biocontrol Science and Technology*, **2**: 127-135.

Kumar, P. S. 2002. Development of a biopesticide for the coconut mite in India, pp. 335-340. In:

Proceedings of the British Crop Protection Conference-Pests and Diseases, Vol. 1 & 2, 18-21 November 2002, British Crop Protection Council, Brighton, UK.

Kumar, P. S. and Singh, S. P. 2000. *Hirsutella thompsonii*: The best biological control option for the management of the coconut mite in India. *Indian Coconut Journal*, **31**: 11-17.

Latgé, J. P., Cabrera Cabrera, R. I. and Prévost, M. C. 1988. Microcycle conidiation in *Hirsutella thompsonii*. *Canadian Journal of Microbiology*, **34**: 625-630.

Lysansky, S. G and Hall, R. A. 1983. Fungal control of insects, pp. 327-345. In: J. E. Smith, D. R. Berry and B. Kristiansen (Eds.). *The filamentous fungi* Vol. 4. *Fungal technology*, Arnold, London, UK.

McCoy, C. W. 1996. Pathogens of eriophyid mites, pp. 481-490. In: E. E. Lindquist, M. W. Sabelis and J. Bruin (Eds). *Eriophyoid Mites– Their Biology, Natural Enemies and Control*. Elsevier Science B. V., Amsterdam, The Netherlands.

McCoy, C. W. and Kanavel, R. F. 1969. Isolation of *Hirsutella thompsonii* from the citrus rust mite, *Phyllocoptruta oleivora*, and its cultivation on various synthetic media. *Journal of Invertebrate Pathology*, **14**: 386-390.

McCoy, C. W., Hill, A. J. and Kanavel, R. F. 1972. A liquid medium for the large-scale production of *Hirsutella thompsonii* in submerged culture. *Journal of Invertebrate Pathology*, **19**: 370-374.

Samson, R. A., McCoy, C. W. and O'Donnell, K. L. 1980. Taxonomy of the acarine parasite *Hirsutella thompsonii*. *Mycologia*, **72**: 359-377.

van der Geest, L. P. S., Elliot, S. L., Breeuwer, J. A. J. and Beerling, E. A. M. 2000. Diseases of mites. *Experimental and Applied Acarology*, **24**: 497-560.

van Winkelhoff, A. J. and McCoy, C. W. 1984. Conidiation of *Hirsutella thompsonii* var. *synnematosa* in submerged culture. *Journal of Invertebrate Pathology*, **43**: 59-68.