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The shelf life of carrier (talc and activated charcoal) and pellet (calcium chloride and calcium gluconate gelled) based formulation of *Trichoderma harzianum* under varying storage environment was investigated. Irrespective of formulation type, storage under cool temperature was always far advantageous to maintain satisfactory population level in time scale. Population decline in cool temperature (4°C) was lowest in calcium gluconate gelled pellet formulation followed by calcium chloride based pellets, whereas, among the carrier based formulations rate of population decline was much faster in activated charcoal. The population decline in pellet based formulations was markedly lower indicating its ability to maintain higher level of population. The results revealed that activated charcoal and talc based formulation must be stored in cool temperature for maintaining desired population, whereas, pellet based formulations can be stored either in cool or room temperature without any significant difference up to 105 days after preparation.

Key words: *Trichoderma harzianum*, formulation, viable population, storage condition

INTRODUCTION

The concept of biological control and use of plant growth promoting microorganisms (PGPMs) has been emerged with immense importance as one of the alternative strategies to chemical dependent crop production and protection system (Whipps, 2004). Biological control of plant disease is generally a popularized form of disease control from more than a decade, as is evident from several classical reviews (Cook, 1996; Jeyarajan and Nakkeeran, 2000; Spadaro and Gullino, 2005). Recently there is an increasing demand of organic cultivation by using biocontrol agents (BCAs) like *Trichoderma* spp., *Gliocladium* spp., fluorescent pseudomonads, *Bacillus* spp., etc. which play a significant role to protect plant directly from pathogen invasion along with their additional attributes in plant growth promotion and induction of disease resistance. In spite of extending continuous effort to develop awareness on utilization of these beneficial microorganasims, several factors such as lack of knowledge on mass production technology, formulation process, delivery system along with their inconsistent performance may be attributed for lower acceptability of the biocontrol agents among the farming community.

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A successful BCA or PGPM must survive in formulation for longer time under appropriate storage condition. It must be a competitive and aggressive colonizer after inoculation (Beatty and Jensen, 2002; Selim *et al*, 2005). Most frequently the dry formulation is preferred over liquid formulation since it extends longer shelf life and become ease in storing and transportation (Lumsden *et al*, 1995). Talc is the most commonly available formulation of bioagents worldwide. However, short shelf of the bioagents in talc formulation is a major impediment in utilization under field condition. Effort, therefore, must be continued to improve the quality of formulation. Nutritional modification was evaluated with various additives to protect the microbial cells from desiccation. Various carrier materials had been tested to validate various delivery systems of the PGPMs in greenhouses and fields. Beside dry and wet formulation, improvement in formulation technology was made with incorporation of mycoherbicide into sodium alginate (Walker and Connick, 1983). Subsequently, Fravel (1985) reported long term survival of microbial bioagents in sodium alginate based pellet formulation where the reaction between aqueous solution of sodium alginate and certain metal cations such as Ca++ in the form of calcium chloride or calcium gluconate to form gels has been used in the process of pellet formulation (Fravel *et al*, 1985). The specific functions of metal ions in sporulation are probably that they act as activators of the various enzyme systems necessary for sporulation (Bruno and Ralph, 1964). However, for successful survival of the encapsulated microbial cells the choice of polymer is very important (Rekha *et al*, 2007). Alginate plus calcium gluconate solidified culture media is reported to be equivalent to agar for culturing of common bacteria such as *Escherichia coli* and fungus like *Aspergillus* sp. and *Penicillium* sp. (Cranston, 1983). However, many factors like medium and inoculum type (Elzein *et al*, 2004), method of drying, addition of protectants (Friesen *et al*, 2006) and environmental condition during storage (Connick *et al*. 1996) affect the viability of the formulation. In the present investigation an attempt was made to study the shelf life of different pellet and carrier based formulations of *T. harzianum* under storage at cool and room temperature.

