Research Article

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Preservation and long-term storage of *Trichoderma* spp. by sodium alginate encapsulation

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Abstract

Trichoderma species have become popular as biocontrol agents and are being used to protect crops from many plant pathogens world over. The experiments carried out to preserve *Trichoderma* species in the form of sodium alginate encapsulated beads revealed that the conidial and mycelial stages in encapsulated form remained viable for more than six years at room temperature. Production of alginate encapsulated *Trichoderma* was relatively a simple procedure but found to be an efficient method for maintaining the pure culture and also for long-term preservation. The survival and conidiation from the encapsulated beads are very much comparable to freshly inoculated culture. The organism can be revived from encapsulated beads by inoculating onto either PDA or organic substrates like vermicompost. On revival, its bio-efficacy remained as in fresh culture, and on soil application the antagonistic potential was sheltered.

Keywords: Black pepper, biological control, encapsulation, foot rot, long-term preservation, *Trichoderma harzianum*

Introduction

Fungal cultures are generally preserved/stored in water at normal room temperature, which is an easy and cost-effective procedure (Castellani, 1939; 1963; McGinnis *et al.*, 1974). But here the stability of the cultures is not ensured. Preservation in soil or on oil (Bakerspiegel, 1953; Buell and Weston, 1947), water-covered slants or agar slants, cryopreservation in liquid nitrogen or at low temperature (-20 and -70°C) (Elliott, 1976) *etc.*, are the methods being followed by the American type culture collection (ATCC, 1991). Although lyophilization is effective for long term storage, it is cumbersome and involves a lengthy procedure, so also it requires sophisticated equipment like Lyophilizer. Storage in liquid nitrogen vapour (above liquid at ≥-130°C) is a more feasible and cost-effective alternative for long-term preservation of living cells, but many times the availability of liquid N_2 is limited.

T. harzianum is one of the most potential biocontrol agents against *Phytophthora* induced foot rot disease of black pepper (Rajan *et al.*, 2002). Significant improvements have been made in culturing and mass multiplication of this potential strain. Several formulations were also available in practice for disseminating the technology to the farmers which include talc-based formulation and liquid formulations (Bhai and Anandaraj, 2014), solid formulation in decomposed coffee husk and farmyard manure (Bhai *et al.*, 1994), besides bio-capsule recently patented at ICAR-Indian Institute of Spices Research (ICAR-IISR, Kozhikode). The technology-based on talc and bio-capsule are being licensed by many entrepreneurs. Bhai and Anandaraj (2014) tested different liquid media based on glycerol (100, 50 and 25%), glucose (100, 50 and 25%), and DMSO (10%) for maintaining the viability of *T. harzianum* spores. They observed that all the above media maintained the viability only up to 75 days, and thereafter, there was a decline in viability. Viability was completely lost in 300 days while sterile deionised water maintained the viability even after

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720 days (Bhai and Anandaraj, 2014). However, all these formulations were made mainly for field-level applications. At ICAR-IISR, Kozhikode, during investigations on disease control, many *Trichoderma* isolates were collected from geographically different locations and maintained in agar slants. These cultures were sub-cultured periodically to maintain their viability, which is cumbersome and needs skilled technicians. Many of these cultures may not survive for long, or even if survived, it would be contaminated through the moist cotton plug. Retrieval of pure culture from these contaminated agar slants after long-term preservation is a difficult task. Therefore, the study was envisaged to develop a suitable preservation method to maintain these potential collections without losing the viability and bioactivity.

There are reports on encapsulation for preserving the viability (Lacey and Undeen, 1986; Axtell and Guzman, 1987) of the mosquito pathogen *Lagenidium giganteum* in calcium alginate. It is an oomycete belong to Lagenidiales, one of the most promising biological control agents against mosquito larvae (Lacey and Undeen, 1986). Alginate encapsulation has been successful with plant pathogens and biofertilizers, including bacteria and fungi (Bashan, 1986; Sangeeth and Bhai, 2010). Entomopathogens *viz.*, Steinernematid and Heterorhabditid nematodes and even entomopathogenic *Metarrhizium* sp. have been encapsulated (Kaya and Nelson, 1985; Liu and Liu, 2009). So, if encapsulated *T. harzianum* retained its viability and bioactivity, it would be more convenient to preserve without contamination and better than the conventional preservation procedures. Therefore, in this study experiments were conducted to develop encapsulated conidial and mycelial stage of *Trichoderma* in sodium alginate and to evaluate the effect of encapsulated beads to ensure long-term survival and bioactivity as well as its biocontrol efficiency.

