

**ASSESSMENT OF DIFFERENT  
FORMULATIONS OF *Trichoderma*  
FOR THEIR SHELF LIFE**

**THESIS**

**Submitted to  
Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola  
in partial fulfilment of the requirements  
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**MASTER OF SCIENCE  
IN  
AGRICULTURE  
(PLANT PATHOLOGY)**

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## DECLARATION OF STUDENT

I hereby declare that, the experimental work and its interpretation of the thesis entitled "**ASSESSMENT OF DIFFERENT FORMULATIONS OF *Trichoderma* FOR THEIR SHELF LIFE**" or part thereof has neither been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis / publication of any University or scientific organization. The source of materials used and all assistance received during the course of investigation have been duly acknowledged.

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**Date:**     /     /2018

Enrolment No. II/1095

## CERTIFICATE

This is to certify that, the thesis entitled "**ASSESSMENT OF DIFFERENT FORMULATIONS OF *Trichoderma* FOR THEIR SHELF LIFE**" submitted in partial fulfilment of the requirements for the degree of "**Master of Science in Agriculture (Plant Pathology)**" of Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola is a record of bonafide research work carried out by **TAYADE MEGHAL SUBHASH** under my guidance and supervision.

The subject of thesis has been approved by the Student's Advisory Committee.

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## D) List of Abbreviations

<b>Abbreviations</b>	<b>Full form</b>
%	: Per cent
/	: Per
@	: At the rate of
°C	: Degree Celsius
Mm	: Milli meter
ml	: Mililitre
C.D.	: Critical difference
Cm	: Centimetre(s)
h	: Hours
CFU	: Colony Forming Unit
Deptt.	: Department
PDA	: Potato dextrose agar
CRD	: Completely randomized design
e.g.	: Exempli gratia (For example)
SE(M)±	: Standard error
et al.	: Et alia (and associates)
etc.	: Et cetera
Fig.	: Figure
g	: Gram
i.e.	: That is
J.	: Journal
BOD	: Biological oxygen demand
spp.	: Species
DAI	: Days after inoculation
viz.,	: Vice licet ( namely )
Min.	: Minutes
No.	: Number
% C.V	: Per cent coefficient of variation
T	: Treatment
Mg	: Miligram
UV	: Ultra violet
S. N.	: Serial number
T. A.	: <i>Trichoderma asperellum</i>
viz.	: Videlicet (namely)
Dr. PDKV.	: Dr. Panjabrao Deshmukh Krishi Vidyapeeth

## **F) Thesis Abstract**

- a) Title of the thesis : “ASSESSMENT OF DIFFERENT FORMULATIONS OF *Trichoderma* FOR THEIR SHELF LIFE ”
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## **ABSTRACT**

*Trichoderma* is a genus of fungi that is present in all soils, where they are most prevalent culturable fungi. Several species and strains of *Trichoderma* have been developed as biocontrol agents against fungal pathogens. *Trichoderma asperellum* is one of the most popular species effective against most of the soil borne plant pathogens.

An experiment entitled “Assessment of different formulations of *Trichoderma* for their shelf life” was undertaken at Department of Plant

Pathology, PGI, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola during 2017-2018. The objective of the investigation to evaluate different formulations of *Trichoderma* for shelf life and their efficacy in management of soil borne plant pathogens.

The soil samples were collected from different locations of Maharashtra viz, Akola, Rahuri, Beed, Ratnagiri (Ta1, Ta2, Ta3, Ta4) for the isolation of *Trichoderma asperellum* on *Trichoderma* Selective Media (TSM) by serial dilution and pour plate technique. Purified cultures were maintained on PDA plates for further study.

Eleven formulations of *Trichoderma asperellum* were evaluated in present investigation, among these four types of tablets formulation viz. talc tablets, charcoal tablets, lignite tablets, fly ash tablets, six capsules formulations viz. talc capsule, alginate capsules, gelatin capsules, alginate + charcoal capsules, gelatin + fly ash capsules, *T. asperellum* frozen culture mass along with medium disc capsules and one sorghum grains formulation were used and observation on shelf life up to 270 days from date of manufacturing at regular intervals of 30 days were taken.

Maximum cfu/g ( $42.00 \times 10^7$ , plate no.17) was obtained in *Trichoderma* treatment T<sub>10</sub> i.e. frozen culture mass along with medium disc even after 270 days of storage followed by T<sub>11</sub>, sorghum grains ( $30.67 \times 10^7$  cfu/g, plate no.18 ), T<sub>6</sub>, *T. asperellum* alginate capsule ( $29.67 \times 10^7$  cfu/g, plate no.13), T<sub>5</sub>, *T. asperellum* gelatin capsule ( $28.67 \times 10^7$  cfu/g, plate no.12), T<sub>8</sub>, *T. asperellum* gelatin + flyash capsule ( $21.00 \times 10^7$  cfu/g, plate no.15), T<sub>7</sub>, *T. asperellum* talc capsule ( $20.00 \times 10^7$  cfu/g, plate no.14), T<sub>9</sub>, *T. asperellum* alginate + charcoal capsule ( $19.33 \times 10^7$  cfu/g, plate no.16), T<sub>4</sub>, *T. asperellum* flyash tablets ( $18.00 \times 10^7$  cfu/g, plate no.11), T<sub>2</sub>, *T. asperellum* charcoal tablet ( $17.67 \times 10^7$  cfu/g, plate no.9), T<sub>1</sub>, *T. asperellum* talc tablet ( $12.33 \times 10^7$  cfu/g, plate no.8) and T<sub>3</sub>, *T. asperellum* lignite tablet ( $11.33 \times 10^7$  cfu/g, plate no.10) after 270 days of storage.

In the present study tablet and capsule delivery system of various formulations was tried. One capsule/tablet from each formulations was dispersed separately in 500 ml of sterilized water enriched with 10 g

jaggery and incubated for 12 h. It is revealed from data presented that the population of *T. asperellum* gradually improved within 12 h of incubation. One gram of capsulated or tablet form of *T. asperellum* culture retained more than sufficient population. It is clear from data that maximum population of *T. asperellum* obtained ( $45.00 \times 10^7$  cfu/ml) in T<sub>10</sub>, *T. asperellum* Frozen culture mass along with medium disc capsule followed by ( $31 \times 10^7$  cfu/ml) in T<sub>6</sub>, *T. asperellum* alginate capsule, ( $21.33 \times 10^7$ cfu/ml) in T<sub>9</sub>, *T. asperellum* alginate + charcoal capsule, ( $20.66 \times 10^7$ cfu/ml) in T<sub>7</sub>, *T. asperellum* talc capsule, ( $20.33 \times 10^7$  cfu/ml) in T<sub>5</sub>, *T. asperellum* gelatin capsule, ( $15 \times 10^7$ cfu/ml) in T<sub>4</sub>, *T. asperellum* fly ash tablet, ( $14.33 \times 10^7$ cfu/ml) in T<sub>8</sub>, *T. asperellum* gelatin + flyash capsule, T<sub>2</sub>, *T. asperellum* charcoal tablets, ( $14.00 \times 10^7$ cfu/ml).