MATERIALS AND METHODS

T. harzianum isolate (UBT -18) was taken from the biocontrol agent repository of Department of Plant Pathology, Uttar Banga Krishi Viswavidyalya, Pundibari, Cooch Behar and then first inoculated in 100ml potato dextrose broth followed by incubation at 28$\pm$1^{0}C for 7 days for mass multiplication. Mycelial mat along with the culture filtrate was poured on the mixer, and blended till the mycelial mat homogenizes with the solution. The homogenized cultures were then used for preparation of pellet formulations [calcium chloride ($CaCl_2$) / calcium gluconate ($C_{12}H_{22}CaO_{14}$) gelled] or mixed with pre-sterilized talc and activated charcoal to prepare carrier based formulations.

Preparation of pellet and carrier based formulation of UBT-18

Two different types of sodium alginate based pellet formulations were prepared and two Ca++ cations ($CaCl_2$ and $C_{12}H_{22}CaO_{14}$) were used as gelling agent. Bentonite clay (30g) along with 250ml sterilized distilled water was added to the already blended solution of 7 day aged mycelial mat of UBT 18 and again grinded in the mixture. To the resultant blended mixer, 1% of sodium alginate (3g) was added and mixed in the mixture. After blending, the derived blended mixture was then pipetted out and dropped into a solution containing hydrated $CaCl_2$ (0.5M) or $C_{12}H_{22}CaO_{14}$ solution using a peristaltic pump. Each drop of the blended mixer was then dripped into a solution containing hydrated $CaCl_2$ or $C_{12}H_{22}CaO_{14}$ solution. Pellets thus formed were harvested from the solution, rinsed in sterilized water and spread in a single layer on a blotting paper. They were then air dried in a laminar air flow. These pellets were initially 3-4 mm diameter which later shrink to 1-3 mm diameter after drying and such dried pellets were then stored in capped jars at room temperature and also in cool chambers (4°C).

For preparation of carrier based formulations, 250 g of talc powder or activated charcoal was uniformly mixed to 100 ml of homogenized broth and left to dry overnight on trays lined with blotting paper. After drying, the bio-inoculated talc powder was stored in a moisture-proof packaging such as aluminium laminate sachets and kept in cool place (4°C) and also at room temperature to study the shelf life of the formulated products.

Determination of shelf life of pellet and carrier based formulation of UBT-18

For enumeration of viable population of UBT-18 in

pellet based formulations, 1g of the alginate pellets was soaked in 9ml phosphate buffer solution for 24 hr. After 24 hr of soaking the pellets were crushed in a mortar with a pestle to form a solution. Later 1ml of resultant solution in a mortar was pipetted out with the help of a micro pipette and serially diluted at different dilutions upto 10^{-7} in sterile distilled water. In case of talc and activated charcoal based formulation serial dilutions upto 10^{-7} in sterile distilled water was also prepared. Shelf life of UBT-18 in pellet and carrier based formulations at different time interval upto 195 days was determined by dilution plating method (Waksman and Fred, 1952) on modified Trichoderma specific medium (Saha and Pan, 1997).

RESULTS AND DISCUSSION

The population dynamics of UBT-18 in different formulations mentioned above stored at two different conditions (cool and room temperature) showed that irrespective of type of formulation, storage in cool temperature had significant effect on viable population of UBT 18 throughout the experimental period (Table 1). The initial population although vary significantly in four formulations being highest in talc based formulation followed by pellet formulation with calcium gluconate as gelling agent, population variation was quite different in later part of the experimental period. At 45 days after preparation viable population in calcium gluconate gelled pellets was significantly at par with talc based formulation when stored at cool temperature and this continued up to 105 days of storage. However, in room temperature calcium gluconate gelled pellets showed significantly higher population as compared to talc based formulation from very initial period of storage.

The initial population of UBT 18 in pellet formulation with calcium chloride was quite low resulted in significantly lower population in comparison to talc based formulation up to 105 days after preparation when stored in cool temperature and up to 75 days after preparation when stored at room temperature. Activated charcoal based formulation on the other hand exhibited more or less stable viable population up to 135 days even at par with talc based formulation when stored at cool temperature but then after viable population reduced significantly. At 135 days after preparation, in cool temperature significantly higher viable population was enumerated in pellet formulation with calcium gluconate followed by activated charcoal based formulation which was further at par with talc but significantly different to calcium chloride gelled pellet formulation. At the end of the experimental period, viable population in activated charcoal based formulation stored in cool temperature was significantly lower even than the population enumerated in calcium gluconate based formulation stored in room temperature. At the same time calcium gluconate based pellet formulation was found to be the most effective one in maintaining the population level significantly differing with talc based and calcium chloride gelled pellet formulations.