Materials and methods

Trichoderma culture

The experiment was started during 2012 at ICAR-IISR, Kozhikode, Kerala, India. *Trichoderma harzianum*, MTCC 5179, a potential biocontrol agent against foot rot of black pepper, maintained, in the Institute's repository was used for encapsulation. The isolate was made into a pure culture on potato dextrose agar (PDA) medium by growing the culture for 72 hrs in the light at 25±1°C. Mycelial plugs of 5 mm size cut from the growing margin were transferred to sterile potato dextrose broth (100 mL) and grown for 5-7 days at 25±1°C. The conidia were extracted and centrifuged, and the pellet was suspended in the required quantity of water.

Pathogen culture

The major pathogens of spice crops *viz.*, *Phytophthora capsici*, *Colletotrichum gloeosporioides*, *Rhizoctonia solani*, *Sclerotium rolfsii* and *Fusarium oxysporum* maintained in the pathology section at ICAR-IISR was used for bioassay tests. These pathogen cultures were preserved in PDA slants and sub-cultured for further use.

Encapsulation

Trichoderma suspension made as above, having a cfu $20x10^{10}$ mL^{-1}, was mixed in 1:4 ratio with 0.6 per cent sodium alginate solution and allowed to drip into $CaCl_2$ (1.5%) taken in a beaker and kept at room temperature for 30 min. The beads (of uniform size of 3 mm) formed in $CaCl_2$ was strained through a sterile muslin cloth and allowed to air dry aseptically. The sodium alginate coated *Trichoderma* beads were then stored under sterile distilled water in plastic bottles and kept at room temperature throughout the experimental period. These beads were taken out as and when required for the assay. The experiments were repeated at least four times.

Purity and viability check

The viability of encapsulated *Trichoderma* was checked periodically at 5, 10 and 20 days after storage and then after one year and thereafter, at alternate years for six years. For viability and purity check, the inoculated beads were retrieved under aseptic conditions by serial dilution plating in PDA to get single pure colonies. Similarly, the encapsulated beads were inoculated onto four different substrates *viz.*, vermicompost, powdered farmyard manure, coir pith and potting mixture. Briefly, the substrates were taken in jam bottles @ 100 g $bottle^{-1}$ and sterilized under 15 lb pressure

for 20 min. After cooling, the substrates were inoculated with encapsulated beads and incubated for 5-7 days. There were three replications for each substrate and was repeated twice to confirm results.

In vitro bio-efficacy check

The encapsulated *Trichoderma* beads after storage at room temperature were tested periodically for bioactivity against major pathogens *viz.*, *P. capcisi*, *C. gloeosporioides*, *S. rolfsii* and *R. solani*, *F. oxysporum* f.sp *vanillae by* dual culture technique. There were three replications for each test, and the dual culture assay was repeated twice and at specific intervals as mentioned above.

In planta bio-efficacy check

Black pepper plants

Plants of variety Sreekara were raised in potting mixture in poly bags of 15 x 10 cm. These plants at the 3-5 leaf stage were used for challenge inoculation and bio-efficacy tests for disease suppression.

Multiplication of *Trichoderma* from encapsulated beads

For *in planta* bio-efficacy testing, the *Trichoderma* was multiplied from encapsulated beads by inoculating into different substrates as above. These sterilized substrates were inoculated @ 5 beads per bottle. After five days of growth, the enriched substrates were applied to black pepper plants raised as above @ 10 g per plant. These plants were challenged with *P. capsici,* 10 days after inoculation with *Trichoderma* enriched substrates. Talc based formulation, as well as a liquid formulation, was also included keeping an absolute control without *Trichoderma*. Observations were recorded on plant infection by *P. capsici*. The experiment was repeated twice for confirmation.

