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Research Paper

Determination of shelf-life of *Trichoderma asperellum* in solid- and liquid-based formulations

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Abstract: *Trichoderma* has gained attention as a promising bio-control agent owing to its effectiveness against a wide array of soil-borne plant pathogens. Successful introduction of bio-control agents to farmers is hindered mostly by unavailability of commercial preparation of *Trichoderma* bio-inoculate with a considerably higher shelf-life. The objectives of this investigation was to study the shelf-life of *T. asperellum* in selected liquid- and solid-based formulations and to find the suitability of cattle manure as a multiplication medium. Different carrier material including liquids and solids were tested for preparation of a commercial biological formulation of *T. asperellum.* The liquid carrier media tested in the present study were 1% sucrose, 1% peptone water, 1% tryptone broth, 1% tryptone soy broth and sterilized distilled water. Talc powder was tested as the solid carrier material. Cattle manure was incorporated with talc-*Trichoderma* formulation to check the suitability of cattle manure as a multiplying substrate. The initial spore count of each liquid formulation was maintained at $1.0x10^6$ cfu/ml. At the end of 4th week, the mean viable spore counts of the tryptone broth, peptone water and tryptone soy broth used were $7.15x10^6$ cfu/ml, $7.26x10^5$ cfu/ml and $7.30x10^5$ cfu/ml, respectively. From the 4th week onwards, the viable spore count of those three formulations could not be calculated due to heavy contaminations. In the 1% sugar solution and sterilized distilled water, spores were countable only till the end of 8th week of preparation and the counts were $2.5x10^4$ cfu/ml and $2.6x10^3$ cfu/ml, respectively. A talc-based formulation was prepared by mixing *T. asperellum* spore suspension ($1.0x10^8$ CFU/ml) with talc powder at three different levels (v/w); 30ml/100 g (T1), 40 ml/100 g (T2) and 50 ml/100 g (T3). Three months after storage, all the three treatments (T1, T2 and T3) yielded mean spore counts of $0.92x10^8$ cfu/g, $1.11x10^8$ cfu/g and $1.21x10^8$ cfu/g, respectively. Talc powder inoculation with cattle manure was done at four different rates, *i.e.* 10 g/kg cattle manure (C1), 15 g/kg cattle manure (C2), 20 g/kg cattle manure (C3) and 25 g/kg cattle manure (C4). Cattle manure was identified as a potential multiplying substrate of the talc-based formulation of *T. asperellum*.

Keywords: Bio-control agents, survival in carrier medium, *Trichoderma*

Introduction

Soil-borne plant pathogenic fungi cause significant economic losses to agricultural crops, in terms of yield quantity and quality. Commonly reported soil borne pathogens are *Phytophthora capsici, Rhyzoctonia solani, Fusarium* spp. and *Pythium* spp. Genus *Trichoderma* represents a widely studied fungi that show antagonistic activity towards soil-borne pathogens (Kelaniyangoda *et al*. 2003). The

results of a previous experiment confirmed the antagonistic activity of *Trichoderma* sp. against *P. capsici* (Kodithuwakku *et al.* 2013). The complete crop losses due to prevailing diseases result in increased uses of agrochemicals and thereby high cost of production. Biological control agents (BCAs) developed on the basis of mycelium and spores of fungi belonging to the genus *Trichoderma* have a long history of successful application in controlling plant diseases (Kolombet *et al*. 2007). Bio-formulations containing *Trichoderma* have emerged as effective alternatives. However, before preparing a bio-formulation, a suitable medium with higher shelf life is a pre-requisite. The cost-effective large scale production, shelf life of formulation and consistency in disease control are the prime concerns with augmentative biological control. Commercial use of *Trichoderma* BCAs must be headed by precise identification (Rajapakse *et al.* 2016). Therefore, the major objectives of this study were to identify *Trichoderma* strain at species level and to evaluate the shelf-life of *T. asperellum* in soild- and liquid-based formulations.