The viable population of UBT 18 in different formulations irrespective of storing temperature revealed that significantly higher colony forming unit maintained throughout the experimental period in pellet formulation with calcium gluconate. Although the population in talc and pellet formulation with calcium chloride was significantly at par throughout the experimental period, however, the pellet formulation showed greater population retention capacity at the later part of the storage period since it had significantly lower initial population. The trend in population decline of the same revealed that pelleted alginate based beads had lower population reduction rate than the carrier based formulations.

Figure 1 showing the percentage of population reduction over initial population in different formulations stored at cool and room temperature is self explanatory of the above findings. However, it has been derived that, although at the end of the experimental period viable population in talc based formulation was significantly higher than calcium chloride based formulation stored in cool temperature yet the population decline in time scale was lower in the later one as shown through linear equation (Table 2). Population decline in cool temperature was lowest in calcium gluconate gelled pellet formulation followed by calcium chloride based pellet formulation, whereas among the carrier based formulations population decline was much higher in activated charcoal. In room temperature, the population reduced rapidly as the storage time progressed comparing with that in cool temperature. In spite of it, population decline in pellet based formulations was markedly lower indicating its ability to maintain higher population level over time.

The effect of storage condition on different formu-

Table 1 : Shelf life of UBT 18 in pellet and carrier based formulations under storage at cold and room temperature

Formulation	Storage condition	Log cfu/g of formulation						
		0 DAP	45 DAP	75 DAP	105 DAP	135 DAP	165 DAP	195 DAP
Pellet with $CaCl_2$	Cold Temp.	8.34	8.28[d]	8.20[bc]	7.97[c]	7.74[c]	7.51[b]	7.10[c]
	Room Temp.		7.99[f]	7.83[d]	7.49[d]	7.13[e]	6.74[e]	6.40[e]
Pellet with Ca-gluconate	Cold Temp.	8.57	8.54[ab]	8.51[a]	8.36[a]	8.17[a]	7.98[a]	7.74[a]
	Room Temp.		8.39[c]	8.10[c]	7.85[c]	7.41[d]	7.00[d]	6.73[d]
Activated charcoal	Cold Temp.	8.51	8.47[bc]	8.36[ab]	8.10[b]	7.93[b]	7.25[c]	6.47[e]
	Room Temp.		8.04[f]	7.33[f]	6.88[f]	6.43[g]	5.41[g]	4.98[f]
Talc	Cold Temp.	8.64	8.59[a]	8.44[a]	8.28[a]	7.85[bc]	7.52[b]	7.39[b]
	Room Temp.		8.17[e]	7.64[e]	7.27[e]	6.90[f]	6.48[f]	6.34[e]
SEm±			0.037	0.059	0.043	0.048	0.086	0.068
LSD (P=0.05)			0.105	0.17	0.123	0.139	0.246	0.196
Formulation								
Pellet with $CaCl_2$			8.13[d]	8.01[b]	7.73[b]	7.44[b]	7.13[b]	6.75[b]
Pellet with Ca-gluconate			8.46[a]	8.30[a]	8.11[a]	7.79[a]	7.49[a]	7.23[a]
Activated charcoal			8.26[c]	7.84[c]	7.49[c]	7.18[c]	6.33[c]	5.73[c]
Talc			8.38[b]	8.04[b]	7.77[b]	7.37[b]	7.00[b]	6.87[b]
SEm±			0.026	0.041	0.03	0.034	0.061	0.048
LSD (P=0.05)			0.074	0.12	0.09	0.098	0.174	0.139
Storage Condition								
Cold temp.		8.51	8.47[a]	8.37[a]	8.18[a]	7.92[a]	7.57[a]	7.18[a]
Room temp			8.15[b]	7.73[b]	7.37[b]	6.97[b]	6.41[b]	6.12[b]
SEm±			0.018	0.03	0.021	0.024	0.042	0.033
LSD (P=0.05)			0.05	0.083	0.058	0.067	0.116	0.092

