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Research Article

Development of capsule formulation of *Beauveria bassiana* (Balsamo) Vuillemin

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ABSTRACT: Capsule is a stable formulation wherein the bioagent is encapsulated in coatings and thus protected from extreme environmental conditions. In this study, various coating materials were tested for their stability based on the time taken for disintegration when kept under ambient conditions both *in vitro* and *in vivo.* The *in vivo* performance was assessed for placement in soil as well as in banana pseudostem. The coating materials tested was Hard Gelatin Transparent (HGT), Hard Gelatin Coloured (HGC) and Hydroxy Propyl Methyl Cellulose (HPMC), while the carrier materials used were talc, chitin, chitosan, sodium alginate and calcium alginate. The entomopathogen encapsulated was *Beauveria bassiana* (Balsamo) Vuillemin and the efficacy of product was assessed against pseudostem weevil, *Odoiporus longicollis* (Olivier). HGT was the best coating material as it was stable under room temperature and normal atmospheric humidity. For soil placement, chitosan based capsules in transparent gelatin coating took only 24 to 48 hr to disintegrate completely under varying soil moisture. Talc based capsules in transparent gelatin coating got disintegrated completely at the end of 24 hr when placed in banana pseudostem, owing to the receipt of sufficient humidity. Placement of capsules in leaf axils or pseudostem sheath can be adopted for prophylactic control of pseudostem weevil and placement in bore holes can be considered for curative application. The ideal moisture content of filler material for fungal encapsulation was determined as 10%. Through this research paper, we would like to disclose about the ideal coating material, carrier material and moisture content for the encapsulation of entomopathogenic fungi.

KEY WORDS: Biocapsule, chitosan, entomopathogenic fungi, talc

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INTRODUCTION

Entomopathogenic fungi have been investigated exhaustively for their potential use as biopesticides owing to their amenability for mass production. The share of biopesticides in worlds' total pesticide market is only 1.3% (Bailey, 2010). However, as per the recent reports by Union Ministry of Agriculture, biopesticides have witnessed 23% increase in usage in comparison with chemical pesticides. Even then, the development of suitable bioformulations still remains as a challenge. Formulations with improved stability, viability and delivery mechanism is essential to enhance the field performance. More often, it is not the lack of effective chemicals or biopesticides that makes plant protection measures ineffective, but the lack of proper delivery systems that ensures proper contact and persistence in the plant. A bioformulation should also maintain the essential criterion of viability and virulence of the active ingredient. Other properties of an ideal formulation include easier handling and application of the product and storage stability (Seaman, 1990).

Capsule is a stable formulation wherein the bioagent is encapsulated in coatings and thus protected from extreme environmental conditions (UV radiation, rain, temperature, etc.), and its residual stability is enhanced due to slow or controlled release. Biocapsules essentially carry an inner core of microorganism and an inert carrier material, surrounded by a coating material (Burges and Jones, 2008). Development of capsule formulations of microbes essentially involves standardization of a coating material and carrier material. The coating material selected should retain stability of the formulation in storage and in the meantime disintegrate readily with controlled release of microbes, when applied in the field. The opted carrier material should be able to maintain the virulence of the organism formulated and should be non-reactive with it.

Germination rate is an indirect attribute of spore viability (Soetoppo 2004; Herlinda, 2010; Faria *et al.,* 2015). A viable bioformulation should retain the microorganism in a dormant state while under storage and when sprayed in

field, it should enhance early germination of conidia. Hence, the formulations are developed in a suitable carrier material which will discourage the ability of spore to germinate under storage, but do not offer hindrance to spores for germination under aqueous environment in field.

Moisture content of the formulation also plays a pivotal role in the viability of the microbe to be encapsulated as well as acts as a critical factor that determines the stability in storage (Moore *et al*., 1996; Derakshan *et al.,* 2008). While developing a capsule formulation, the moisture content of the active ingredient is retained in a manner in which the coating remains stable and the viability of microorganism is also assured.