Materials and Methods

Molecular identification of the *Trichoderma* strain:
Trichoderma strain isolated previously from black pepper rhizosphere was dispatched to the biotechnology unit of the Industrial Technology Institute (ITI), Colombo, Sri Lanka for molecular identification. Fungal strain characterization was done by DNA sequencing of ITS regions. The PCR product of the sample was sequenced by Macrogen Inc. Korea and resulting sequence was analyzed using National Centre for Biotechnology Information (NCBI) genebank data base.

Preparation of *T. asperellum* liquid formulation:
One week-old cultures of *T. asperellum* grown on potato dextrose agar (PDA) were used for the preparation of spore suspensions. The liquid media used for the study was 1% sucrose solution, 1% peptone water, 1% tryptone broth, 1% tryptone soy broth and sterilized distilled water. The prepared medium was added gradually into the culture plates and spores were extracted into each medium using a fine brush.

The initial spore count of each liquid formulation was maintained at $1.0x10^6$ CFU/ml. Spore suspension was poured into 500 ml screw capped glass bottles (400 ml was filled). Loosely capped glass bottles were stored at 25 °C. The initial spore count was taken using a haemacytometer. Dilution plating technique was followed at weekly intervals for the measurement of viable spore count during the storage period. The physical appearance and odour of each solution were also recorded. The test as repeated twice with three replicates. The data presented thus represent six replicates.

Preparation of talc-based formulation:
Talc powder was evaluated as the carrier material to produce bio-formulation of *T. asperellum.* Carrier material (250 g) was placed in 150 gauge polypropylene bags and autoclaved at 121 °C, 15 psi for 15 minutes. A spore suspension ($1.0x10^8$ CFU/ml) was prepared using 7 days old cultures of *T. asperellum* which were grown on PDA medium. The spore suspension was prepared in sterilized distilled water and mixed with talc at three different levels (v/w); 30 ml/100 g (T1), 40 ml/100 g (T2) and 50 ml/100 g (T3). Five replicates were maintained for each treatment. Then, each formulation was allowed to dry under room temperature for a few days. Air dried powder (25 g) was packed in 150 gauge polypropylene bags, sealed and then stored under room temperature (25 °C – 30 °C). The formulations were tested at 30-day intervals to measure the spore concentrations and pH values.

Mass culturing of *Trichoderma* in cattle manure:
Dried cattle manure (10% moisture content) was moistened with tap water (100 ml tap water : 1 kg cattle manure) and then 250 g of medium was placed in polypropylene bags (25" x 15", 150 gauge). Autoclaving was done at 121 °C under 15 psi for 15 min. Talc-*Trichoderma* formulation, which was prepared three months before (T1) was used for inoculation of autoclaved cattle manure. Inoculation was done at four different rates; 10 g/kg cattle manure (C1), 15 g/kg cattle manure (C2), 20 g/kg cattle manure (C3) and 25 g/kg cattle manure (C4). Cattle manure and talc formulation was mixed thoroughly in polypropylene bags,

sealed with rubber bands and then incubated for two weeks under room temperature (25 °C – 30 °C). After two weeks of incubation, spore count was measured by dilution plating technique. Five replicates were maintained for each treatment.

Results and Discussion

Molecular identification of the *Trichoderma* strain:
Based on the results of Macrogen Inc. Korea, ITS sequences of the sample was similar to *T. asperellum* sequence information deposited in the NCBI data bank (ITI Test Report, Reference No. CTS 1709084).

Preparation of *Trichoderma* liquid formulation:
At the initial stage, colour of all the formulations was light green to light yellow. The mean viable spore count in sterilized distilled water and 1% sucrose remained comparatively higher (>10^6 cfu/ml) during the first 4 weeks of the study period (Figure 1). Decreasing trends of spore counts of *T. asperellum* were observed in the liquid formulations. Similarly, increasing trends of contaminations were observed in peptone and TSB formulations. At the end of 4th week, the viable spore counts of 1% tryptone broth, 1% peptone water and 1% tryptone soy broth were 7.15x10^6 cfu/ml, 7.26x10^5 cfu/ml and 7.30x10^5 cfu/ml, respectively. Heavy contaminations in these formulations from the 4th week onwards and hence spore counts were not take. The reason for heavy contamination could be the presence of carbohydrate and proteins in the formulations. Bidochka *et al.* (1987) reported that the highest biomass production of *Beauveria bassiana* in glucose-peptone-yeast extract than peptone, peptone-glucose and glucose. In 1% sucrose solution and sterilized distilled water, spore count was countable only at the end of 8th week of preparation (2.5x10^4 cfu/ml and 2.6x10^3 cfu/ml, respectively; Figure 1).