Values followed by different letters differ significantly according to Duncan's multiple range test at P = 0.05.

lations (Figure 2) revealed that the advantage of storage in cool temperature was always higher in activated charcoal followed by talc based formulation, whereas, pellet based formulations did not show marked variation up to 135 days of storage. With further increase in storage period, calcium chloride based formulation exhibited better advantages with regards to population maintenance over calcium gluconate gelled pellet formulation. The above findings revealed that activated charcoal and talc based formulation must be stored in cool temperature for maintaining desired population, whereas, pellet based formulations can be stored either in cool or room temperature without any marked difference up to 105 days after preparation.

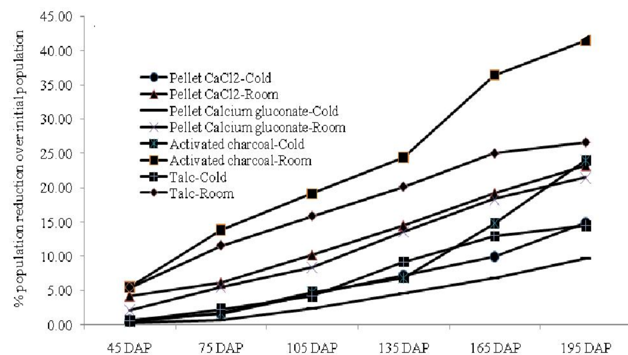

Fig. 1 : Population reduction of UBT 18 in different formulations in time scale under cool and room temperature

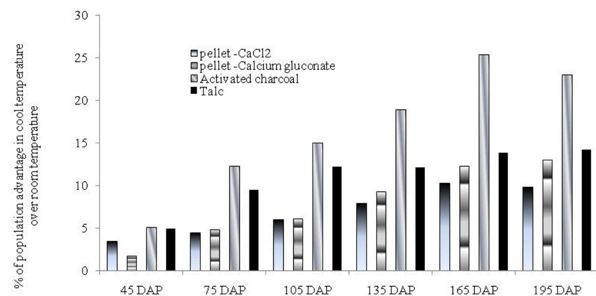

Fig. 2 : Effect of storage condition on shelf life of UBT 18 in different formulation

For commercialization, greater shelf life is an advantage for any bio-inoculants (Fages, 1992). Formulation of biocontrol agents depends upon biomass production and maintaining viability at the end of the process (Adenkule *et al*, 2001). Calcium gluconate gelled pellets of *T. harzianum* in the present study had viable population significantly at par with talc formulation up to 3-4 month storage in cool temperature, however in room tem-

perature calcium gluconate gelled pellets showed a clear advantage in maintaining viable propagule. Talc based formulation was found to be significantly better in comparison to calcium chloride gelled alginate pellets after six and half months of storage. Chemically talc is magnesium silicate, which has very low moisture equilibrium, hydrophobicity and water absorption which provide a suitable niche for the bioagents to escape from desiccation since most of the microbial propagules having thin cell wall. The population decline trend also revealed that alginate pelleted formulation had lower population decline rate as compared to carrier based formulations. Fravel *et al* (1985) exhibited long term survival in pellets made with $C_{12}H_{22}CaO_{14}$ than those made in calcium chloride which was in harmony with findings of Sanyal *et al* (2003). Talc as a carrier used in most of the biopesticide formulation does not promote multiplication or even immobilized the bioagents during storage rather long term storage results in decrease of viability (Rangeshwaran *et al.* 2010). Charcoal based formulation was not a suitable carrier for long term survival of biocontrol agents (Shahid *et al.* 2011). The rate of population decline was also much rapid in room temperature as compared to cool temperature. The advantage of storage at cool temperature was found to higher in carrier based formulation particularly in activated charcoal based formulation, whereas pellet based formulations can be stored either in cool or room temperature without any marked reduction in viable population up to three and half months of storage. Low temperature storage and a moisture-free environment slows the metabolic rate of the viable propagules, and prevents accumulation of toxic metabolites and depletion of nutrients. Low temperature (4 to 15°C) storage has been successfully used for many fungal and bacterial BCA (Lewis, 1991; McIntyre and Press, 1991; Chakravarty and Kalita, 2011, Mishra *et al*, 2011). The viable population in talc formulation over time recorded in the present investigation was in harmony with observation of Sen *et al,* (2012). Viability of encapsulated *T. harzianum* remained high for at least six months when stored at 5°C (Harman *et al.* 1989). In conclusion, it can be summarized that calcium gluconate gelled pellets is comparatively better since the BCAs in that particular formulation showed lower population decline rate. Calcium chloride gelled pellet formulation is qualitatively at par with talc based formulation when both stored in cool temperature but in case of activated charcoal based formulation it must be stored in cool temperature for long term survival.

