Journal of Spices and Aromatic Crops
Vol. 23 (2) : 243–249 (2014)
www.indianspicesociety.in/josac/index.php/josac

Indian Society for Spices

Enhancing shelf life of *Trichoderma harzianum* by conidial storage in sterile deionized water

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Received 18 May 2013; Revised 01 February 2014; Accepted 28 April 2014

Abstract

In this study *T. harzianum* conidial suspension was made in different concentrations of cryoprotectants *viz*., glycerol, glucose, DMSO and deionized distilled water and stored at two different temperatures *viz*., 25± 1°C and 28-32ºC for more than 720 days for evaluating the shelf life in order to develop a ready to use liquid carrier formulation. The results showed that submerged conidia formulation in cryoprotectants like glycerol, glucose and DMSO preserved the viability for 75 days only when compared to the formulation in sterile deionized water which at 28-32°C retained the viability for 480 days with a viable count of $\log_e$= 6.671 (3.2×10^6) and for more than 720 days with a viable count of $\log_e$=5.54 (2×10^5) and in 480 days at 25±1°C with a viable count of $\log_e$=6.3911 (6.5×10^6) and with a viable count of $\log_e$ =5.40 (2×10^5) for more than 720 days. The viability was tested *in vitro* by recovery in TSM and effectiveness was tested *in vivo* by drenching the suspension on plants and challenge inoculating with *Phytophthora capsici* after seven days. Challenge inoculation of black pepper plants with *P. capsici* after drenching with diluted stored suspension gave a disease reduction of 66.67%. Hence, sterile deionized water can be used as a liquid storage medium for the long term storage of *T. harzianum* culture without loosing the viability and effectiveness of the spores.

Keywords: biological control, black pepper, conidial suspension, cryo-preservatives, foot rot, liquid formulation, *Phytophthora capsici*, *Trichoderma harzianum*

Introduction

Replacement of chemical fungicide with *Trichoderma* or integration of *Trichoderma* with chemicals reduce the input of chemicals into agricultural soils. However, its successful commercial use depends upon mass production methods that provide appropriate formulations that have good activity, better shelf life and adaptation to local farming practices and have economic viability. Delivery systems that enhance/retain the activity of the bio protectant are critically important. Demand for organic plant protection measures has brightened the importance of *Trichoderma* in a wide variety of crops. *T. harzianum* has been demonstrated for its biocontrol activity against *Phytophthora capsici* causing foot rot disease of black pepper (Rajan *et al*. 2002). They have abilities like rhizosphere competence and antibiosis which permit long term protection of roots against

pathogens (Paul *et al*. 2005). Different commercial formulations of *Trichoderma* are available in the market. Most of these commercial products are talc based. There are also formulations based on organic carriers such as neem cake, cow dung, tea waste, coffee husk (Bhai *et al*. 1994) sorghum grains (Sarma *et al.* 1998) etc. But these formulations/ products have certain limitations. In talc based formulations, the viability of the spores are less and population comes down with storage. Even though organic based formulations maintain the viability of spores, they are always prone to spoilage by insects and other microbes in the long term. Moreover these formulations are too bulky and difficult to transport in large quantities. In general, the major obstacle to the commercialization of such products is the development of a shelf-stable formulated product that retains biocontrol activity similar to that of the fresh product (Janisiewicz & Jeffers 1997). There are only a few published research concerning the impact of growth and viability of a liquid formulated biocontrol agent. Hence, study was inidiated with objectives to test the viability of *T. harzianum* conidia in different cryoprotectants, to test the effect of different storage conditions and to test the effectiveness of stored product for the biological control of *P. capsici*.