Results and discussion

T. harzianum, the most used bioagent against *P. capsici*, the foot rot pathogen, was tested for long term preservation in sodium alginate by encapsulation, which otherwise is being preserved on agar slants at 4°C. The encapsulated beads of *T. harzianum* were stored under sterile distilled water kept at normal temperature (Fig. 1). Viability and bioactivity of the encapsulated beads were tested periodically for six years. After encapsulation of *Trichoderma* (in 2012), re-isolation was done at 5, 10 and 20 days and after 1, 2, 4 and 6 years (up to 2018). Initially, the beads were taken out, crushed and serial dilution plating was undertaken to get single and pure colonies (Fig. 2 a and b). The single colonies were tested for *in vitro* bioassay. The recovery showed abundant growth and sporulation of *Trichoderma* in 48hrs as seen in freshly inoculated culture, and bioassay showed inhibition of *P. capsici* (Fig. 2c and d) revealing the retention of bioactivity of alginate embedded *Trichoderma*. The encapsulated *Trichoderma* also showed abundant growth in dried farmyard manure and vermicompost (Fig. 3; Table 1). The beads stored during December 2012 remained viable even in 2018 (6 years) without losing the bioactivity or bio-efficacy as observed from the growth and inhibitory activity of retrieved culture in different years, *i.e.*, 2012-2018 (Fig. 4-6; Table 2 and 3). No contamination was observed at any time of sub-culturing indicating

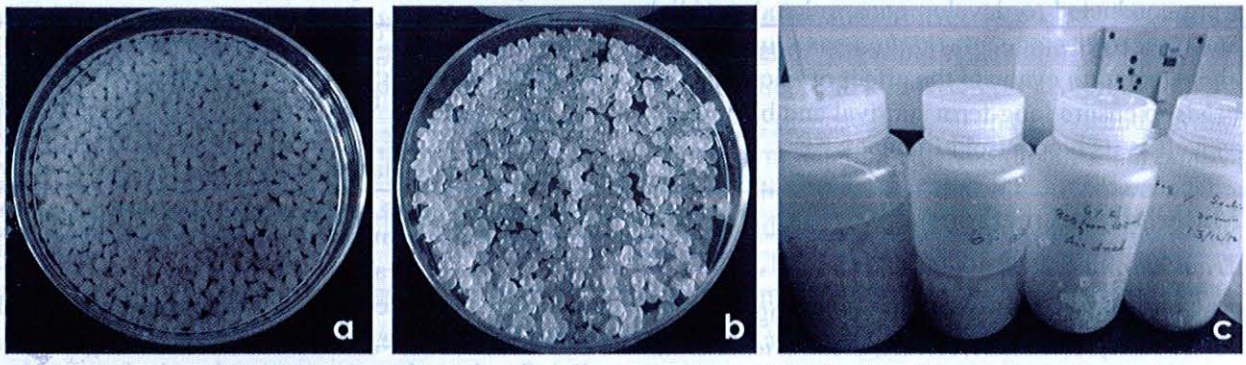

Fig. 1. Encapsulation of *T. harzianum*: a) Formation of beads in 1.5% $CaCl_2$, b) Drying of encapsulated beads, c) Storage of beads in water at room temperature