The antagonistic efficacy of *Trichoderma asperellum* against the soil born plant pathogens viz., *Sclerotium rolfsii*, *Rhizoctonia bataticola* and *Fusarium solani* were tested. The maximum per cent inhibition (79.5%) and minimum radial growth (18.40 mm) of *sclerotium rolfsii* was observed in isolate Ta2. In case of *Rhizoctonia bataticola* the minimum radial growth (18.20 mm) with maximum per cent inhibition (79.7%) was recorded in isolate Ta4 collected from Ratnagiri district. Antagonism between *Trichoderma asperellum* and test pathogen *Fusarium solani* showed maximum growth inhibition (82.58%) in Ta2 isolate.

## CHAPTER I

### INTRODUCTION

#### 1.1 Background information

The most widely used biocontrol agents in the world belong to the fungal genus *Trichoderma*. In particular isolates of *Trichoderma harzianum*, *Trichoderma asperellum*, *Trichoderma hamatum* are used against plant diseases in wide variety of economically important crops. They have been used with successes against soil-borne, seed-borne and phyllo-sphere diseases and against storage rots (Chet 1987, Papavizas 1985, Tronsmo, 1986).

*Trichoderma* is a ubiquitous, easy to isolate and cultured, grown rapidly on many substrate and affect wide range of pathogens. One of the popular species *Trichoderma asperellum* is effective against soil-borne fungal pathogens like *Phytophthora*, *Fusarium*, *Pythium*, *Rhizoctonia* and have been exploited on about 87 different crops and about 70 soil-borne and 18 foliar pathogens (Sharma *et al.*,2014).

The status of bio-pesticides shows that, India has the capacity to produce 1850 MT of *Trichoderma* formulation per annum. There are around 150 registered manufacturers of *Trichoderma* in India, out of which *Trichoderma asperellum* has a major share (Sriram *et al.*, 2013).

*Trichoderma* is prepared by various methods, solid and liquid state fermentation is used to make carrier based or liquid based formulations. Shelf life of the formulated product of a biocontrol agent plays a significant role in successful marketing. At PDBC, Bangalore work on increasing shelf life of talc formulations of *Trichoderma* using various ingredients (chitin and glycerol) in production medium and heat shock at the end of log phase of fermentation was carried out, which can extend the shelf life of talc formulation up to one year (Sriram *et al.*, 2011). Bhat *et al.* (2009) reported shelf life of talc based formulation up to 180 days under ambient condition. The rate of desiccation was less up to the first 75 days of storage as compared to that observed from 75 to 180 days of storage.

The formulation retained good numbers of viable propagules (above  $10^6 \times 10^7$  cfu/g) after more than 150 to 180 days of storage.

Major research on biocontrol is centered with the use of spores of *Trichoderma* directly to seed. Though *Trichoderma* has a very good potential in the management of diseases, it could not be used as spore suspension under field conditions. Thus, the culture of *Trichoderma* should be immobilized in certain carriers and should be prepared as formulations for easy application, storage, commercialization and field use (Kumar *et al.*, 2014).

Characteristics of an ideal *Trichoderma* formulation should have increased shelf life and non-phytotoxic to the crop plants, it should tolerate adverse environmental conditions and well dissolving in water, it should be cost effective and should give reliable control of plant diseases, it must be compatible with other agrochemicals and lastly, the carriers should be cheap and readily available for formulation development.

*Trichoderma* sp. is now the most common fungal biological control agents that have been comprehensively researched and deployed throughout the world. Several fungal cell wall degrading enzymes such as chitinase and glucanase seem to play an important role in the antagonistic action of *Trichoderma* against a wide range of fungal plant pathogens. *Trichoderma* sp. that secrete extra-cellular lytic enzymes (chitinase and glucanase) having higher potential of antagonistic efficiency. The lytic enzymes break down cell wall polysaccharides into short oligomers and by this way facilitate the hyper-parasite to penetrate into the cytoplasm of the target fungi (Kucuk and Kivanc, 2008). More than 50% crop losses are due to soil inhabiting microorganisms, such as *Fusarium*, *Sclerotium*, *Rhizoctonia*, *Verticillium*, *Phytophthora*, *Pythium* (Elad *et al.*, 1982).

*Trichoderma* belongs to subdivision Deutromycotina which are heterogenous group of fungi in which sexual stages (perfect stages) are not known or rarely found and reproduction is limited to the production of conidia (Vinale, 2007). The Mycoparasitism, antibiosis, competition for nutrients, tolerance to stress through enhanced root and plant development, induced resistance, solubilization and sequestration for

inorganic nutrients and inactivation of the pathogen enzymes are important mechanisms of *Trichoderma* sp. against plant pathogens (Harman, 2000).

## 1.2 Importance of study

Biological control of plant diseases appears to be an effective and ecofriendly approach being practiced world over. Further biological control strategy has a major role to play as a component of integrated pest management (IPM). Large scale production, along with shelf life and establishment of bioagent in targeted *niche*, determine the success of biological control (Lakshmi Tewari and Chandra Bhanu,2004).

Shelf life of the formulated product of a biocontrol agent plays a significant role in successful marketing. In general, the antagonists multiplied in an organic food base have longer shelf life than the inert or inorganic food bases. Shelf life of *Trichoderma* in coffee husk was more than 18 months. Talc, peat, lignite and kaolin based formulation of *Trichoderma* have a shelf life of 3 to 4 months (Sankar and Jeyarajan, 1996). Studies on the storage of *T. asperellum* formulation in poly propylene bags of various colours revealed that the population of *T. asperellum* was maximum in milky white bags of 100 micron thickness (Ramanujam *et. al.*,2010).

Inoculum is prepared by solid state and liquid state fermentation method such as tablet and capsule. The capsule preparation has the advantages that, it can be as long as about 3 years in shelf life and efficiency. It is simple in preparation process, greatly improve disease resistant effect, it has easy transportation, environmental friendly and reduce a chances of contamination. It has good effects of controlling *Sclerotinia*, wilt and seedling blight of vegetables, fruit trees, flowers and other crops (*Trichoderma* biocontrol capsule microbial inocula and preparation method there of CN 101496528 B).