Table 2 : Linear equation of population decline of UBT 18 in pellet and carrier based formulation

Formulation	Linear equation	R^2
Pellet with $CaCl_2$	Y= -0.25x+8.70	0.922
Pellet with $C_{12}H_{22}CaO_{14}$	Y= -0.23x+8.92	0.974
Activated charcoal	Y= -0.46x+9.17	0.964
Talc	Y= -0.31x+8.97	0.991

REFERENCES

Adenkule, A.T., Cardwell, K.F., Florini, D.A. and Ikotun, T. 2001. Seed treatment with *Trichoderma* species for control of damping-off of cowpea caused by *Macrophomina phaseolina*. *Biocontrol. Sci. Techn.* **11:**449-457.

Beatty, P.H. and Jensen, S.E. 2002. *Paenibacillus polymyxa* produced fusaricidin-type antifungal antibiotics active against *Leptosphaeria maculans*, the causative agents of blackleg disease of canola. *Can. J. Microbiol.* **48:**159-169.

Bruno, J.K. and Ralph, A. 1964. Trace metal requirement of sporulation of *B. megatherium. J. Bacteriol.* **88:** 821–830.

Chakravarty, G. and Kalita, M.C. 2011. Comparative evaluation of organic formulations of *Pseudomonas fluorescens* based biopesticides and their application in the management of bacterial wilt of brinjal (*Solanum melongena* L.). *Afr. J. Biotechnol.* **10:** 7174-7182.

Connick, W.J., Lewis, J.A. and Quimby, P.C. 1990. Formulation of bicontrol agents for use in plant pathology. In-*New Directions is Biological Control: Alternatives for suppressing Agricultural pests and diseases*. (eds. R.R. Baker and P.E. Dunn), Altar liss, New York 345-372pp.

Cook, R.J**.** 1996. Assuring the safe use of microbial biocontrol agents: A need for policy based on real rather than perceived risks. *Can. J. Plant Pathol.* **18:**439-466.

Cranston, P.M. 1983. Alginic acid derivatives as a solidifying agent for microbiological nutrient suspensions. *Food Technol. Aust.* **35:**134-136.

Elzein, A., Kroshel, J. and Mueller-Stoever, D. 2004. Effects of inoculum type and propagules, concentration on shelf life of pasta formulations containing *Fusarium oxysporum* Foxy2, a potential myco-herbicide agent for Striga spp. *Biol. Control* **30:**203-211.

Fages, J. 1992. An industrial view of *Azospirillum* inoculants: formulation and application technology. *Symbiosis* **13:**15-26

Fravel, D.R., Marios, J.J., Lumsden, R.D. and Connick, Jr. W.J. 1985. Encapsulation of potential biocontrol agents in an alginate-clay matrix. *Phytopathol.* **75**:774-777.

Friesen, T.J., Holloway, G., Hill, G.A. and Pugsley, T.S. 2006. Effect of conditions and protectants on the survival of *Penicillium bilaiae* during storage. *Biocontrol Sci. Techn.* **16:** 89-98.

Harman, G.E., Taylor, A.G. and Stasz, T.E. 1989. Combining effective strains of *Trichoderma harzianum* and solid matrix priming to improve biological seed treatment. *Phytopathol.* **73:** 631-637.