Therefore in this study we have focused on these three imperative aspects of encapsulation, *viz*., selection of suitable coating material and carrier material for capsule formulations, assessment of germination rate as a determinant of viability of propagule and assessment of ideal moisture content of formulation for developing biocapsules of the entomopathogenic fungi, *B. bassiana* which is a broad spectrum mycoinsecticide.

MATERIALS AND METHODS

The present study on development of biocapsules of *Beauveria bassiana* (Balsamo) Vuillemin was carried out during the period 2016-17 at Biocontrol laboratory for crop pest management, College of Agriculture, Vellayani, Kerala. Different coating materials *viz.,* Hard Gelatin Transparent (HGT), Hard Gelatin Coloured (HGC) and Hydroxy Propyl Methyl Cellulose (HPMC) were tested for their suitability for encapsulation, based on their stability under ambient conditions (31.5 to 33°C and 60 to 80% Relative Humidity (RH)) of storage. The carrier materials evaluated were talc (Industrial Grade), chitin (crude), chitosan (crude), sodium alginate (LR grade) and calcium alginate (LR grade). The capsules used for the study were of size '00' which can accommodate 500 to 700 mg of the content.

Empty capsules were filled with fixed quantities of each of the carrier materials, without incorporating the entomopathogens, using a 100-holed GMP model manually operated capsule filling device. The quantity used per capsule was 300 mg in the case of talc based ones, 500 mg for chitin based 350 mg for chitosan 350 mg for sodium alginate and 400 mg for calcium alginate capsules. The quantity of carrier materials varied depending on their density.

Assessment of stability under ambient conditions of storage

To study the stability of different coating materials, capsules of different coatings filled with carrier materials alone were kept under ambient conditions (up to 75% RH and temperature range of 32 to 33.5°C) in Petri plates of 9 cm diameter, lined with filter paper. The extent of disintegration was observed at 24 hr interval for a period of one week. In order to study the disintegration at higher humidity levels (80 to 85%) with temperature 27.5 to 30°C that can be expected during rainy season, a humid chamber was created by filling the base of a container with lid with moist cotton and adjusting the moisture percentage to 80 to 85% by trial and error method. The desired moisture content was ensured throughout the experimental period by checking the same at 12 hr interval and maintaining the desired level by wetting cotton at the base of humid chamber. A thermo hygrometer fitted inside the chamber was used for this purpose. Observations were recorded at 24 hr interval on the time taken for disintegration and extent of disintegration of capsules.

Assessment of degradability in soil

In order to manage the soil dwelling pests, it is necessary to apply the capsules in the planting pit. Therefore, it is essential to study the time taken for disintegration at varying levels of soil moisture. For this, the capsules were evaluated for their disintegration at five different moisture levels *viz.,* Permanent Wilting Point (5 and 15%), Available Water Holding Capacity (20 and 25%) and Field Capacity (30%). Permanent Wilting Point is defined as the moisture content of soil when most plants in that soil wilt and fail to retain their turgor upon rewetting. Available Water Holding Capacity is the portion of water that can be absorbed by plant roots. Field capacity is the amount of water remaining in the soil a few days after having been wetted and after free drainage has been ceased. The capsules were placed in plastic Petri plates of 9 cm diameter, filled with 30 g of red soil, the moisture content of which was adjusted to the desired level by adding measured volume of water throughout the observational period by trial and error method and the moisture level was determined using a moisture analyser (AXIS, Model ATS 60). The atmospheric temperature ranged from 27 to 33.5˚C during the experimental period. Observations were recorded on the time taken for disintegration of quarter, half, three-fourth and full capsules.

Assessment of degradability in plant

Time taken for degradation was therefore assessed by placing the capsules to the vulnerable points of entry of pseudostem weevil such as leaf sheath, leaf axils and bore holes of seven month old plants of banana variety, Nendran. The atmospheric temperature and humidity that prevailed during the experimental period were collected from the Department of Agricultural Meteorology, College of Agriculture, Vellayani, Kerala, India.