Materials and methods

Experiment was conducted at Indian Institute of Spices Research (IISR), Kozhikode during 2007–2010. *T. harzianum* strain IISR 1369 (MTCC-5179) used against *Phytophthora* foot rot disease of black pepper was used in the experiment. For spore production, the culture of *T. harzianum* was grown in PDA for 72 h and 5 mm mycelia plugs cut from the edge of 72 h old culture were inoculated to 100 mL PDA slant in Roux bottles and incubated for 30 days. Spores were extracted from this flask with sterile distilled water after straining through four layers of cheese cloth and distributed in equal quantities to pre weighed centrifuge tubes of 30 mL capacity and centrifuged at 5000 rpm for 10 min at 4°C. The supernatant was poured off and the pellet was air dried and weighed. The pellet at this concentration contained about 1.5 g of spores. The cryoprotectants *viz*., glycerol, glucose and DMSO were prepared in different concentrations (Table 1) in sterile deionized distilled water. All the preparations were made under aseptic conditions. The pelleted conidial mass was dispensed in cryoprotectants solutions (1.5 g in 250 mL) in Tarsons reagent bottles. The solutions were made in duplicate. The initial spore count in these solutions just after preparation was estimated using hemocytometer. The viable spore count as estimated by dilution plating using *Trichoderma* Selective Medium (Elad & Chet 1983) also showed a cfu of 108×10^6 which revealed that almost all spores are viable (Table 1). These suspensions were incubated at two different conditions *viz*., 25±1°C and 28-32°C. The viability of the spores in different formulations estimated at different intervals by dilution plating is given in Table 2.

Pathogen *P. capsici* isolated from infected plants and maintained in IISR repository (IISR 07-08) was used for inoculation studies. At the end of the incubation period (720 days), the formulation (T10) that showed the maximum viable spore count (CFU) in dilution plating was diluted and applied to black pepper rooted cuttings raised in polythene bags. 2 mL of the stock solution having a cfu of 5×10^8 mL^{-1} was diluted to 1 L and applied @10 mL $plant^{-1}$. After seven days these plants were challenge inoculated with *P. capsici*. There were 30 plants $treatment^{-1}$ with 10 plants $replication^{-1}$. The disease incidence was observed after 15 days of pathogen inoculation.

Statistical Analysis

Data were analyzed using analysis of variance procedure (ANOVA) and treatment means were separated according to Fisher's protected least significant difference (LSD) test ($P<0.05$) using SAS 9.3 software (Cary.NC).

Results and discussion

The pellets having spore load of 1.5 g was dissolved in 250 mL of each cryoprotectant solution. The initial spore count in these solutions soon after preparation was estimated using hemocytometer. The average count was

91×10^5 cfu mL^{-1} (7.9-8.4 $\log_e$ cfu mL^{-1}) (Table 1). The viable spore count as estimated by dilution plating using *Trichoderma* selective medium showed a population count of 11×10^7 cfu mL^{-1} which revealed that almost all spores are viable.

The overall effect of different treatments is given in Table 1. Fifteen days after incubation, the two treatments *viz*., glycerol 100% (T1) and glycerol 50% (T2) showed a sharp decline in viable spore count from 24×10^8 to 17×10^7 and 31×10^7 cfu mL^{-1} [9.08 (av.) to 4.67 and 6.04 $\log_8$ cfu mL^{-1}], respectively where as the other treatments showed only slight decline. On incubation up to 75 days, all the treatments maintained the same viability and thereafter T1-T6 (Glycerol 100, 50 and 25 and Glucose 100, 50 and 25%) showed complete loss in viability whereas the treatments T7-T9 (Glucose 10%, DMSO 5% and DMSO 10%) showed a sharp decline from 75 days onwards. Glucose 10% showed decline from 70×10^7 to 3×10^2 (6.13-0.827) in 300 days and completely lost its viability after 300 days. DMSO 5% and 10% also showed viable spore count even after 720 days but declined by 95-98%. However, sterile deionized water (T10) maintained the viability even up to 720 days but with slight decline after 480 days which showed that the spore suspension in that particular condition (in sterilized deionized) can be stored up to 480 days without much loss in viability, which clearly indicated that this treatment is effective for the long term storage and preservation of viable conidia of *Trichoderma* (Table 2). Similarly, among the two conditions under which the conidia were preserved, it was found that storing at 25±1°C is much more effective in maintaining the viability than storing at 28-32°C (Table 3). However, in the case of sterile deionized water (T10), conidia can also be stored even at 28-32°C without much significant difference in the spore viability. This is supported by the *in vitro* studies conducted on the effect of temperature on the growth of *T. harzianum* (Smitha 1999). They found that the ideal temperature for growth *in vitro* was 25-32°C. Our result also showed that sterile deionized water preserved the colour of the conidia which play a key role for the acceptance of the product.