Alginate prill have been used to deliver several biocontrol agents (Fravel *et al.*,1986). Sodium alginate is commonly used in many food products any residues in plant or soil should not be toxic (Connick, 1979).

Soil borne fungal pathogen such as *Rhizoctonia bataticola*, *Fusarium udum*, *Sclerotium rolfsii*, *Pythium*, *Phytophthora* etc. causes greater reduction in crop yield. The use of fungicides for control of these pathogens has met with moderate success and their future use is a question due to increased regulatory restrictions. In addition, a number of currently used fungicides, such as mercurials have been withdrawn from the market. One means of overcoming these problems is to use an Integrated Disease Management (IDM) system which would include bio control strategies also. A component of such strategies might include those of non-pathogenic soil borne fungi which secrete low molecular weight protein compound that may be toxic to the pathogen. The discovery of new biological control agents and the demonstration of their value is reducing disease incidence and sever it have opened new promising avenues for practical applications in agriculture and for promoting environmental safety. Since the pioneering work of Weindling and Fawcett (1936) the use of *Trichoderma* strains to control damping off caused by *Rhizoctonia solani* in citrus, considerable attention has been focused over the past 25 years on effective as chemicals in suppressing fungal pathogens.

### **1.3 Objective of the study**

To evaluate different formulations of *Trichoderma* for shelf life and their efficacy in management of soil borne plant pathogens.

### **1.4 Scope and limitations**

Mass production is broad aspect of successful biological control techniques which include establishment of products, formulation and delivery system of fungi where used as an efficient disease control (Seema Solanki *et al.*,2016).

But some of the limitation in their use is carrier material have shelf life upto 4 to 5 month and contamination occurs during delivery method.

## **1.5 Hypothesis**

*Trichoderma* is a promising antagonistic organism for the biological control of plant pathogenic fungi. In present investigation efforts have been made to improve the awareness and application technology along with shelf life of different formulation of *Trichoderma* culture. The shelf life of the different formulations has been tested upto nine months from date of manufacturing at regular intervals of 30 days and result should be obtained considering this possibilities, the present study was undertaken.

## CHAPTER II

### REVIEW OF LITERATURE

In present investigation entitled “Assessment of different formulations of *Trichoderma* for their shelf life” literature available on various aspects were received during the period under study reviewed here.

#### **2.1 Collection of soil samples and Isolation of *Trichoderma* sp.**

Papavizas *et al.* (1987) reported that, a semi selective medium, previously developed for the isolation and enumeration of *Trichoderma* sp. from soils was not effective with soils containing rapidly spreading mucorals. The medium was used effectively to recover *Trichoderma* sp. from soil by the addition of alkylaryl polyether alcohol at 2.0 ml/l alone or in combination with sodium propionate. The basal medium contained V-8 juice, 200ml; water, 800ml; agar, 20g; and glucose 1g and improved medium designated TME-SA, contained antimicrobial agents like neomycin sulfate, bacitracin, penicillin G and chloroneb,100; chlorotetracycline hydrochloride, 25; nystatin, 20; and sodium propionate 500. Both modified media allowed *Trichoderma* sp. to grow on agar surface and suppressed other fungi like Rhizopus.

Rahman *et al.* (2011) quantified and characterized the native population of *Trichoderma* species collected from different habitats. The identified strains were *T. harzianum*, *T. pseudokoningii* and *T. virens*. Colony forming units (cfu's) of *Trichoderma* species varied significantly ( $P = 0.05$ ) in different habitats and were positively correlated with the physicochemical characteristics of the habitat. In testing for an efficient bioconversion agent, *T. harzianum* (IMI-392432) was found to be the most effective in kitchen waste decomposition. It provided the highest volume (31.80%) and weight (30.80%) losses in waste treated with spore suspension.

Sreedevi *et al.* (2011) isolated *Trichoderma* species by using *Trichoderma* selective medium (TSM) and then subcultured on potato dextrose agar. Based on the conidiophore morphology, the isolates were designated as T1, T2, T3, T4, and T5. Under scanning electron microscope the fine structure of *T. harzianum* conidial surfaces and conidial masses on a phialide was observed. The ability of *Trichoderma* isolates to inhibit the mycelial growth of *M. phaseolina* in dual culture was determined on PDA medium. Among the five isolates *T. harzianum* (T3), *T. viride* (T1) had maximum antifungal activity against *M. phaseolina*. They reduced mycelial growth by 61.1 and 64.4%, respectively.

Kumar *et al* (2012) isolated twelve isolates of *Trichoderma* sp. from different locations of South Andaman and were characterized for their cultural, morphological and antagonistic activity against soil borne and foliar pathogens. The sequencing of these isolates showed seven different species. The isolates showed differential reaction patterns against the test pathogens viz., *Sclerotium rolfsii*, *Colletotrichum gloeosporioides* and *Colletotrichum capsici*. However, the isolates, TND1, TWN1, TWC1, TGD1 and TSD1 were most effective in percent inhibition of mycelial growth of test pathogens.

Mridula Khandelwal *et al.* (2012) isolated fungal species *Trichoderma viride* from soil samples by using potato dextrose agar (PDA) medium. Samples were inoculated over plates by multiple tube dilution technique (MTDT) and the plates were incubated at 26°C for 4 days. The fungal colonies which were picked up and purified by streaking and incubated at 26°C for 7-8 days. Green conidia forming fungal bodies were selected and microscopic observation was identified to be *Trichoderma viride*.

Arumugam *et al.* (2013) were isolated *Trichoderma viride* and *Pseudomonas fluorescens* from the rhizosphere of rice fields by the serial dilution method and studied their morphological characters and evaluated the antagonistic activity.

Khang *et al.* (2013) isolated 21 *Trichoderma* strains from 24 soil samples collected from different locations of Provinces as Can Tho,

Hau Giang, Vinh Long, Dong Thap, Ca Mau and Dong Nai belong to Mekong Delta. The *Trichoderma* strains that were isolated from DT3 showed strong inhibitory effect against *Phytophthora*. Among these the *Trichoderma* sp. strain of 1.1, 5.2, 8.1, 8.2 and HG1 showed to remarkably inhibit the growth of *Phytophthora* but had no significant difference compared with control strain *T. viride*.

Gonzalez *et al.* (2016) obtained three *Trichoderma* isolates from sandy soils collected at the “Gran Desierto de Altar” in the northwest of Mexico and characterized by morphological and molecular analyses as *Trichoderma harzianum* (8.4), *Trichoderma asperellum* (12-2) and *Trichoderma asperellum* (BP60). Isolate *T. asperellum* (BP60) inhibited *Setophoma terrestris*, grew above 50°C, and produced chitinases and siderophores, therefore it was chosen to obtain enough biomass and conidia for field applications.