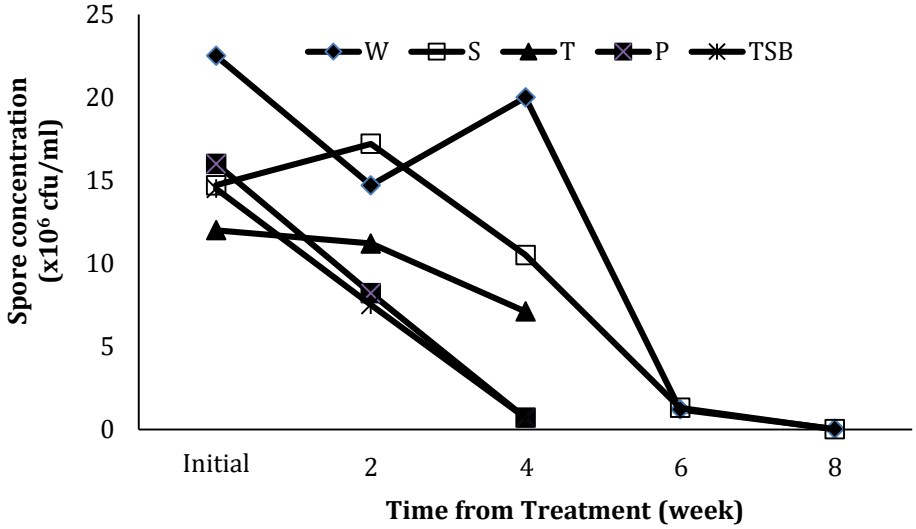

Figure 1. The mean *T. asperellum* spore count in different liquid formulations taken at 2-week intervals (W = distilled water, S = 1% sugar solution, T = 1% tryptone broth, P = 1% peptone water, TSB = 1% tryptone soy broth) at 25 °C (n=15)

Preparation of the talc-based formulation of *T. asperellum*:
The initial colour of all talc formulations was dull white and during the three months study period, no colour change was observed in any of the formulations. Three months after preparation, all the talc formulations (T1, T2 and T3) yielded a mean spore count of 0.92x10^8 cfu/g, 1.11x10^8 cfu/g and 1.21x10^8 cfu/g, respectively (Figure 2). Kumar *et al.* (2013) reported a spore count of 96.67x10^9 cfu/g in a talc-based formulation of *Trichoderma* after 120 days preparation. The pH value of all talc

preparations ranged from 7.5 to 8.5 throughout the study period. Devi and Paul (2008) reported that the alkaline condition inhibits multiplication of *T. harzianum* in soil. Therefore, further studies on multiplication and survival of *Trichoderma* in talc-based medium are necessary to have conclusive results in bio-inoculum production.