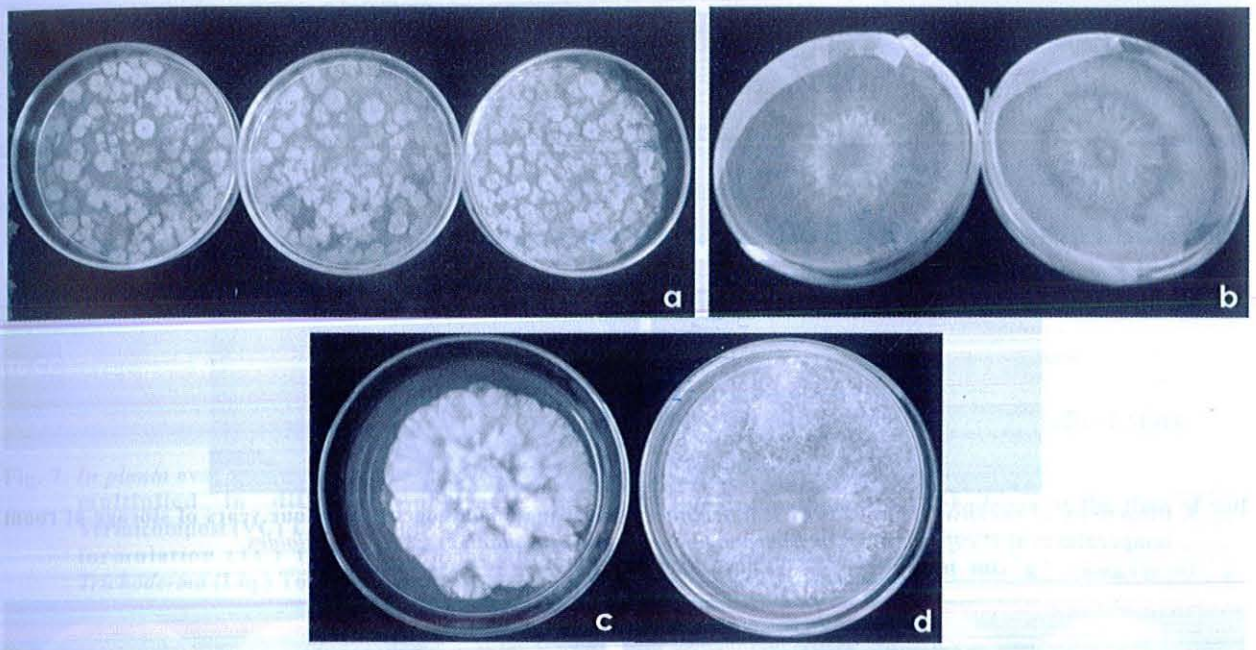

Fig. 2. Viability and bioactivity of *T. harzianum* encapsulated beads 20 days after storage: a) Growth of *Trichoderma* from encapsulated beads in dilution plates, b) Pure culture of *T. harzianum* retrieved from beads, c) *P. capsici* growth in control, d) Inhibition of *P. capsici* by *T. harzianum* in dual culture

the purity of the encapsulated culture. The alginate beads of *Trichoderma* in water can be stored for more than six years without contamination, which is an effective system for long term culture storage.

The bioactivity of the encapsulated *Trichoderma* was tested by dual culture method against pathogens, such as *P. capsici*, *S. rolfsii*. *C. gloeosporioides*, *R. solani* and *F. oxysporum* f.sp. *vanillae*. The results showed an inhibitory effect on the range of 48 to 88 per cent as seen in fresh culture (Table 3). The pathogen inhibition *in planta* by encapsulated *Trichoderma* is comparable to the liquid and talc-based formulations. Complete

Table 1. Growth of *Trichoderma* from encapsulated beads in different substrates

Sl. No.	Treatments	10th day	20th day
1.	Potting mixture	5.05^{d} (11 x 10^{4})	6.75^{d} (6 x 10^{6})
2.	Dried and powdered FYM	9.16^{b} (14 x 10^{8})	8.76^{c} (6 x 10^{8})
3.	Vermicompost	10.84^{a} (69 x 10^{9})	11.93^{a} (9 x 10^{11})
4.	Decomposed coir pith	6.97^{c} (93 x 10^{5})	9.08^{b} (1 x 10^{9})
CV (%)		0.48	0.57
SE(d)		0.027	0.036
LSD at 5%		0.0598	0.0795

*Figures in parenthesis are original values. Values followed by the same letters are not significantly different in DMRT test at P=0.05

Fig. 3. Growth of encapsulated beads in different substrates: a) Coir pith compost (CC), b)Vermicompost (VC), c) Farmyard manure (FYM), d) Potting mixture (PM)

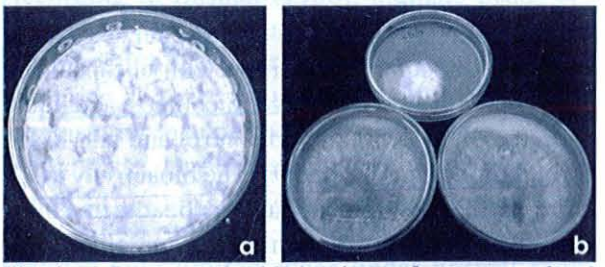

Fig. 4. a) Pure growth of *T. harzianum* from encapsulated beads after storage for one year, b) *In vitro* antagonistic activity of encapsulated *T. harzianum* against *P. capsici* after storage for I year at room temperature