Chandra Sekhar *et al.* (2017) ten *Trichoderma* isolate were isolated, among the ten isolates of *Trichoderma* spp. Considerable variations were observed regarding total growth, growth rate colony characters and sporulation. Isolates colony showed dark green to dark bluish green sporulation, colony reverse was amber or uncoloured.

## **2.2 Testing of viability of *Trichoderma asperellum* in different formulations**

Ward (1977) revealed that, gelatin is a mixture of peptides and proteins produced by partial hydrolysis of collagens extracted from the skin, bones and connective tissues of animals such as domesticated cattle, chicken, pigs and fish. It is commonly used as a gelling agent in food, pharmaceutical drugs, vitamin capsules, photography and cosmetic manufacturing.

Rafael and Fernando (1987) were described the production, properties, uses and main applications of agar in the publication of McHugh, D.J. 1987. According to the US Pharmacopeia, agar is a hydrophilic colloid extracted from certain seaweeds of the Rhodophyceae class. It is insoluble in cold water but soluble in boiling water. It is a mixture

of polysaccharides whose basic monomer is galactose. These polysaccharides can be sulphated in very variable degrees but to a lesser degree than in carrageenan. For this reason the ash content is below those of carrageenan, furcelleran and others.

Karunanithi *et al.* (2001) tested different carriers among them gypsum and talc were significantly superior in supporting the survival of *T. viride* and these showed mean populations of  $15.0 \times 10^7$  and  $14.53 \times 10^7$  cfu  $g^{-1}$  product respectively. All the carriers tested showed decreasing trend in retaining the viability of *T. viride* with increasing period of storage. The mean population of *T. viride* gradually decreased from  $24.5 \times 10^7$  cfu  $g^{-1}$  product on the day of preparation to  $3.7 \times 10^7$  cfu  $g^{-1}$  product 150 days after storage.

Sathiyaseelan *et al.* (2009) were used five different carriers T1: paraffin oil + glycerol (1:1) ratio, T2: paraffin oil + soybean oil (1:1) ratio, T3: soybean oil + glycerol (1:1) ratio, T4: paraffin oil and T5: soybean oil. The formulations were kept in room temperature and results were recorded in terms of cfu against control and the shelf life study revealed that the survivability of *Trichoderma viride* in paraffin oil was better than other formulation with  $28 \times 10^8$  Cfu/ml followed by 8 soya bean oil with  $6 \times 10^8$  Cfu/ml at 49 day. There was a 7 th steep declain of population level in formulation T1, T2 and T3 at 14 day. In all the formulation, sedimentation or buoyancy was recorded.

Sriram and Savitha (2011) estimated the possible 'number of conidia per cc' and 'number of conidia per 'g' of *Trichoderma harzianum* spores (NBAll Th10-MTCC 5584). The results showed that even at higher dilutions ( $>10^{18}$  to  $10^{20}$ ) colonies of *Trichoderma* appeared on plates. It is proposed that while enumerating the viable propagules in a formulation, the tips should be changed for each dilution without fail and dilution should be restricted to  $10^9$  or  $10^{10}$ . The limitations of the serial dilution beyond  $10^{10}$  and precautions to be taken were discussed.

Anis Muhammad *et al.* (2012) reported that when sunflower seeds coated with Na-alginate at 1, 2 and 4% w/w with *Trichoderma viride*, *T. reesei*, significant increase in germination was recorded when seeds

were coated with *Trichoderma* species using Na alginate at different concentrations as a sticker. Plant length and weight was significantly higher in treated seeds as compared to non treated control. Seeds coated with *T. viride* using 2% Na-alginate as a sticker showed maximum increase in plant length and weight followed by seed coated with *T. viride* in combination with 1% Na alginate. Maximum vigor index were observed in sunflower seeds treated with *T. viride* using 2% Na-alginate.

Singh and Nautiyal (2012) NBRI-1055 (*Trichoderma harzianum*) spores scrapped from potato dextrose agar plates were used to prepare a concentrated formulation after optimizing carrier material, the formulation had an initial 11–12 log<sub>10</sub> CFU g<sup>-1</sup>. Its concentrated form reduces its application amount by 100 times (10 g 100 kg<sup>-1</sup> seed) and provides 3–4 log<sub>10</sub> CFU seed<sup>-1</sup>. Shelf life of the product was experimentally determined at 30 and 40°C. Stable and effective formulation of *Trichoderma harzianum* NBRI-1055 was obtained by a simple scrapping method for the production of concentrated, stable, effective and cost efficient formulation of *T. harzianum* was validated for seed application.

Four solid and liquid substrates viz; wheat bran-vermiculite, sorghum grain flour, molasses-yeast and potato dextrose broth were used for the mass multiplication of *Trichoderma* sp. Among all the four substrates, wheat bran-vermiculite substrate was found to be best in respect of colony forming units compared to other substrates. The mean population was highest in wheat bran-vermiculite (3.56 x 10<sup>8</sup> cfu/g) followed by molasses yeast medium (3.03 x 10<sup>8</sup> cfu/g), sorghum grain flour (2.46 x 10<sup>8</sup> cfu/g) and potato dextrose broth (2.06 x 10<sup>8</sup> cfu/g). Highest mean population was recorded in wheat bran-vermiculite (3.56 x 10<sup>8</sup> cfu/g) and the lowest mean population (2.06 x 10<sup>8</sup> cfu/g) was recorded in potato dextrose broth by Hari krishna and Reddi kumar (2013).

Kumar *et al.* (2013) studied shelf-life of *Trichoderma viride* in talc and charcoal based formulations. Potato dextrose broth talc based formulation was prepared by adding three different volumes of biomass of *T. viride* along with medium @ 30, 40 and 50 ml/g talc. The initial mean CFU of *T. viride* at 30, 40 and 50 ml/100 g talc on 0 day was 227.2 x 10<sup>9</sup>,

256.00 × 10<sup>9</sup> and 291.03 × 10<sup>9</sup> CFU/g sample, respectively. It gradually declined and at 120 days of storage the population came down to 70.33 × 10<sup>9</sup>, 80.67 × 10<sup>9</sup> and 96.67 × 10<sup>9</sup> CFU/g which in terms of reduction in viability were 69.04, 68.48 and 66.78% respectively.