Table 1. Viability of conidia of *T. harzianum* in different treatments at different intervals

Treatments	Direct count No. of spores mL^{-1} ($\log_7$ mL^{-1})	Initial cfu	15d	30d	75d	150d	250d	300d	360d	480d	720d	Mean
Glycerol 100%	8.05	8.64	4.672	5.345	4.528	0.500	0.000	0.000	0.389	0.000	0.000	1.715
Glycerol 50%	8.06	8.09	6.041	6.723	4.659	1.586	0.000	0.000	0.314	0.000	0.000	2.147
Glycerol 25%	8.11	9.42	7.411	6.929	6.254	3.185	1.075	0.075	0.000	0.000	0.000	2.770
Glucose 100%	8.54	9.10	7.090	6.397	5.222	0.651	0.000	0.000	0.000	0.000	2.695	2.451
Glucose 50%	8.07	9.19	7.184	7.072	6.339	0.500	0.000	0.000	0.000	0.000	0.000	2.344
Glucose 25%	7.97	9.47	7.463	7.162	5.484	0.000	0.000	0.000	0.000	0.000	0.075	2.243
Glucose 10%	8.11	8.93	7.424	7.205	6.126	2.214	2.044	0.869	0.000	0.000	0.000	2.876
DMSO 5%	8.04	9.15	7.145	7.344	6.687	3.627	1.860	2.427	2.068	1.962	0.175	3.699
DMSO 10%	7.97	9.93	7.923	7.841	6.166	1.270	0.500	1.524	0.889	0.708	0.525	3.039
Control	8.45	8.87	7.120	7.346	6.601	6.810	6.991	6.822	6.741	6.671	5.464	6.287

CD ($P<0.05$); Treatments=0.1962; Time interval=0.1861; Treatments × time interval=0.5885

Table 2. Overall effects of time intervals on the viability of conidia

Days of preservation	25±1°C	28-32°C	Mean
15	6.913	6.981	6.947
30	7.031	6.843	6.937
75	6.037	5.557	5.807
150	2.602	1.066	1.834
250	1.593	0.051	1.047
300	1.234	1.110	1.171
360	0.958	1.124	1.040
480	0.911	0.957	0.934
720	0.694	1.093	0.893

CD (P<0.05); Time interval=0.1861; Temperature=0.0877; Time interval × Temperature=0.2632

Table 3. Overall effects of incubation conditions on the viability of conidia of *T.harzianum*

Cryopreservatives	25±1ºC	28-32ºC
T1- Glycerol 50%	2.3775	1.9162
T2- Glycerol 25%	2.9062	2. 6337
T3- Glucose 100%	2.2839	2.6172
T4- Glucose 50%	2.4074	2.2803
T5- Glucose 25%	2.4249	2.0605
T6- Glucose 10%	3.5228	2.2294
T7- DMSO 5%	4.1105	3.2882
T8- DMSO 10%	3.0803	2.9967
T9- Control (sterile deionized water)	6.3911	6.1797

CD (P<0.05); Treatments=0.1962; Temperature=0.0877; Treatments × Temperature=0.2774

Among the cryoprotectants, DMSO was found to be comparatively better than glycerol and glucose. DMSO at 5% and 10% preserve the viability up to 75 days with 28-37% reduction in viability, but there was drastic difference between initial and final viable conidial count with 73-85%, 79-93% and 95-98% at 300, 480 and 720 days, respectively which indicated the poor performance of this cryoprotectants in long term storage. But it was very clear that the spores preserved in sterile deionized water maintained the viable spore count under the same conditions with only 25% reduction and for almost two years with less than 40% reduction (Fig. 1).