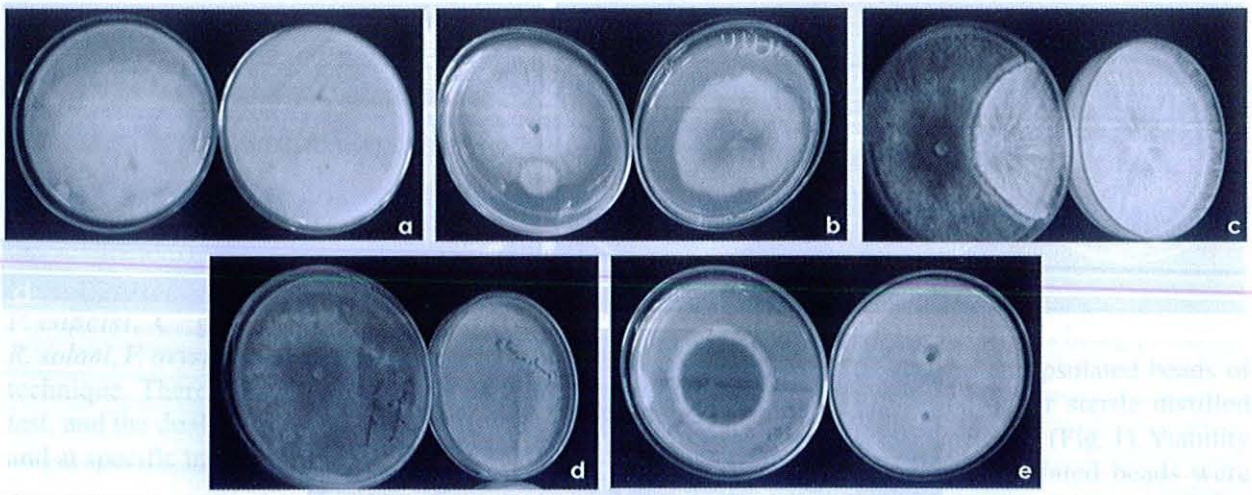

Fig. 5. Bioactivity of encapsulated beads of *T. harzianum* against major pathogens after four years of storage at room temperature: a) *P. capsici*, b) *F. oxysporum*, c) *S. rolfsii*, d) *R. solani*, e) *C. gloeosporioides*