Table 1. Treatments used for the assessment of stability and degradation of capsules

Capsule coating	Carrier materials
Hard Gelatin Transparent (HGT)	Talc
	Chitin
	Chitosan
	Sodium alginate
	Calcium alginate
Hard Gelatin Coloured (HGC)	Talc
	Chitin
	Chitosan
	Sodium alginate
	Calcium alginate
Hydroxy Propyl Methyl Cellulose (HPMC)	Talc
	Chitin
	Chitosan
	Sodium alginate
	Calcium alginate

The above three experiments were conducted in Completely Randomized Design (CRD) with 15 treatments, each replicated thrice. The details of the treatments are provided in Table 1. Superior coating material selected from this experiment was used for further studies to standardize the moisture content.

Preparation of primary powder (Active ingredient)

Beauveria bassiana (Bb5) originally sourced from National Bureau of Agricultural Insect Resources (NBAIR) and maintained in the Biocontrol laboratory for crop pest management, College of Agriculture Vellayani, Kerala, India was utilized for encapsulation studies. Conidial suspension of 14-day old cultures raised in Potato Dextrose Broth (PDB) and incubated at 25°C for 7 to 10 days was centrifuged in a Rotek centrifuge (REMI R-8C) at 4000 rpm for 20 min. to obtain the fungal pellet. The spore pellet, after removing the mycelial fragments by gentle washing with sterile distilled water, was mixed with equal quantity of chitosan (crude) to obtain primary powder with increased spore count at the rate of 10^{10} spores g^{-1}. Chitosan was selected to prepare primary powder as it has anti- microbial properties.

Preparation of filling material

The ratio of primary powder and carrier material was fixed as 1:20 based on trial and error method maintaining the spore load at the rate of 10^{10} spores g^{-1}. Two superior carrier materials selected from the preliminary experiment was used in the filling material.

Fig. 1. *Beauveria* capsules

Preparation of capsule formulations

Empty capsules were filled with the filling material mentioned above using a capsule filling device mentioned earlier. The biocapsules of *B. bassiana* is shown in Figure 1. Moisture content of the filling material tested for suitability were 5, 8, 10 and 15% which was determined using a moisture analyser (AXIS, Model ATS 60).

Viability of capsule formulations

To obtain firsthand information on viability of spores, germination assay was carried out using the primary powder before encapsulation. Germination assay was carried out by 'Hanging drop technique' (Pimpalgaonkar & Chandel, 2014). A stock solution of primary powder was prepared by dissolving one gram of the same in one mL of sterile water. The sample (100 µL) was placed on a coverslip and it was then inverted onto the concave depression of cavity slide to produce a 'hanging drop'. The slide was fixed with lactophenol-cotton blue stain and was observed under high power (45X) of a compound microscope (Motic, Model: BA210LED). Spore suspension prepared from 14-day old

culture broth served as the control. Observations were recorded on total number of spores and number of spores germinated till 100% germination was noted.

Assessment of moisture content of the formulation

Ideal moisture content of the formulation was determined based on stability of the coating *vis-a-vis* viability of the fungal propagules used in the formulation. Ideal moisture content of a microbial formulation specified by Central Insecticide Board and Registration Committee (CIB&RC) being less than 12%, the moisture regimes lesser and greater than that *viz.* 5, 8, 10 and 15% were tested in this experiment.

Capsules formulated in the above mentioned moisture levels were subjected to viability studies for a period of one month. It was determined based on the number of colony forming units in 10^{-5} dilution. Serial dilution and agar plating technique using Rose Bengal Agar (RBA) suggested by Aneja (1996) was followed. The tabulated data were subjected to statistical analysis using WASP 1 (Web Assisted Statistical Package) software, developed by ICAR - Central Coastal Agricultural Research Institute, Goa, India.

