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Effect of chitin on the shelf-life of *Trichoderma harzianum* in talc formulation*

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Trichoderma spp have been recognized as potential bioagents for the management of soil-borne plant diseases. Although, conidia of *Trichoderma* derived from solid substrate fermentation are highly tolerant to adverse situation than the propagules or biomass derived from liquid fermentation, wettable powder formulations based on liquid fermentation are common in India though they are not so desiccation tolerant (Singh *et al.* 2006). All the *Trichoderma* formulations registered in India are wettable powder formulations that are based on liquid fermentation. From the commercial producers' point of view, liquid fermentation has the advantages of control over the production process, short time for production, requirement of less space and labour, control over contamination level etc. (Deshpande 2006). *Trichoderma* spp are well known for their utilization of chitin because of their ability to produce hydrolytic enzymes like chitinases and glucanases. Chitin incorporation in formulations of *Trichoderma* has been found to increase the bioefficacy of the strain. Yang *et al.* (2002) observed that chitin addition enhanced the efficacy of *T. harzianum* against *Fusarium* wilt of cotton (*Gossypium hirsutum*). In the present study the objective was to add the chitin in the talc formulation or to add the more utilizable form, ie colloidal chitin, in the production medium to study their effect on the shelf-life of the formulation derived from the liquid fermentation.

T. harzianum (PDBC-Th10) maintained on potato dextrose agar at National Bureau of Agriculturally Important Insets, Bangalore was used. *T. harzianum* was grown on potato dextrose agar for 96 hr and the conidia from the colonies were picked off using sterile inoculation needle and transferred to sterile distilled water. The conidial population was adjusted to 1×10^7 conidia/ml using a haemocytometer and transferred to potato dextrose broth so that the final conidial population would be 1×10^5 conidia/ml and incubated in shaker culture at 28°C and 200 rpm for 48 hr. This 48 hr-old culture was used as inoculum for the main culture (liquid fermentation for production purpose) at the rate of 100 ml/litre.

To study the effect of addition of colloidal chitin in the production medium on the shelf-life of talc formulations of *Trichoderma* spp, colloidal chitin was prepared (Berger and Reynolds 1958) and amended in the media used for the production of *Trichoderma*.

Molasses yeast extract medium (30 g molasses, 10 g yeast extract in 1 litre of distilled water) and a synthetic medium (consisting of $MgSO_4$, 0.2 g; KCl, 0.15 g, KH_2PO_4, 0.9 g; NH_4NO_3, 3.0 g; glucose 3.0 g/litre) were prepared along with colloidal chitin 0.2% (w/v) and tested for their suitability for colloidal chitin amendment.

These 2 broths were inoculated with *T. harzianum* and incubated in a shaker at 28°C for a week. The biomass was mixed with the talc powder (Mesh No. 300, 53 mm size, white coloured) at 1 : 2 ratio (v : w). Twelve replications were maintained for each treatment. For each replication 1 litre medium was used. The initial population and the population of viable conidia after 1 month of storage were enumerated in these 2 formulations by plating one gram of sample serially diluted to 10^{-6} dilution by adopting pour plate method using *Trichoderma*-specific medium (Elad *et al.* 1981).

To study the effect of the addition of colloidal chitin in production medium on the shelf-life, *T. harzianum* was grown on malasses yeast extract broth amended with 0.1, 0.2 or 0.5% colloidal chitin in shaker culture at 200 rpm for 7 days at 28°C. One set of culture grown on malasses yeast extract broth without colloidal chitin served as control. Four replications were maintained for each treatment. After 7 days of growth the biomass was mixed with talc formulation and dried to have 10% moisture content. The formulations were kept in storage at room temperature and populations in terms of CFUs (colony forming units) were enumerated as described earlier at monthly intervals till the population in all treatments was less than 2×10^6, the minimum recommended level of CFUs/g prescribed by Central

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Insecticides Board Act, Government of India. The formulations were filled in a polypropylene covers (density 0.90 g/cc) and stored at room temperature in closed storage cabinets. The ambient temperature varied from 20 to 35°C in a year in the place where experiment was conducted.