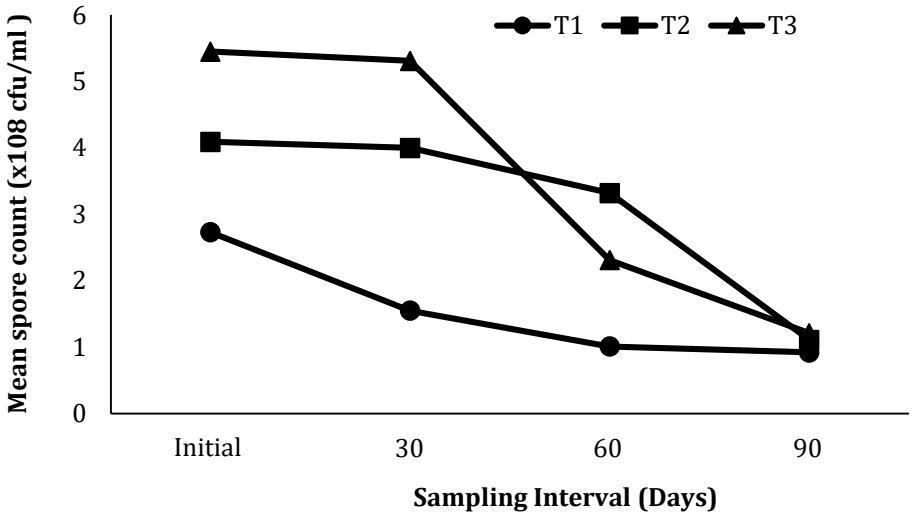

Figure 2. The mean *Trichoderma asperellum* spore count of different talc-based formulations. Samples were taken at 30 days intervals; T1 = 30 ml spore solution/100 g talc powder, T2 = 40 ml spore solution/100 g talc powder, T3 = 50 ml spore solution/100 g talc powder, at 25 °C (n=15)

Mass culturing of *Trichoderma* in cattle manure:

Trichoderma growth was clearly seen on the surface of inoculated cattle manure as yellowish green colour masses (Figure 3). The mean *T. asperellum* spore count of the talc-based formulation after two weeks of incubation period are shown in Table 1.

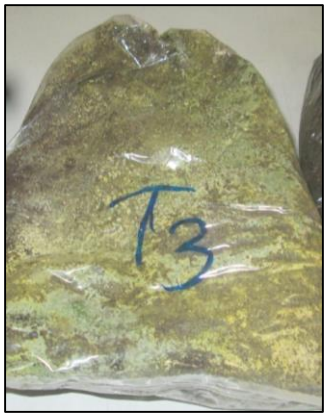

Figure 3. *Trichoderma* growth on talc-based formulation inoculated with cattle manure

The results revealed that higher populations of *Trichoderma* spores (>10^8 cfu/ml) in all talk + cattle manure formulations after 2 weeks of incubation (Table 1). There was no relationship between the initial inoculum levels and final spore concentration of *Trichoderma* bio-control agent in the talc + cattle manure medium.

Table 1. The mean *T. asperellum* spore count incorporated with cattle manure after two weeks of incubation period (n=20)

Cattle manure treatment (g/kg)	Mean spore count (cfu/g)
10 (C1)	$2.0x10^8$
15 (C2)	$2.0x10^8$
20 (C3)	$4.0x10^8$
25 (C4)	$2.0x10^8$

The talc-based formulation is relatively cheap, easy to prepare and had a considerably higher shelf-life. As the spores are lying in a dormant stage in the formulation, it is essential to provide an organic substrate with favourable growth conditions (pH, moisture and temperature) for spore proliferation. The maximum multiplication of *Trichoderma* has been found under 30-35% moisture, 25 °C temperature and 5.4-6.6 pH (Devi and Paul, 2008). However, there were no microclimatic data and organic matter content in media collected in these studies. Hence, further investigations are necessary to identify the methods to increase shelf-life further. Commercial preparation of talc-based formulation could be developed and introduced to farmers as an environmentally-friendly bio-control agent. Zaidi and Singh (2004) reported that cattle manure is a proven multiplication substrate for *Trichoderma* in carrier medium. Investigations on potential use of other available organic substrates such as compost are also important in the production of *Trichoderma* inoculum at a commercial scale.

Conclusion

Trichoderma strain isolated previously from black pepper rhizosphere was highly homologous to *T/ asperellum*. Liquid medium such as 1% sucrose solution, 1% peptone water, 1% tryptone broth, 1% tryptone soy broth and sterilized distilled water were not suitable for multiplication of *Trichoderma* due to heavy contaminations. Spores of *T. asperellum* could be preserved for three months in a talc-based formulation stored under 25 °C – 30 °C in polypropylene bags without adding any preservatives. Cattle manure is a good multiplying substrate for a commercial formulation. Spore preservation technique in a talc-based formulation could be useful in advancing the commercial preparations of the bio-inoculate.

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