Assessment of efficacy of capsule formulations

The promising capsule formulations were kept within holes made on the pseudostem pieces of 15 cm length (four holes per pseudostem) using a glass rod and third instar grubs of *O. longicollis* @ one hole^{-1} were released into the same. The pseudostem pieces were split opened every day and examined for symptoms of morbidity and mortality of grubs, for a period of five days.

RESULTS AND DISCUSSION

Stability of capsule coating for storage under ambient conditions

Observations under ambient conditions revealed that up to 75% RH and a corresponding temperature range of 32 to 33.5°C, all the coatings (HGT, HGC, HPMC) were stable irrespective of the carrier materials used, till the end of experimental period (one week). When the RH was increased to 80 to 85% with the corresponding temperature, 27.5 to 30˚C, all the coating materials were stable for 48 hr. Thereafter, HGT and HPMC capsules started disintegrating from the surface of coating. This indicated the suitability of all the coatings under ambient conditions of temperature and humidity, which is the usual storage condition.

An ideal capsule should be stable under storage but degradable under field conditions. The results pertaining to its degradability in field is depicted below.

Degradability in soil

All the capsule coatings filled with different carrier materials retained stability when kept in soil at 5 and 15% moisture (Permanent Wilting Point).

At 20 to 25% moisture (Available Water Holding Capacity), it was observed that chitosan was the carrier material that was susceptible to easy and complete disintegration, (within 48 hr) irrespective of coating material used. Talc filled capsules took more time *i.e.,* 48 to 96 hr. Those talc based capsules coated with HPMC exhibited more resistance for degradation. Chitin was the carrier that took long time for degradation (48 to 168 hr).

At 30% soil moisture (field capacity) also, chitosan filled capsule degraded faster (24 hr), chitin filled capsules took 48 hr and talc filled ones took 60 hr. Among the coating materials HGT was susceptible to complete degradation within 24 hr, while HGC and HPMC took 48 and 60 hr, respectively. Hence it is concluded that for quick release of biopesticide which is often meant for curative treatment, HGT is the ideal coating and chitosan is the ideal carrier. For slow release requirement (prophylactic treatment), HGC or HPMC coatings can be selected with chitin or talc as carrier.

The analysis of capsule coatings and carrier materials based on storage stability and the extent of disintegration in soil and plants is the first of its kind in the field of agriculture. A similar work carried out by Pina and Brojo (1996) in the field of pharmaceuticals proved that the hard gelatin capsules disintegrate readily within the gastro intestinal tract of humans resulting in fast release of contents. The qualities of HPMC were highlighted by Charan (2015), who reported that it has more physical strength, offers protection from moisture and microbial contamination and is compatible with carrier materials. Hard gelatin coatings were successfully used for encapsulating Plant Growth Promoting Rhizobacteria (PGPR) by Anandaraj (2016) and claimed that the technology offers easiness in storage and delivery of the microbes under field conditions. Similar were the findings by Gola, *et al*. (2019) who have used gelatin as coating for developing myco-capsules of *B. bassiana*.

Degradability in plant

HGT, HGC and HPMC coated capsules remained intact for three days on a normal sunny day with no rainfall or irrigation (RH - 70%, temperature - 33°C), irrespective of the carrier material filled. But on a rainy day, (RH - 77%, rain fall - 6.76 mm), capsules disintegrated completely within the leaf axils as well as beneath the leaf sheaths within 48 hr, whereas talc based capsules disintegrated on the first day itself.

When inserted into the bore holes of pseudostem, there was complete disintegration of all capsules within 24 hr. Therefore, placement of capsules in leaf axils or within pseudostem sheath can be adopted for prophylactic control as there is slow release, while placement in bore holes can be considered for curative application, as the disintegration is faster. It can be concluded from above experiments that talc filled HGT capsules are well suited for application in plants to control pseudostem weevil. Furthermore, chitosan filled HGT capsules can be employed for controlling rhizome weevils in soil.