Baghel *et al.* (2014) evaluated the viability in different formulations of *Trichoderma viride* and also assessed the moisture present in using formulations of *Trichoderma viride*, seven different carrier based formulations used such as chalk, talc, charcoal powder, chalk tablet, talc tablet, charcoal tablet with 3 concentrations 5%,10% and 15%. It was found that, the storability is maximum in *Trichoderma viride* 15% in charcoal powder with high moistures, one capsule formulation *T. viride* and culture of *T. viride* (ISO-1) were evaluated on different period to observe their viability. Different formulations of *T. viride* maximum (80 × 10<sup>7</sup> cfu/g) was recorded in 15% charcoal powder followed by capsules (78.67 × 10<sup>7</sup>cfu/g) upto 260 days storage.

Cumagun (2014) studied the method of mass multiplication and formulation to stabilize the product during storage and to facilitate its delivery to the plant. Solid and liquid formulations were used to produce suitable quantities of active and viable inocula of *Trichoderma*. Three kinds of propagules used in formulations – hyphae, clamydospores and conidia. Their research was on development of formulation and delivery systems of *Trichoderma* to enhance its biocontrol efficacy against plant pathogens. The advances in *Trichoderma* formulation have promoted biological control of plant pathogens for sustainable agriculture.

Kumar *et al.* (2014) reported that, the popularization of bio-pesticides is very slow as compared to chemicals. The potential *Trichoderma* isolates are formulated using different organic and inorganic carriers solid and liquid fermentation technology. They are delivered either through seed treatment, bio-priming, seedling dip, soil application and foliar spray. Research should be concentrated to increase the shelf life.

Saili *et al.* (2014) Were studied the potential of lignocellulolytic *Trichoderma* fungi for the use of rapid composting of oil palm empty fruit bunches fibers. Fifty-two of *Trichoderma* isolates from

Sabah and seven isolates from Antarctic were examined for *in-vitro* lignocellulolytic activity. The best six Sabah *Trichoderma* isolates (5D, 10L2, 10P, 5E, 10X, and 10E2) were found to be potential lignocellulolytic agents. In contrast, Antarctic *Trichoderma* isolates consistently exhibited lower lignocellulolytic activities. Most of the *Trichoderma* isolates were found to synthesize polyphenol oxidase, endoglucanases and were able to hydrolyze starch to glucose in the three different media. Thus, the finding showed the potential of these isolates for use in large-scale composting of oil palm empty fruit bunches.

Rai and Tewari (2016) studied total nine types of *Trichoderma* based formulations prepared using formulating materials viz. Dextrin, talc, gypsum, paraffin oil and soybean oil. Shelf life of formulations was tested up to 6 months at room temperature (15-35°C) and 11 months at refrigerator (4°C). Among different formulation dextrin based formulation TF. paste8 retained maximum viability (26.10%;  $4.33 \times 10^7$  CFU/ g) after 6 months of storage at room temperature (15-35°C). The formulations stored at 4°C retained viability (2.06- 16.06 %) up to 11 months during storage. Maximum viability was observed in TF. paste8 (16.06%;  $2.67 \times 10^7$  CFU/ g). Their study showed that there is potential in using of *Trichoderma* paste and liquid formulations for improving shelf life of bioformulation as well as in biological control.

Kose and Totawar (2017) were prepared four types of *T. viride* tablets viz. talc, charcoal, lignite, flyash, and also tried different six types of capsule of *T. viride* viz. talc, gelatin, alginate, alginate + charcoal, gelatin + flyash and frozen mass culture along with medium disc capsules and compared with sorghum grain based carrier as control. They revealed that maximum cfu g<sup>-1</sup> ( $76.33 \times 10^7$ ) was obtained in *Trichoderma* treatment of frozen culture along with disc capsule even after 180 days of storage followed by sorghum grains ( $75.67 \times 10^7$ ).

Kose and Totawar (2017) were tried six types of capsules of *T. viride* viz. talc, gelatin, alginate, alginate + charcoal, gelatin + flyash and frozen mass culture along with medium disc capsules also four types of tablets of *T. viride* viz. talc, charcoal, lignite and flyash for delivery system

and application and revealed that when one capsule/tablet from each formulation dispersed separately in 500 ml capacity conical flask containing 250 ml sterilized water enriched with 10 g jaggery and incubated for 12 h given maximum population of *T. viride* ( $77.66 \times 10^7$  cfu/ml) in capsule of frozen culture mass alongwith medium disc followed by alginate capsule ( $53.00 \times 10^7$  cfu/ml), alginate + charcoal capsule ( $36.66 \times 10^7$  cfu/ml), talc capsule ( $31.00 \times 10^7$  cfu/ml), gelatin capsule ( $27.66 \times 10^7$  cfu/ml), flyash tablet ( $27.33 \times 10^7$  cfu/ml) and ( $24.66 \times 10^7$ ) cfu/ml in charcoal tablet. Also in opinion that increased cell count by this technology is due to composition of hard gelatin capsule which is made up of peptides and proteins produced by partial hydrolysis of collagen extracted from skin, bones and tissues of animals (stegemann, 2002).

### **2.3 Mass production of *Trichoderma***

Solid media containing carboxy methyl cellulose (CMC) were developed to detect C<sub>x</sub> cellulase-producing micro-organisms. Hydrolysis of CMC was seen as a clear zone around colonies after flooding plates with 1 % aqueous hexadecyltrimethyl- ammonium bromide. Tests with ten bacterial and four fungal species showed that the degree of substitution (DS) of the CMC affects both growth and enzyme production. Most of the organisms produced more C<sub>x</sub> cellulase on CMC with a DS of 0.9, but CMC with a DS of 0.4 was better for one fungus. Solid media containing CMC provided a more rapid assay of C<sub>x</sub> cellulase production than a medium containing native cellulose was observed by Hankin and Sandra L. Anagnostakis (1977).

Lakshmi Tewari and Chandra Bhanu (2004) evaluated three categories of agro-industrial wastes viz. 1) cellulosic waste 2) cereal brans 3) organic manures for growth response and conidial yield of biocontrol agent *Trichoderma harzianum*. All the substrates supported fairly good growth and sporulation of bioagent, the optimum level of chickpea flour supplementation for paper waste was recorded as 12 per cent (w/w), thereby showing maximum conidiospore production ( $17.07 \times 10^8$  cfu/g) in 20 days, paper based mixed substrate combination containing paper waste (80 per cent), rice bran (8 per cent) and chickpea flour (12 per cent)

showed higher conidial yield ( $11.73 \times 10^9$  cfu/g) after 10 days incubation period.

