Evaluation of *Trichoderma Asperellum* Mass Production and Shelf Life in Talc Formulation

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Abstract**—** *Anthracnose of chilli has been reported to cause more than 50 % of yield loss in Malaysia. The popular approach for disease management of chilli is by the integration of several practices such as cultural, mechanical, chemical and biological control. With the current awareness of the importance of healthy and natural product, more chilli farmers and consumers are showing interest on safer pest and disease control practices. In this study, Trichoderma asperellum has been identified as an excellent biocontrol agent against Colletotrichum spp, the causal agent of anthracnose disease. To be able to be used in the field, it was mass-produced and then formulated in talc. Studies on liquid substrates for mass production of T. asperellum and shelf life for the talc formulation were conducted to evaluate the spore viability. Results showed formulation added with 5% glycerol gave highest initial colony forming unit (CFU) (2.33x 10^8) of T. asperellum and retained the spore count at 1.12 x 10^6 after 120 days of storage.*

Keywords— Mass production, shelf life, talc formulation, Trichoderma.

I. INTRODUCTION

Plant diseases are one of the major concerns in cultivation worldwide consequential in loss of billions of dollars of farm produce. There is an urgent need to manage diseases to ensure a steady and constant supply of marketable products for the escalating world population. In disease management, the amplified use of chemicals has caused negative impact on environmental quality and resulted in the upward trend of many living forms which are resistant to the chemicals [1]. *Trichoderma* as a powerful fungal biocontrol agent against a range of phytopathogens has attracted considerable scientific attention. Several Trichoderma species such as *T. harzianum* have been used as BCA against *Colletotrichum*, the causal agent of anthracnose in greenhouse and field conditions [2]. Commercial success of biocontrol agent depends on its bioefficacy or shelf life but also the substrate for multiplication. The production of adequate quantities of high-quality inoculum is a key component of the biological control program [3]. Growth and sporulation of Trichoderma on cheap and suitable substrates would provide an economical method for mass production of biocontrol agents. A formulated product should be easy to be prepared and stable during transportation and storage. It should have abundant viable propagules with good shelf life. Various substances like talc have been used to formulate the biocontrol agents. As most of the previous studies have emphasized on use and mass production of *T. harzianum* and *T. viride* for the control of pathogens, whereas, other Trichoderma species have received comparatively less such as *T. asperellum*. Therefore, the purpose of this study is to determine the effect of different liquid substrate on mass production and shelf life of the talc powder formulation of *T. asperellum* at different time storage to ensure the viability of the spore.

II. MATERIALS AND METHODS

2.1 Biocontrol agent

Trichoderma asperellum was isolated from a healthy chilli plant rhizosphere cultivated in MARDI Organic Farm, Serdang, Selangor, Malaysia and was used against anthracnose disease of chilli in an *in vitro* experiment. The culture was maintained on potato dextrose agar slant. The identification of the *T. asperellum* was done through DNA sequencing and the sequence was submitted to NCBI GenBank with the given accession number (MW148407).

2.2 Mass multiplication of *T. asperellum*

Two types of broth as the liquid medium; Potato Dextrose Broth and Molasses-Brewer yeast were tested for mass production of *T. asperellum*. The broths were prepared in triplicates each in 250 ml Erlenmeyer flask. The medium was inoculated with 2 discs of 5 mm of 7 days old culture of *T. asperellum* and incubated in an incubator shaker for 14 days at 27±1°C. The spore content in the broths were evaluated on the final day by using hemocytometer. Liquid medium with higher spore concentration was chosen to be used in mass-producing *T. asperellum* before being added to the talc powder.

2.3 Formulation development and shelf life of the final product

Upon harvest, the broth was added with glycerol before being mixed with the talc powder and carboxymethyl was added as the sticker agent. The talc was air-dried for less than 8% of moisture content. The final formulated products packed in a polythene bag of 500 g/ pack and then stored at room temperature. The spore count of the formulated products was evaluated every 30 days and assessed by serial dilution technique. The product was serially diluted up to 10^8 concentration and 1 ml was poured in sterilized petri plates. Thereafter, a selective medium (*Trichoderma* Selective Media) was poured at 20 ml/plate. Plates were rotated horizontally for uniform distribution of inoculum and incubated at 28±1 °C for 48 hours and the numbers of the colony were counted [5].

2.4 Statistical Analysis

All data were subjected to a one way analysis of variance (ANOVA). Treatment means were compared using Duncan Multiple Range test at a significance level of $P<0.05$ using SAS.

III. RESULTS AND DISCUSSION

Table 1 showed molasses-brewer yeast broth yielded the highest concentration of *T. asperellum* (10^8) compared to the potato dextrose broth (10^7) after 14 days of the incubation period. This is similar to a study by [6] where molasses yeast extract broth was found beneficial for the maximum growth of *T. viride* compared to the potato dextrose broth. Our results were also in accordance with a study by [7] who found out the maximum colony growth of *T. polysporum* was obtained on sucrose followed by dextrose, glucose and maltose amended media. According to [8] cane molasses has the potential as a cost-effective carbon source that could serve as nutrients for industrial enzyme-producing microorganisms, especially filamentous fungi. [9] also produced biomass of fungal antagonists by liquid fermentation by using molasses and brewer's yeast medium. Yeast is known to be a superior nitrogen source for *Trichoderma* spore formation and it was chosen instead of an inorganic nitrogen. Yeast is required for microbial cultivation as it offers some additional growth factors like vitamins and amino acids [10]. Submerged liquid fermentation is the best way to mass produce *Trichoderma* for large scale production compared to the solid medium. Maximum growth and a greater percentage of survival during storage are important benefits for the commercial marketing of biocontrol agents. Many factors like medium and inoculum type [11], method of drying, the addition of protectants [12] and environmental conditions during storage [13] affect the viability of the formulation derived from liquid fermentation.