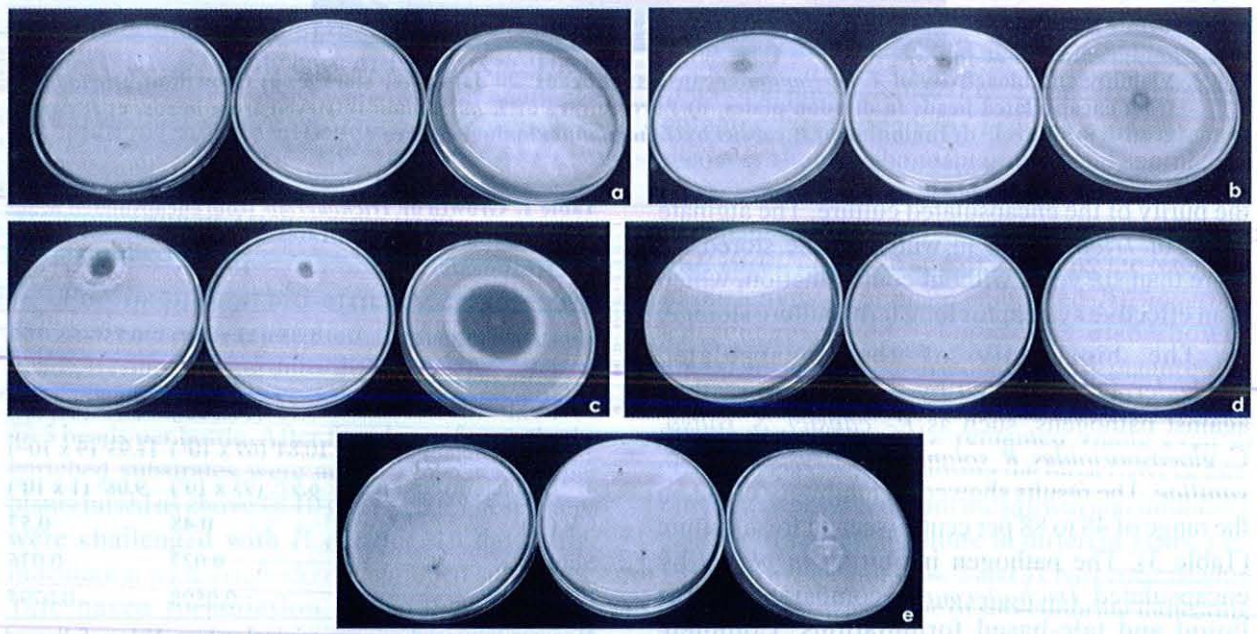

Fig. 6. Bioactivity of encapsulated *Trichoderma* against major pathogens after six years of storage at room temperature: a) *P. capsici*, b) *F. oxysporum*, c) *C. gloeosporioides*, d) *S. rolfsii*, e) *R. solani*

suppression of infection was observed under challenge inoculated condition with encapsulated beads in farmyard manure, vermicompost and liquid formulation as compared to control and talc-based formulation (Fig. 7, 8 and 9). The bioactivity assay showed that five encapsulated beads in 100 g vermicompost are sufficient to get a population of $>10^{11}$ cfu g^{-1} in 10 days which is equivalent to >10 kg of talc-based formulation (Table 1). Thus the results of viability as well as bioactivity tests revealed that the encapsulated formulation could retain the viability up to >6 years at room temperature and when incorporated with farmyard manure or vermicompost, could multiply as usual (Table 2). There was no loss in viability from initial storing as evidenced from its luxuriant growth in organic media as well as in potato dextrose agar media.

Production of alginate encapsulated *Trichoderma* is a relatively simple procedure. Mycelia, along with conidia or even conidia alone,

Fig. 7. *In planta* evaluation of encapsulated *Trichoderma* multiplied in different substrates. T1-Vermicompost (VC) T2-Control, T3-FYM, T4 Talc formulation (TC) T5-Liquid formulation of *Trichoderma* (Liq.) T6-Coir compost (CC)

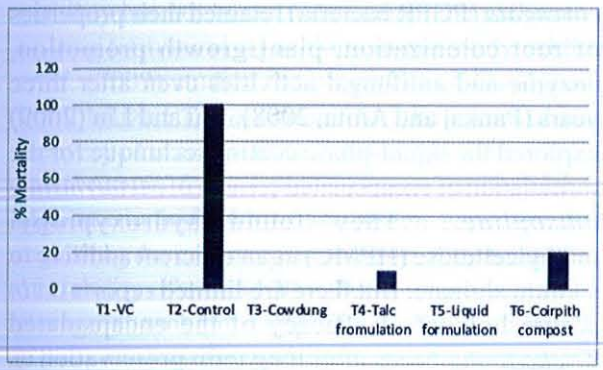

Fig. 8. *In planta* bio-efficacy of the formulation after four years

can be stored by this method. The encapsulated beads remain viable for more than six years under normal room temperature. This is found to be an efficient system for maintaining pure culture and also for long term preservation, especially bio-agents like *Trichoderma* spp. Apart from viability, the culture also retained its bioactivity as evidenced from *in vitro* inhibition of pathogen at various stages of retrieval as well as disease suppression by challenge inoculation. The bioactivity was comparable to freshly prepared talc-based or liquid formulation. The encapsulation in the present experiment was done under sterile conditions, and no contamination was observed during storage and also at the time of revival during different periods. The encapsulation can be achieved with minimum inputs.