Drying the product and maintenance in dry environment or suspension in oil are common approaches that allow microbial agents to be

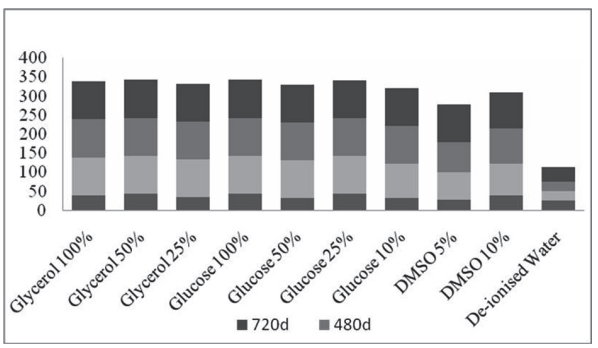

Fig. 1. Reduction (%) of viable conidia of *T.harzianum* at different periods

handled in commercial channels of distribution and storage (Rhodes 1993). In case of *T. harzianum* for the biocontrol of foot rot disease, the feasibility of formulating the bioagent in sorghum based solid fermentation medium has already been standardized (Sarma & Anandaraj 1998). Its shelf life lasts for about 6 months but the disadvantage of the product is that, being in a fermentation state, it is attracted by ants and flies which lead to contamination and spoilage *in situ* thereby losing the purity and quality of the product. Moreover the transportation of the product also is cumbersome. This is supported by the work done by Sibi *et al.* (2008). In their studies they found that organic materials increased the population of introduced *T. harzianum* and also increased population of fungi, bacteria, microarthropods, and nematodes. In case of sorghum, there was a sudden spurt in the population of saprophytic nematodes and mycophagus mites within 15 days followed by a succession of predatory mites and nematodes. The unspent carbohydrates and mycelia of *Trichoderma* in sorghum perhaps helped in the population build up of soil microarthropods and nematodes, which in turn affect the biocontrol efficiency of the introduced organisms. Hence it is advisable to use

formulations where the unspent organic matter is least and the fungus is present in the conidial or chlamydospore form, as these spores have better ability to tide over adverse conditions. Another formulation is based on talc which can be stored for up to 270 days. But a liquid formulation which can be preserved and stored without contamination and loss of viability is an alternative to the solid state fermentation medium or talc based formulation. The increasing demand can also be satisfied by making liquid formulation which can retain its viability for a long period and can be stored just like fungicides. Though the conidia are preserved in water, there was no germination of conidia which lead to mycelial clump formation which is a disadvantage in the case of a liquid formulation. Since this formulation retained the viability for more than 720 days, it was used for bioefficacy test *in vivo* in comparison with one month old talc based formulation holding a cfu of 10^8 g^{-1}. The test result (Fig. 2) showed that liquid formulation is effective in reducing the disease incidence under challenge inoculated conditions. The disease incidence was 33.33% when compared to 80% in control. So a disease reduction of 66.67% was achieved. Thus, the results clearly indicated that the conidial suspension in mere sterile de-ionised water is a promising medium for the long term storage and preservation of viable conidia of *Trichoderma* with biocontrol potency for field application. The method is very simple and highly feasible as a commercial formulation for large scale field application. The advantages of this formulation are (1) Easy to formulate (2) No synthetic chemicals are required which otherwise will deteriorate the quality of conidia (3) The green colour is retained, so the appealing character for *Trichoderma* is maintained (4) Preservation can be done in unbreakable bottles, so that it can be easily transported. Since no chemicals are used, the formulation is environmentally safe. Besides all these, the formulation has a long shelf life.