TABLE 1
COLONY FORMING UNIT (CFU) OF *T. ASPERELLUM* IN DIFFERENT LIQUID MEDIUM

Type of liquid medium	*T. asperellum* spore concentration (x 10^8/ml)	
	7 days	14 days
Molasses and Brewer yeast	0.34	1.03
Potato Dextrose Broth	0.36	0.46

To avoid possible contamination during storage, the whole biomass of *T. asperellum* were dried after mixing with talc (Figure A). The fungal biomass has to be desiccation tolerant besides having high spore viability. Glycerol can induce trehalose production and provide the desiccation tolerance in the osmoticum of the production medium. Table 2 showed talc formulation added with 5% glycerol had highest yield of cfu/g (2.34 x10^8) compared to formulation without glycerol and talc formulation retained its colony forming unit (cfu) on *Trichoderma* Selevtive Agar (TSM) of at least 3.6x 10^6 at room temperature after 120 days of storage (Figure B). Gradually decline of CFU in talc formulation were also determined in a similar study by [14] where the CFU count of *T. harzianum* in talc formulation was initially at 3.2 x 10^8 and gradually decline to 3x10^7 after 120 days of storage. [15] also found decreased CFU of *T. harzianum* from 2.0 x 10^8 cfu/g to 2.6 x 10^6 cfu/g after 80 days of storage at 10°C

temperature. Talc formulation of *T. harzianum* yielded at 2.2 x10^7 after 60 days of storage and this was found similar to our result where CFU of *T. asprellum* resulted at 2.1 x 10^7 at 60 days of storage at the room temperature [16]. The addition of glycerol in production medium prolonged the shelf-life of talc formulation as it helps to maintain a high moisture content in the formulation and protects the viable propagules from reduced water activity during the shelf life [17]. In their study, shelf life of formulation was extended to 7 and 12 months by addition of 3% and 6% glycerol to the production media, respectively, compared to formulation without glycerol which only prolonged the shelf life up to 4 to 5 months. A seed treatment of chickpea was carried out with *T. harzianum* with talc, kaolin and bentonite as carriers [18]. The study concluded the fungus had longer shelf life in talc however a significant decline of *Trichoderma* population also reported after 120 days. *T. harzianum* in talc-based powder formulation remained viable at temperatures ranging between 0 to 40ºC for 180 days [19]. The viability of formulated products during the first 6 months at room temperature would be sufficient for use under realistic conditions of storage, humidity and delivery as reported by [20]. Our *T. asperellum* talc formulation retained the spore viability at 10^6 at 120 days of storage and it complies to the minimum population of fungal bioagent in formulation for seed treatment which is more than 10^6 cfu/g [21]. A study by [22] also stated *Trichoderma* conidia in biocontrol products typically range from 1×10^5 to 1×10^9 colony-forming units (CFU) per gram of product.

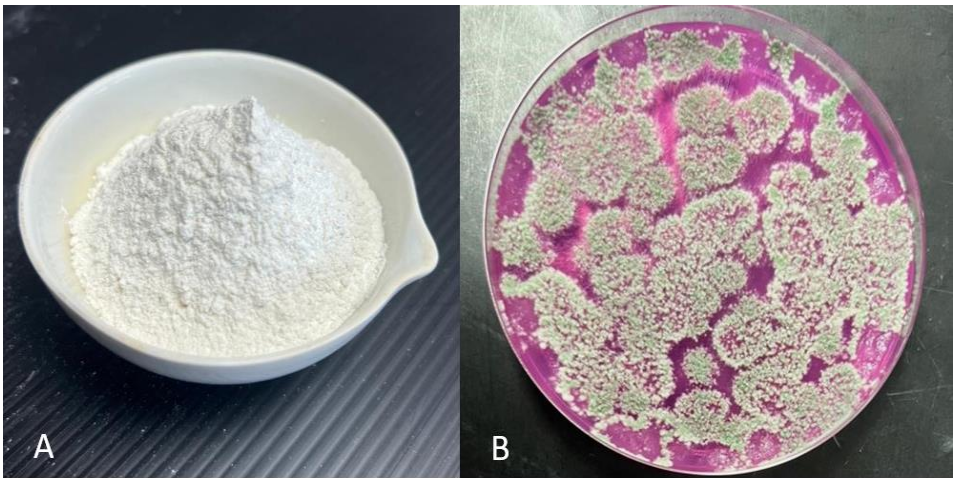

FIGURE: A) Talc formulation of *T. asperellum*; B) The colony of *T. asperellum* on TSM agar

TABLE 2
SHELF LIFE OF *T. ASPERELLUM* TALC POWDER FORMULATION AT DIFFERENT TIME STORAGE (x10^7)

	Day 0	Day 30	Day 60	Day 90	Day 120
Control	2.4b	1.4b	0.3b	0.18a	0.12a
3% glycerol	8.6b	5.8b	1.2a	0.26a	0.14a
5% glycerol	23.4a	11.6a	2.1a	0.84a	0.36a

Means with the same letter in a column are not significantly different (P<0.05) as determined by DMRT

IV. CONCLUSION

The present study indicated the suitability of different liquid medium and talc powder as carrier materials for the commercial preparation of *Trichoderma asperellum*. Microbial count for talc-based formulations of *T. asperellum* was highest initially at ambient temperature but a gradual decline was recorded with the increase in the storage time.

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