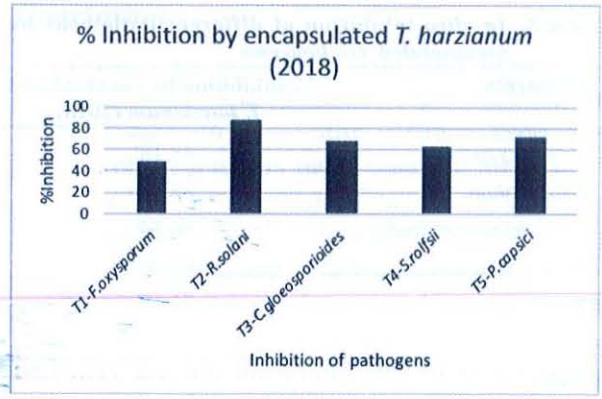

Fig. 9. Bio-efficacy of the formulation after 6 years

Table 2. Population of *Trichoderma* at the time of soil application in in planta evaluation

Treatments	Count mL^{-1} g^{-1}	Log cfu mL^{-1} g^{-1}
T1 - Vermicompost	3×10^8	8.46^c
T2 - Coir compost	6×10^4	4.77^e
T3 - Decomposed FYM	7×10^8	8.86^b
T4 - Talc	5×10^7	7.69^d
T5 - Liquid formulation	5×10^{10}	10.69^a
T6 - Control		-
General mean		8.10
p-value		
CV (%)		1.26
SE(d)		0.083
LSD at 5%		0.185

Values followed by the same letters are not significantly different in DMRT test at P=0.05

There are reports on the production of encapsulated beads using alginate to preserve cultures. Alginate encapsulation has been already reported as successful with certain chemical, plant pathogenic bacteria and fungi (Bashan, 1986). According to Bashan (1986), alginate beads are synthetic, dry, simple to use, uniform in size, biodegradable by soil microorganisms, and non-toxic and also provides a slow release of the organism for long periods and may be produced on a large scale. Dry beads containing bacteria can be stored at room temperature over a long period without loss of bacterial content and also storage requires only limited space under normal room temperature. *Lagenidium giganteum* is one of the most promising biological control agents against

Table 3. *In vitro* inhibition of different pathogens by encapsulated *Trichoderma*

Treatments	% inhibition by encapsulated *T. harzianum* (2018)
T1 - *F. oxysporum*	48.18[c]
T2 - *R. solani*	88.15[a]
T3 - *C. gloeosporioides*	68.84[b]
T4 - *S. rolfsii*	62.96[b]
T5 - *P. capsici*	71.67[b]
General mean	67.96
p-value	
CV (%)	9.15
SE(d)	5.076
LSD at 5%	11.309

Values followed by the same letters are not significantly different in DMRT test at P=0.05

mosquito larvae *Anopheles quadrimaculatus* (Lacey and Undeen, 1986; Rueda *et al.*, 1991). Axtell and Guzman (1987) preserved the viability of *L. giganteum* in calcium alginate where floating calcium alginate capsules of *L. giganteum* with 1 per cent ground cork gave higher levels of control of the larvae than sinking capsules containing no cork. However, they found no comparable difference between the cork capsules and sinking capsules in the infection of larvae after storage at 15 ^{0}C for 57 days (Axtell and Guzman, 1987). Similarly, potential biocontrol agents such as *Penicillium oxalicum*, *T. viride*, *Pseudomonas cepacia, etc.* were encapsulated in the alginate-Pyrax matrix using 0.25 per cent $CaCl_2$ or 1 M calcium gluconate. Here except *P. cepacia*, other fungi remain viable after pellet formation with Ca gluconate. But a decline in population was noticed during the testing period itself with a loss of 10-100 folds after four weeks (Fravel *et al.*, 1985).

Viveganandgan and Jauhri (2000) reported the effectiveness of alginate formulations over charcoal formulations in maintaining the population of phosphate solubilizing bacteria. Incorporation of charcoal-soil in 3:1 proportion adversely affected the initial filling of these bacteria in alginate. Still, alginate alone supported maximum survival at higher temperature. Bashan *et al.* (2002) also developed alginate beads for preserving *Azospirillum brasilense*.