Rini and Sulochana (2007) were evaluated substrates as cow dung, neem cake, coir pith, sorghum grains, saw dust, and rice bran, either alone or in certain combinations, with or without additives such as jaggery and wheat flour for mass production of *Trichoderma harzianum* and *T. viride*. pre-boiled sorghum grains, coir pith + neem cake (1:1), cow dung + neem cake (1:1) + wheat flour (10%) maintained high populations of *T. harzianum* and *T. viride* within 10 days of inoculation. Jaggery and wheat flour served as nutritional supplements and enhanced the conidial yield from  $23.66 \times 10^8$  to  $34 \times 10^8$  and  $45.6 \times 10^8$  colony forming units  $g^{-1}$  respectively. Although highest initial population of *Trichoderma* spp. was observed in sorghum grains and propagule viability was low in that compared to other substrates. Coir pith + neem cake (1:1) at 35% and 45% moisture gave longer shelf life for *Trichoderma* propagules.

Rosane *et al.* (2008) evaluated low-cost substrates to produce *Trichoderma* spores. Rice, corn bran, and wheat bran were used as solid substrate to grow *Trichoderma harzianum*, *T. viride*, *T. koningii* and *T. polysporum*. No external nutrient sources were added to the solid substrate that was only moisturized with deionized water, sterilized, inoculated, and cultivated at 30 °C for 7 days. Wheat bran showed to be the most suitable substrate to produce *Trichoderma* spores for all strains that were evaluated. High spore counts were obtained for *T. harzianum* ( $28.30 \times 10^8$ /gds) and *T. viride* ( $24.10 \times 10^8$  spores/gds).

Ramanujam *et al.* (2010) reported that *Trichoderma* has gained maximum attention as biocontrol agent. There are two major methods of inoculum production of *Trichoderma* sp. viz., solid state fermentation and liquid state fermentation. Seedlings of horticultural crops and rice were treated by dipping the roots in *Trichoderma* suspensions before planting. Granular or pellets preparations and *Trichoderma* enriched FYM have been used for soil application directly and have provided effective control of diseases in both nurseries and field conditions.

Shafa Khan *et al.* (2011) indicated that, the vermi-compost, de-oiled castor cake and farm yard manure formulation supported the growth of *Trichoderma viride* during storage, which is a major advantage for the marketing of these biocontrol agents. Vermiculite based fermenter biomass of formulation with an initial population of  $205 \times 10^6$  cfu/g stored in milky white bags showed an exponential phase up to 30 days ( $309 \times 10^6$  cfu/g). Temperature of 20-30°C was optimum for the storage of the formulation at which even after 75 days, the product contained  $206-271 \times 10^6$  cfu/g. A fermenter biomass in vermicompost formulation had longer growth and survival rate as compared to other formulations. Growth of *T. viride* was continued in vermicompost formulation up to 195 and retained good viability for 255 days. Thereafter, a reduction in colony forming unit (cfu) was recorded.

Sobita Simon (2011) evaluated various agricultural byproducts such as tea leaves, pea husk, rice husk, wheat bran, vegetable wastes, fruit waste, FYM etc. for mass production of *T. viride* and *T. harzianum*. Among the substrates used, waste of brinjal, papaya, banana, guava, spinach, sugarcane of solid and liquid was found supported maximum spore production as compared to carrot, cucumber, potato, radish, cabbage, FYM. The moderate sporulation obtained in the substrates of wheat bran was 0.79(s), 0.83(L), in raddish 0.68(S); 0.84(L)  $\times 10^6$ cfu/ml for *T. harzianum*. In case of *T. viride* the sporulation was noted in wheat bran 0.81(s); 0.84 (L) and in radish 0.46(s); 0.69(L)  $\times 10^6$ cfu/ml. *T. harzianum* sporulation showed slow in FYM 0.27(S), 0.32(L) and rice husk 0.20(S), 0.41(L), Poultry manure 0.26(S); 0.27(L)  $\times 10^6$ cfu/ml. In case of *T. viride* the sporulation rate in FYM 0.16(S); 0.19(L); rice husk 0.23(S); 0.37(L) and in poultry manure 0.13(S); 0.16(L)  $\times 10^6$ cfu/ml respectively.

Yadav (2012) were evaluated various agricultural residues and by products such as rice husk, saw dust, maize husk and wheat bran for mass production of *Trichoderma viride* and *Trichoderma harzianum*. Among the substrates used maize husk was found supported maximum spore production for *T. viride*  $2.0 \times 10^8$  per gram substrate and  $1.2 \times 10^8$  per

gram substrate for *T. harzianum* as compared with other substrate wheat bran, rice husk and saw dust respectively.

Nagur Babu and Pallavi (2013) reported the use of household waste, vegetable waste, other wastes and assessment for their suitability as substrates for mass multiplication of *Trichoderma candidum*. The results indicated that the substrates evaluated were able for production of *Trichoderma candidum*. The sugarcane baggase yield high amount of mycelia, spore & higher CFU count was recorded i.e.  $101.3 \times 10^8$  on solid medium & on liquid medium  $94.7 \times 10^8$ . The both solid & liquid state can be confirmed for the production *T. candidum*. High spore count was recorded on sugarcane baggase media. The formulated CFU count with inert carrier sodium silicate was  $41.5 \times 10^8$ .

Kumar *et al.* (2014) used six agro based substrates such as sorghum grain, wheat grain, rice bran, rice husk, maize flour and saw dust were evaluated for mass production of three *Trichoderma* sp. Amongst, highest population was recorded in sorghum grain after 30 days of storage period of all three species i.e. *T. harzianum* ( $58.68 \times 10^6$  cfu/g) *T. viride* ( $54.24 \times 10^6$  cfu/g) and *T. hamatum* ( $52.51 \times 10^6$  cfu/g), The population of different species remained constant for a period of 60 days after which it started to decline slowly, the rate of decline varied with different substrates. The lowest population was observed in saw dust on *T. harzianum* ( $2.32 \times 10^6$  cfu/g).

Patel and Deepika Patel (2014) prepared the talc based powder of the bioagent (*Trichoderma viride* (FGCC#2437) (spore+mycelium) 1.0% w/w + talc 98.5% w/w+0.5% carboxyl methyl cellulose) and used for shelf life and bioefficacy studies. The talc based bioformulation was stored in LDPE pouches. The powder was dull white in color, pH 7.0, moisture 8% and cfu of  $29.7 \times 10^6$ . It was found that the bioformulation has good shelf life up to six months and then the spores started declining. Talc-based bioformulation was found to be the best material to retain maximum number of viable propagules i.e.,  $29.7 \times 10^6$  cfu/g at 180 days of storage.