In another experiment, the effect of addition of chitin in formulation was studied. The biomass and talc formulation of *T. harzianum* were obtained as mentioned earlier. One set of talc formulations was mixed with pure chitin at different concentrations, viz 1, 2 or 5% immediately after mixing the biomass with talc powder before drying the formulation to have a final moisture content of 10%. In another set of formulations, chitin was added after drying the formulations. Formulations without chitin addition served as control. Four replications were maintained for each treatment. The initial population and population of viable conidia after 1 month were enumerated as mentioned earlier.

The biomass and talc formulation of *T. harzianum* were obtained as mentioned earlier. The talc formulation was mixed with pure chitin (Hi-Media, India) at 1, 2 or 5% after drying the formulation obtained by mixing the biomass with talc to 10% moisture content. One set of formulations without addition of chitin was maintained as control. The initial population and population of viable conidia at monthly intervals were enumerated as mentioned earlier. For each treatment, 4 replications were maintained.

All the experiments were statistically analyzed using IRRISTAT and the differences among the treatments were assessed based on ANOVA (Gomez and Gomez 1984). In each experiment, the sample size was 1 kg/sample. The CFUs values were transformed using logarithmic transformation before analysis. Completely randomized design was used for the analysis of data.

Initial population of *T. harzianum* in the formulation obtained from the synthetic medium and molasses yeast extract medium was 2.2×10^6 and 3.6×10^6 CFUs/g respectively. The CFUs in the formulations developed from biomass grown on synthetic medium with colloidal chitin amendment at 0.1, 0.2 and 0.5%, were 1.7×10^6, 1.66×10^6 and 1.33×10^6, respectively. Addition of colloidal chitin helped in getting more CFUs/g in the formulations obtained from the treatments where colloidal chitin was added in molasses yeast extract medium (6.04×10^6, 5.33×10^6 and 1.17×10^7, respectively, with 0.1, 0.2 and 0.5% colloidal chitin addition compared to 3.66×10^6 CFUs/g in control), indicating that molasses yeast extract medium was more amenable for addition of colloidal chitin than synthetic medium.

Addition of chitin in the media resulted in enhanced initial population in the talc formulation of *T. harzianum*. When 0.2% colloidal chitin was added in the medium, the CFUs were higher (2.23×10^7) compared to the control (1.43×10^7). Colloidal chitin addition at 0.1% or 0.5% resulted in initial CFUs of 1.7×10^7 and 1.9×10^7/g respectively (Table 1). The CFUs in the formulations derived from the treatments where colloidal chitin was added at 0.1, 0.2 and 0.5% reached the minimum required level (2×10^6) after 6 or 7 months, while in the control without colloidal chitin addition in production medium it reached the minimum required level in the 5th month.

Table 1 Effect of the addition of colloidal chitin in the molasses yeast extract medium of *Trichoderma harzianum* on shelf-life (viability in terms of CFUs/g)

Storage time (months)	% colloidal chitin added in the medium			
	0%	0.10%	0.20%	0.50%
Initial	1.43×10^7	1.67×10^7	2.23×10^7	1.87×10^7
	7.155	7.223	7.348	7.272
1	1.23×10^7	1.54×10^7	2.19×10^7	1.78×10^7
	7.090	7.188	7.340	7.250
2	1.53×10^7	1.83×10^7	2.40×10^7	2.03×10^7
	7.185	7.262	7.380	7.307
3	1.27×10^7	1.77×10^7	2.23×10^7	1.63×10^7
	7.104	7.248	7.348	7.212
4	1.03×10^7	1.70×10^7	2.00×10^7	1.23×10^7
	7.013	7.230	7.301	7.090
5	5.66×10^6	1.63×10^7	1.80×10^7	9.67×10^6
	6.753	7.212	7.255	6.985
6	1.33×10^6	1.77×10^7	1.77×10^7	6.33×10^6
	6.124	7.248	7.248	6.801
7	3.30×10^5	2.00×10^6	2.00×10^6	1.00×10^6
	5.522	6.301	6.301	6.000
CD (P=0.05)	0.021	0.063	0.173	0.171

Data analyzed with completely randomized design. Figures in the parentheses are $\log_{10}$ transformed values

Addition of chitin at the time of mixing the biomass with the carrier (talc) resulted in more contamination by bacteria and the *Trichoderma* propagules count was reduced drastically from 2.0×10^7 to 1.1×10^6 at the end of 1 month storage time. High moisture content facilitated the other fungal and bacterial contaminants that could utilize chitin as carbon source. But addition of chitin in formulation after drying it to 10% moisture level did not reduce the propagules count significantly.