Jeyarajan, R. and Nakkeeran S. 2000. Exploitation of microorganisms and viruses as biocontrol agents for crop disease management. In: Upadhyay R., Mukherji, K. and Chamola B. (eds.) *Biocontrol potential and its Exploitation in Sustainable Agriculture. Crop Disease, Weeds and Nematodes* **1:** 95-116.

Lewis, J.A**.** 1991. Formulation and delivery system of biocontrol agents with emphasis on fungi *Beltsville symposia* in Agricultural Research. In: Keister, D. L. and Cregan, P.B. (eds). *The Rhizosphere and Plant Growth* 279-287 pp.

Lumsden, R.D., Lewis, J.A. and Fravel, D.R. 1995. Formulation

and Delivery of Biocontrol Agents for Use against Soilborne Plant Pathogens. In: *Biorational Pest Control Agents: Formulation and Delivery*, Hall, F.R. and J.W. Barry (Eds.). American Chemical Society, Washington. 125-139pp.

McIntyre, J.L. and Press, L.S. 1991. Formulation, delivery systems and marketing of biocontrol agents and plant growth promoting rhizobacteria (PGPR). *In* D. L. Keister, and P. B. Cregan, (*eds*.), *The Rhizosphere and Plant Growth*. Kluwer, The Netherlands. 289-295pp.

Mishra, D.S., Gupta, A.K., Prajapati, C.R. and Singh, U.S. 2011. Combination of fungal and bacterial antagonists for management of root and stem rot disease of soybean. *Pak. J. Bot.* **43:**2569-2574.

Rangeshwaran, R., Vajid, N.V., Ramanujam, B., Sriram, S., Bhaskaran, T.V. and Kumar, S. 2010. Additives in powder based formulation for enhanced shelf life of *Pseudomonas fluorescens* and *Bacillus* sp. *J. Biol. Cont.* **24:**158-163.

Rekha, P.D., Lai, W., Arun, A.B. and Young, C. 2007. Effect of free and encapsulated *Pseudomonas putida* CC-FR2-4 and *Bacillus subtilis* CC-pg104 on plant growth under gnotobiotic conditions. *Bioresour. Technol.* **98:**447-451.

Saha, D.K. and Pan, S. 1997. Qualitative evaluation of some specific media of *Trichoderma* and *Gliocladium* and their possible modifications. *J. Mycopath. Res.* **34:**7-13.

Sanyal, B., Sengupta, C., Poi, R., Dasgupta, B. and Sen, C. 2003. Survival potential of *Trichoderma harzianum* in alginate prills. *J. Biol. Cont.* **17:** 69-73.

Selim, S., Negrel, J., Govaerts, C., Gianinazzi, S. and van Tuinen, D. 2005. Isolation and partial characterization of antagonistic peptides produced by *Paenibacillus* sp. strain B2 isolated from the sorghum mycorrhizosphere. *Appl. Environ. Microb.* 71: 6501-6507.

Sen, S., Biswas, G., Basu, S.K. and Acharya, K. 2012. Management of leaf spot disease of *Stevia rebaudiana* Bertoni with antagonistic bacteria. *Aust. Crop Sci.* **6:** 350-356.

Shahid, M., Singh, A., Srivastava, M., Mishra, R.P. and Biswas, S.K. 2011. Effect of temperature, pH and media for growth and sporulation of *Trichoderma longibrachiatum* and self life study in carrier based formulations. *Ann. Plant Prot. Sci.* **19:**147-149.

Spadaro, D. and Gullino, M.L**.** 2005. Improving the efficacy of biocontrol agents against soil borne pathogens. *Crop Prot.* **24:**601-613.

Waksman, S.A. and Fred, B. 1952. A tentative outline of the plate method for determining the number of micro-organisms in the soil. *Soil Sci* **14:**27-28.

Walker, H.L. and Connick, W.J. 1983. Sodium alginate for production and formulation of mycoherbicides. *Weed Sci* 31: 333-338.

Whipps, J.M. 2004. Prospects and limitations for mycorrhizas in biocontrol of root pathogens. *Can. J. Bot.* **82:**1198–1227.