Suitability of chitosan as a carrier material for biocontrol fungi was earlier suggested by Palma-Guerrero, *et al.* (2007). They reported the degradability of chitosan by biocontrol fungi like *B. bassiana*, *Lecanicillium psalliotae* (Treschew) Zare and Gams, *Pochonia chlamydosporia* (Goddard) Zare and Gams and *Trichoderma harzianum* Rifai. The faster degradation of chitosan filled capsules in soil observed in this study may be attributed to the enzymatic action of soil-inhabiting fungi. However, various capsule coatings filled with chitin and alginates were firm towards disintegration. This may be attributed to the poor solubility of chitin in water which limits its application in the field of agriculture, as observed by Dutta and Dutta (2009).

Viability of capsule formulations

Germination rate of spores is an important attribute of its viability. The rate of germination of *B. bassiana* spores in aqueous suspension of primary powder used for formulating capsules was 35.70% after 24 hr, 49.33% after 72 hr and 100% after 120 hr. The corresponding germination rates noted in 14-day old pure culture were 48.06, 100 and 100%, respectively. Though there was a significant reduction in spore germination (78.28%) in the primary powder when observed after 24 hr, it was equivalent to that in the pure culture after 120 hr, indicating that the formulation process did not affect the viability, except for the delay it took to imbibe moisture when dispersed in water. This may be due to the fact that aqueous spores germinated faster as they are naked, while those incorporated in carrier material encountered a certain limit of dryness during the initial hours. Later on, as and when they absorbed moisture, they germinated. This observation gives a positive indication on the storage stability of the proposed formulation which can eventually remain dormant in storage and can readily germinate in a spray solution.

Viability of *B. bassiana* capsules at varying moisture contents (5,8,10 and 15%) was assessed in the capsules prepared using selected carriers *viz*. chitosan and talc (Table 2). The results revealed that on 7th and 21st Days After Storage (DAS), the number of cfu recorded at various moisture levels, did not vary significantly in chitosan based capsules.

Table 2. Effect of moisture content on the viability of *B. bassiana* capsules formulated in chitosan and talc

Moisture content (%)	*Mean number of cfu (107 mL-1)			
	Storage period			
	7 DAS	15 DAS	21 DAS	30 DAS
Formulated in chitosan				
5	2.57 (1.60)	2.26 (1.51)b	2.23 (1.49)	1.14 (1.07)b
8	2.62 (1.62)	2.42 (1.54)b	2.30 (1.52)	2.23 (1.49)a
10	2.72 (1.65)	2.62 (1.62)ab	2.30 (1.51)	2.27 (1.51)a
15	2.86 (1.69)	2.74 (1.65)a	2.41 (1.55)	2.27 (1.51)a
CD (0.05)	NS	0.088	NS	0.112
Formulated in talc				
5	1.52 (1.23)	1.39 (1.18)	0.63 (0.79)c	0.42 (0.64)c
8	1.54 (1.24)	1.41 (1.24)	0.87 (0.93)b	0.76 (0.87)c
10	1.56 (1.25)	1.48 (1.22)	1.31 (1.16)a	1.21 (1.06)b
15	1.64 (1.29)	1.59 (1.27)	1.39 (1.18)a	1.31 (1.15)a
CD (0.05)	NS	NS	0.041	0.047

Note: * Mean of four replications, DAS – Days After Storage, Figures in parantheses are values after √x+1 transformation

On the 15th DAS, capsules with 15% moisture level exhibited maximum viability with cfu value.