Rajput *et al.* (2014) nine organic substrates viz. rice, sorghum, wheat, millet grains, wheat straw, rice husk, cow dung, saw dust and poultry manure were used for mass multiplication of *Trichoderma harzianum*. The highest population of *T. harzianum* was observed on sorghum grains ( $100.3 \times 10^8$  cfu g<sup>-1</sup>) followed by millet grains ( $75.23 \times 10^8$  cfu g<sup>-1</sup>). The poultry manure appeared to be the most unsuitable substrate and produced the lowest *T. harzianum* population ( $1.03 \times 10^8$  cfu g<sup>-1</sup>) followed by cow dung ( $2.07 \times 10^8$  cfu g<sup>-1</sup>) and saw dust ( $2.5 \times 10^8$  cfu g<sup>-1</sup>). Rice grains, wheat grains, wheat straw and rice husk performed moderately well and produced  $27.27 \times 10^8$ ,  $23.27 \times 10^8$ ,  $20.4 \times 10^8$  and  $17.35 \times 10^8$  cfu g<sup>-1</sup> of *T. harzianum*, respectively.

Singh *et al.* (2014) selected six organic substrates eg. spent mushroom compost, farmyard manure, vermicompost, sorghum grain, wheat grain and broken maize grain based *Trichoderma harzianum* T5 based formulation were selected. Of these sorghum grain substrate had maximum population of *T. harzianum* T5 ( $22.5 \times 10^7$  cfu/g) followed by broken maize grain and wheat grain while vermi-compost ( $12.7 \times 10^7$  cfu/g) substrate had minimum. *T. harzianum* T5 population showed an increasing trend in all the six substrates up to 30 DAI and thereafter, it exhibited decreasing trend up to 240 DAI. The population of *T. harzianum* T5 at 30 DAI was recorded highest again in sorghum grain ( $64.0 \times 10^7$  cfu/g) followed by SMC ( $53.0 \times 10^7$  cfu/g), wheat grain ( $51.0 \times 10^7$  cfu/g) and others.

Emerson and Mikunthan (2015) carried out the experiment to screen out suitable liquid wastes and other liquid media as suitable substrates for small scale production of *T. viride*. Black gram soaked water, coconut water, rice mill effluent from the red pericarp variety, 5% distillery spent wash and other liquid substrates such as 1% palmyrah jaggery solution, 5% palmyrah toddy and 1% palmyrah fruit pulp extract, 10% Cow urine, 10% *Gliricidia sepium* and 10% *Thespesia populnea* leaves extracts were individually investigated. Among these substrates higher growth and sporulation of *T. viride* was recorded in black gram soaked water ( $35.9 \times 10^7$  spores/ml) followed by 1% Jaggery solution ( $30.0 \times 10^7$  spores/ml), Coconut water ( $28.8 \times 10^7$  spores /ml) Rice Mill Effluent ( $28.7 \times 10^7$  spores/ml) and 1%

Palmyrah fruit pulp extract ( $27.1 \times 10^7$  spores/ml) after 14 days of incubation in dark room at 30°C. The study revealed that locally available liquid substrates are potential source for liquid fermentation of *T. viride*.

Rajput and Shahzad (2015) in this study they used nine different organic substrates viz, Rice grains, sorghum grains, millet grains, wheat grains, wheat straw, saw dust, rice husk, poultry manure and cow dung etc for mass multiplication of *T. polysporum*. cereal grains were found more appropriate for the mass production of antagonistic fungus *T. polysporum* as significantly high populations were recorded on cereal grains. However, the highest population *T. polysporum* was observed on sorghum grains ( $53.2 \times 10^8$  cfu g<sup>-1</sup>) followed by millet grains ( $47.433 \times 10^8$  cfu g<sup>-1</sup>). The poultry manure appeared to be the most unsuitable substrate and showed the lowest *T. polysporum* population ( $1.06 \times 10^8$  cfu g<sup>-1</sup>). Cow dung ( $1.76 \times 10^8$  cfu g<sup>-1</sup>) and saw dust ( $2.26 \times 10^8$  cfu g<sup>-1</sup>) were also not suitable. Rice grains, wheat grains, wheat straw and rice husk performed moderately and produced  $29.30 \times 10^8$ ,  $23.3 \times 10^8$ ,  $20.33 \times 10^8$  and  $12.60 \times 10^8$  cfu g<sup>-1</sup> of substrate, respectively.

#### **2.4 Antagonistic efficacy of *Trichoderma asperellum* isolates against different soil born fungi**

Al-Saeedi and Al-Ani (2014) were evaluated two isolates of *T. harzianum* (T1 and T2) against seven soil borne pathogenic fungi isolates (*Acremonium* sp., *Alternaria* sp., *Aspergillus* sp., *Penicillium* sp., *Pythium* sp., *Rhizoctonia* sp. and *Verticillium* sp.) in dual culture techniques *in vitro* condition. Isolate T2 showed highest inhibitory effect of mycelial growth of pathogens (45.99%). Maximum effect noticed in *Alternaria*-T2 interaction (51.18%), maximum growth inhibition noticed against *Rhizoctonia* sp. by T2 isolates (53.66%).

Meenakshi Kushwaha and Verma (2014) showed significant antagonistic activity of *Trichoderma* against isolated pathogenic fungi *Colletotrichum*, *Alternaria*, *Paracercospora* and *Fusarium* species. From this result it is found that the *Trichoderma* is a potential biocontrol agent against pathogenic fungi.

Srivastava *et al.* (2015) reported that, the effect of *Trichoderma* on soil borne pathogens is higher as compared to fungicides and it persists in soil for longer period after application. Data obtained from the studies have provided many clues for future studies. They clearly showed that, this fungus can be efficiently used as biocontrol agent. The genes present in this fungi have the ability to enhance host plant's resistance against many soil-borne pathogens.

Srivastava *et al.* (2016) reported that, *Trichoderma* sp. that are common saprophytic fungi found in soil was investigated and it has been emerged as a potential biocontrol agents because of their ability to reduce the incidences of disease caused by plant pathogenic fungi.

#### **2.4.1 Antagonistic efficacy of *Trichoderma asperellum* isolates against *Sclerotium rolfsii***

Rao and Kulkarni (2003) recorded maximum inhibition of mycelial growth of *S. rolfsii* by *T. harzianum* (60.7%) and was superior to other antagonists tested in dual culture technique. While minimum inhibition of 18.5 percent mycelial growth was noticed in *Penicillium* sp. All the antagonists, viz. *T. harzianum*, *T. viride*, *T. koningii* and *T. virens* inhibited the mycelial growth of *S. rolfsii*.