The population of *T. harzianum* was reduced from 1.13×10^7CFUs/g to 2.0×10^6 CFUs/g within 4 months in control where there was no chitin addition in the formulation (Table 2). In chitin-amended formulations, there was a significant increase in the initial population of *Trichoderma* after 1 month. During the shelf-life studies, it was observed that addition of chitin (2 or 5%) helped in maintaining the high CFUs in the *T. harzianum* formulation for up to 6 months. In chitin amended formulations up to 6th month the CFUs could be maintained above the crucial level and by 7th month the CFUs got reduced below 2.0×10^6 CFUs/g that is minimum required as per the Central Insecticides Board Act, India. The addition of chitin helped in extending the shelf-life by 2 months.

Table 2 Effect of the addition of pure chitin in talc formulation on the shelf-life of *T. harzianum* (viability in terms of CFUs/g)

Storage time (months)	Percentage of pure chitin added in the formulation			
	0	1	2	5
1	1.13×10^7	1.0×10^7	1.03×10^7	1.07×10^7
	7.053	7.000	7.013	7.029
2	1.10×10^7	1.13×10^7	1.50×10^7	2.07×10^7
	7.041	7.053	7.176	7.316
3	2.66×10^6	6.33×10^6	9.33×10^6	1.13×10^7
	6.425	6.801	6.970	6.053
4	2.00×10^6	4.00×10^6	6.00×10^6	6.33×10^6
	6.301	6.602	6.778	6.801
5	1.66×10^6	2.66×10^6	4.00×10^6	3.68×10^6
	6.220	6.425	6.602	6.566
6	1.00×10^5	1.66×10^6	2.00×10^6	2.00×10^6
	5.000	6.220	6.301	6.301
7	1×10^5	1.00×10^5	1.00×10^5	1.00×10^5
	5.000	5.000	5.000	5.000
CD (*P*=0.05)	0.049	0.167	0.476	0.112

Initial population of *T. harzianum* at the time of packing: 8.9×10^7 CFUs/g of sample. Data analyzed with completely randomized design. Figures in the parentheses are $\log_{10}$ transformed values

Addition of chitin in the soil as field application has been found to reduce disease incidence. Sid-Ahmed *et al.* (2003) observed that the antagonistic activity of *T. harzianum* was stimulated by chitin when applied against *Phytophthora capsici* and *R. solani* in pepper plants. Pavlyushin *et al.* (2005) immobilized *T. viride* cells on chitin, chitosan or chitin and chitosan carriers and found that these preparations were able to preserve the cells with viability for longer time in storage. This enhanced the seed vigour and germination rate and reduced *F. oxysporum* infection in cucumber seedlings.

In India, it is estimated that more than 1 lakh tonne of shrimp processing waste is being wasted annually which could be gainfully utilized for manufacturing chitin, a high value industrial product. Crab shells and lobster shells are also raw materials for chitin/chitosan production. The estimated availability of crab shells is 30 000–40 000 tonnes in the Indian waters (http://www.vuatkerla.org). If processed properly, these chitin sources can be utilized for the production of *Trichoderma* formulations. Chitin sources have to be checked for anti-microbial activity and if they are added in formulations of *Trichoderma* they have to be processed by hydrolysis with acid treatment, followed by thorough washing with water.

The results of the present study would be relevant for the extension of shelf-life in liquid fermentation-based commercial production of *Trichoderma* in India. Compared to normal shelf-life of 4–5 months, 2 months, additional shelf-life indicates 30–50% increased shelf-life in talc formulations.

SUMMARY

Effect of addition of chitin in the production medium or in the formulation, on the shelf-life of talc formulation of the bioagent *Trichoderma harzianum* was studied. Colloidal chitin was added at the rate of 0.1, 0.2 or 0.5% (w/v) in molasses yeast extract medium used in liquid fermentation. Similarly, different concentrations of pure chitin were added to the talc formulation of *T. harzianum*. Addition of colloidal chitin at 0.2% in production medium enhanced the shelf-life by additional 2 months compared to control. Addition of pure chitin in talc formulations of *T.harzianum* too enhanced the shelf-life by additional 2 months. The effect of these treatments on shelf-life of talc formulations of *T. harzianum* is discussed.

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