2.74×10^7 mL^{-1} at the end of the experimental period (30 DAS), formulations with 15, 10 and 8% moisture content exhibited same degree of viability and those with 5% moisture content exhibited least viability (1.14×10^7 cfu mL^{-1}). With regard to talc based capsules, during the initial two weeks of storage, there was no statistical variation in the number of cfu observed in those formulated at 5, 8, 10 and 15% moisture level. At the end of one month, formulation with 15% had maximum cfu (1.31×10^7 mL^{-1}) and at 10 and 5% moisture content, cfu counts were on par with each other, with values being 0.76×10^7 mL^{-1} and 0.42×10^7 mL^{-1}. Even though the microorganism exhibited greater viability at 15% in both chitosan based and talc based capsules, 10% moisture content was chosen for preparing capsule formulations, as stability of capsule coating was not maintained at 15%.

In general, *Beauveria* exhibited higher spore viability at 10 and 15% moisture and least viability at 5%. Moisture content of 10% was determined as the ideal moisture content of filler material used for encapsulating the fungus, considering the stability of coating as well as viability of fungal propagules. Derakshan, *et al.* (2008) propounded that the viability of the entomopathogenic fungus *Lecanicillium lecanii* (Zimmermann) Zare and Gams in talc formulations at 5 and 10% were on par and was significantly higher than that at 15%. The percentage viability at room temperature under 5% moisture was 28.37 after two months and 75.03 after six months and at 10% moisture content, it was 27.59 after two months and 76.03 after six months. Similarly, at 15% moisture, the cfu values ranged between 21.86 and 71.56, after two months and six months, respectively. However, Posada-Florez (2008) reported that 10% moisture was high to maintain viability of *B. bassiana* spores and that 5% can be considered as ideal moisture content. Still lower moisture content of less than 4% was used by Satyasayee, *et al.* (2008) for preparing tablets of *Beauveria*, *Metarhizium*, *Paecilomyces*, *Lecanicillium* and *Nomuraea* to get a dry composition.

The present study revealed that HGT, HGC and HPMC coatings are stable under ambient conditions of temperature and humidity. However, for quick release of biopesticide, HGT is the ideal coating which is indicated by greater tendency of HGT coating to disintegrate readily under atmospheric conditions in the field. Furthermore, for slow release application, HGC or HPMC coatings can be selected. Considering the degradability in soil and plant, chitosan and talc are the ideal carrier materials, which when encapsulated in HGT coating can be adopted for prophylactic treatment. Meanwhile, HGC or HPMC coatings with chitin or talc as carrier can be adopted for curative treatment. It was further concluded that talc filled HGT capsules are well suited for application in plants to control pseudostem weevil and chitosan filled HGT capsules can be employed for controlling rhizome weevils in soil. Spore germination assay revealed that within 24 hr, spores in primary powder exhibited greater rate of germination than those in pure culture suspension. However, the germination was completed in both after 120 hr. Regarding the moisture content of filling material used for encapsulation, 10% moisture content was determined as ideal moisture for encapsulating the fungus.

Efficacy of capsule formulations

Placement of chitosan based capsules of *B. bassiana* witnessed disintegration of capsule coatings after 48 h of placement within banana leaf axil and reduced the feeding activity of *O. longicollis* grubs. Moreover, the infected grubs were also found to be sluggish. Mortality was observed on the third day after treatment (72 HAT). When talc based capsules were applied, it was observed that the coating got disintegrated entirely after 24 h of placing the capsules. The grubs exhibited aversion in feeding at 72 HAT, resulting in mortality at 84 HAT.

Pathogenicity of *B. bassiana* to *O. longicollis* noted in this study is in line with the findings of many researchers including Irulandi *et al.* (2012) and Prabhavathi (2012).

The production cost involved is Rs. 0.5 per capsule. Meanwhile, the cost of single application of chlorpyriphos, a conventional chemical used for pseudostem weevil management is Rs. 0.24 only. But, this high cost incurred during plant protection using biocapsules can be compensated with the higher market price fetched by the 'safe-to-eat' organic products. Evaluation of *Beauveria* capsules in farmer's field by Balakrishnan (2020) validated its efficacy in managing pseudostem weevils both prophylactically and curatively.

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