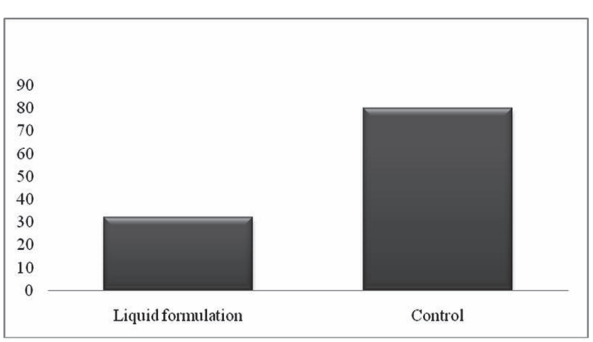

Fig. 2. Biocontrol efficacy (%) of stored liquid *T. harzianum*

The erstwhile Project Directorate of Biological Control (PDBC) has developed commercial formulations of *Trichoderma* by different methods. A new wheat bran powder based formulation of *Trichoderma harzianum* was developed at PDBC and evaluated under field conditions against some important soil borne pathogens. This formulation was found to support maximum viable propagules and also helps in bioagents proliferation in soil. At PDBC kaolin also was identified as suitable carrier for *Trichoderma* species in addition to the widely used talc based carrier. Conidial formulation retained optimum amount of viable propagules (>10^6 cfu g^{-1}) even after 80 days of storage at room temperature but in submerged conidia formulation, viable propagules reduced to less than 10^6 by 150 days.

Biocontrol potential and shelf life of *Trichoderma* species have been evaluated by many workers. Jayaraj *et al.* (2006) evaluated seven different formulations *viz*., talc, lignite, lignite + fly ash-based powder formulation, wettable powder, bentonite paste, polyethylene glycol-paste and gelatin-glycerin-gel for *Trichoderma* for seed treatment to reduce the incidence of damping-off disease of tomato. The shelf life of the formulations was evaluated under storage at 24°C up to nine months and population of propagules was optimum in all the formulations up to three months of storage. Raoof *et al.* (2006) studied the shelf life of *T. viride* in talc based formulation for the management of castor wilt. They reported that the talc based formulation retained the viability up to 270 days ($\log_e$ 6.952= (8.95 × 10^6) at low temperature (10±2°C) in a refrigerator and up to 240 days ($\log_e$ 6.519= (3.30 × 10^6) under ambient conditions. Here they macerated the culture

along with the medium for formulating the product in talc. Batta (2004) from Israel made a formulation of *T. harzianum* in an invert emulsion (water-in oil formulation) based on coconut and soybean oils and found the viability to be 36 months with 50% reduction in viability after 5.3 months at 20±1°C and compared with dry non- formulated conidia which have a viability of only up to 2.7 months.

A similar study conducted by Simpfenderfer *et al*. (1996) with *P. cladenstina* showed that sterile water storage in suspension to liquid nitrogen for the long term storage of the fungus without losing the pathogenicity. They also tested glycerol 10% and DMSO 5% which retained the viability for 10 months and three months respectively.

The effect of various amendments on the preservation of the viability and competitiveness, *in vitro*, of fungal mycelium and spores in a liquid paste was determined (Kolombet *et al*. 2008). The amendments with greatest effect were the addition of starch as a food base, reduction of metabolic activity by lowering the pH of the biomass paste and the addition of small amounts of copper. Oxygen availability was also shown to be important in maintaining biomass viability and competitiveness. Optimization of these factors produces a biomass paste formulation of *T. asperellum* that remains active, *in vitro*, for at least 6 months at room temperature. In our studies it is very clear that deionized distilled water can retain the viability and functionality of the spores for 720 days showing its potential as a viable carrier medium.

Hence, from our study it was found that the submerged conidia formulation in cryoprotectants like glycerol, glucose and DMSO preserved the viability only up to 75 days. But its preservation in sterile deionized water retained the viability up to 720 days at temperature range of 25-32°C. The average loss in viability from initial storing till 720 days was only 25%. Hence, this formulation can be commercialized.

Acknowledgements

The authors are thankful to Mrs. P.K. Chandravally for technical assistance and Mr. K. Jayarajan for statistical support.

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