In our study, initial bioactivity test with encapsulated *Trichoderma* by dual culture method against pathogens showed inhibitory effect up to the range of 48 to 88 per cent. The bioactivity on pathogen was as normal as that of the liquid or talc-based formulation. Disease suppression was 100 per cent under challenge inoculation compared to control or talc-based formulation. Five encapsulated beads were enough to attain a population of $>10^{11}$ cfu g^{-1} in 10 days which is almost equivalent to >10 kg talc based formulation (Table 1). As demonstrated by Rekha *et al.* (2007), encapsulated bacterial isolates enhanced plant growth as that of fresh cells and would be a novel and useful technique for implementation in agriculture. This is quite in tune with our study. It is also supported by the fact that sodium alginate formulations of *Bacillus subtilis* and *Pseudomonas corrugata* (PGPR bacteria) retained their properties of root colonization, plant growth promotion, enzyme and antifungal activities even after three years (Pankaj and Anita, 2008). Liu and Liu (2009) explored the liquid-phase coating technique for the formulation of encapsulated conidia of *Metarrhizium anisopliae*. They found hydroxypropyl methylcellulose (HPMC) as an efficient additive to sodium alginate. But there are limited reports on *in planta* biocontrol efficacy of the encapsulated *Trichoderma* beads after long term preservation as done in our study by challenge inoculation.

Power *et al.* (2011) showed that alginate-based beads are effective storage as well as a delivery system for PCB degrading *Pseudomonas fluorescens*. Complete recovery of viable cells was possible even after storage for 250 days. Sangeeth and Bhai (2010) standardized suitable conditions for preserving encapsulated beads of biofertilizer (Sangeeth and Bhai, 2010). The beads in water showed higher bacterial counts indicating the release of bacteria. Here water served as a triggering medium for the release of bacteria (Charpentier *et al.*, 1999). But the characteristics of beads like size, colour or disintegration varied with substrates. In the case of *Trichoderma* encapsulation, the beads are green in colour, and that too depends on the concentration of the suspension. The technology offers an efficient system for the application of biocontrol fungi, with the protection of conidia from

abiotic and biotic stresses. Sodium alginate is a biopolymer and is produced by living organisms and contains proteins, polysaccharides and nucleic acids. There are reports of *Trichoderma* encapsulation using alginate.

For soil application, as early as 1985, Lewis and Papavizas (1985) made pelletized conidia, chlamydospores and fermented suspension of *Trichoderma* and *Gliocladium viride*, in alginate gel using wheat bran and Kaolin clay. But the viability of the formulation declined to <10 per cent after 24 weeks at 25°C (Lewis and Papavizas, 1985). Fariz *et al.* (2012) formulated micro-encapsulated *T. harzianum* to enhance the viability of the effective biological control agent isolated from healthy groundnut roots. They formulated the capsules with alginate-montmorillonite clay and tested the character of the beads and storage conditions for viability but not bioactivity against the pathogens, or long term survival as done in our study. Akhtar *et al.* (2009) studied the properties of Ca-alginate immobilized *T. harzianum* for the removal and recovery of uranium from streams. Mancera-Lópeza *et al.* (2018) formulated *Trichoderma* in calcium alginate in both white and green capsules. But regarding the conidia preservation, they found loss of viability of around 30 per cent in two years after storage at ambient temperature. Knudsen and Li (1990) made pelleted sodium alginate formulation of *Trichoderma* with an intend to develop methods to quantify influences of temperature, matric potential, and nutrient source on the hyphal growth potential of pelletized *T. harzianum*. They used 2-8 week old pellets for their study; however, it remained viable for several weeks. In the present study, our idea was to protect and preserve conidia under static conditions to ensure lasting action against a series of pathogens damaging the crops, especially black pepper. The sodium alginate encapsulation preserved the viability and protects the bio-efficacy of *T. harzianum* even after six years at room temperature. Hence, *Trichoderma* encapsulation in sodium alginate would be a useful storage method for the preservation of cultures. The increasing demand can also be met with this type of easy to make formulations which can retain its viability for long periods and can be stored for long just like existing fungicide preparations.

Conclusion

Preservation of potential and beneficial microbial cultures is of utmost importance for the future. Long-term preservation of strains, especially biocontrol agent is very much essential for their in-depth study. During preservation, it is necessary to ensure viability, bioactivity and stability of living cells. *Trichoderma* spp. is of worldwide occurrence and became a popular biological tool to protect crops against plant pathogens. Recent studies showed that they are not only parasites of fungal plant pathogens but also can produce beneficial antibiotics like trichodermin. The present study is an attempt to preserve these valuable collections for the future.

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