Srinivasulu *et al.* (2005) isolated and screened three antagonists, *Trichoderma viride* Pers. Fr., *T. harzianum* Rifai and *T. hamatum* (Bon.) Bain. *T. hamatum* inhibited the radial growth of *S. rolfsii* to an extent of 52.22 per cent followed by *T. viride* (44.11 %) and *T. harzianum* (38.87%). All the three *Trichoderma* sp. were very effective in producing volatile and non-volatile metabolites that were suppressive to *S. rolfsii*. Viability tests on sclerotia of *S. rolfsii* parasitized by *Trichoderma* sp. revealed complete colonization and replacement of sclerotial contents by 7<sup>th</sup> day of parasitisation in case of *T. hamatum* and by 10<sup>th</sup> day in case the of *T. viride* and *T. harzianum*.

Bagwan N.B. (2011) studied forty six isolates of *Trichoderma* sp. belonging to viz., *viride*, *harzianum*, *hamanatum*, *ressei* and *koningii* species groups were screened for their modes of biocontrol ability against

*Sclerotium rolfsii* Sacc., *Aspergillus niger* van Teighem and *A. flavus* Link ex Fries. The isolates T005, T043, T095, T49, T126, T144, T166, T191, 250, 390 and T425 gave maximum inhibition of mycelial growth of *S. rolfsii* in dual culture and killed the sclerotia. Isolates T144 and T390 were effective against *S. rolfsii* and *A. flavus*. The results of this study indicate that the two strains (T043 and T425) had the potential for biological control of *S. rolfsii*, *A. niger* and *A. flavus*.

Prajapati *et al.* (2015) selected five species of *Trichoderma* viz., *T. viride*, *T. harzianum*, *T. virens*, *T. asperellum* and *T. atroviride* and screened by dual culture technique. Among the *Trichoderma* sp. tested, *T. asperellum* showed maximum mycelial growth inhibition i.e. 61.48, 75.00 and 73.33 per cent at 4, 6 and 8 days of incubation, respectively. Whereas, *T. harzianum* gave significantly highest per cent inhibition in sclerotia production i.e. 98.44, 85.22 and 78.56 per cent at 4, 6 and 8 days of incubation, respectively. However, the LCF (Liquid culture filtrate) study of *T. harzianum* and *T. viride* after 4, 6 and 8 days of incubation revealed complete inhibition of mycelial growth and sclerotia production of *S. rolfsii*. In present *in vitro* study, *T. asperellum* found effective in inhibiting the mycelial growth as well as sclerotia production of *S. rolfsii* followed by *T. harzianum*.

Swathi *et al.* (2015) Observed *Trichoderma* isolates viz., *T. harzianum* Th4 with fast radial growth, simultaneous lysis and over growth on *Sclerotium rolfsii* and *T. virens* Tv5 with slow radial growth over *S. rolfsii* and subsequent lysis were evaluated for their antagonistic potential *in vitro*. Volatile metabolites of Tv5 isolate were more effective against *S. rolfsii* growth (54.6% inhibition) compared to Th4 (16.3% inhibition) while non volatile metabolites of Th4 were more effective against *S. rolfsii* with 100% growth inhibition at 60 and 80% concentration compared to 69.3% inhibition at 80% concentration of Tv5 culture filtrate.

#### **2.4.2 Antagonistic efficacy of *Trichoderma asperellum* against *Rhizoctonia bataticola***

Melo and Faull (2000) showed that, the fungus *Trichoderma* has been to be particularly effective in the control of the pathogen. Thus,

this research was carried out to screen fourteen *Trichoderma* strains against *R. solani* *in vitro*. All strains tested inhibited the growth of *R. solani*. Three *T. koningii* strains produced toxic metabolites with strong activity against *R. solani*, inhibiting the mycelial growth by 79%. *T. harzianum* Th-9 reduced the viability of sclerotia of *R. solani* by 81.8% and *T. koningii* TK-5 reduced by 53%. Electron microscopic observations revealed that, all *T. harzianum* strains interacted with *R. solani*. Th-9 grew toward and coiled around the host cells, penetrating and destroying the hyphae. Penetration of host cells was apparently accomplished by mechanical activity.

Seventeen isolates of *Trichoderma* were tested against *R. solani* *in vitro*, which is the major fungus responsible for damping off and root rot diseases. All strains including *T. harzianum*, *T. viride* and *T. aureoviride* were tested, inhibited the growth of *R. solani*. Light microscopic observation on dual culture assay showed that the hyphae of all *Trichoderma* isolates could grow parallel to the hyphae of *R. solani*. However, *Trichoderma* isolates coiling around the hyphae of *Rhizoctonia solani* and forming appresoria and hook-like structures observed by Shalini and Kotasthane (2000).

Shalini *et al.* (2006) the dual culture technique was used to test the antagonistic ability of *Trichoderma* against *Rhizoctonia solani*. The host fungus and *Trichoderma* were grown on potato dextrose agar (PDA) for a week at room temperature ( $28\pm 2^{\circ}\text{C}$ ). Small blocks of the target fungus (*R. solani*) cut from the periphery were transferred to the Petri dish. After two days of growth, *Trichoderma* was transferred aseptically in the same plate at about 3 cm distance and were incubated at room temperature with alternate light and darkness for seven days and observed periodically. The interaction between *R. solani* and *Trichoderma* sp. were observed.

Kumar *et al.* (2011) found that, among all different bioagents evaluated *in vitro* against *Rhizoctonia bataticola*, *Trichoderma harzianum* showed the highest mycelial growth inhibition (58.9%) followed by *T. viride* (54.45%).

Manjunatha *et al.* (2011) observed that the *Trichoderma viride* arrested highest mycelial growth (78.50%) of *Rhizoctonia bataticola* in chickpea.

Sangeetha (2011) found that bioagent *Trichoderma koningii* arrested the highest mycelial growth of *Rhizoctonia bataticola* (25.90%) followed by *T. viride* and *T. harzianum* with the inhibition of 23.25% and 23.15% respectively in soybean.

Khan *et al.* (2012) observed that, *Trichoderma viride* arrested the mycelial growth (81.96%) followed by *T. harzianum* against *Rhizoctonia bataticola* in chickpea.

Sara Mayo *et al.* (2015) reported isolates of *Trichoderma* i.e T003, T004, T006, T020, T022, T012, T013, T025, T016, T007, T024, T005, and T010 inhibited *R. solani* growth by more than 75%, with the highest inhibition produced by T003, T004, T006, T020, and T022 (86.70%). T019, T008, T002, T021, T001, T018, and T023 showed a remarkable inhibition (75–40%). Finally, T015, T014, T017, T011, and T009 inhibited *R. solani* growth by less than 40%, and T009 showed the lowest percentage (15.82%). T021 was the *Trichoderma* isolate showing the highest percentage of inhibition (72.77%) in the direct confrontation assays (Table 3, Figure 1B), whereas T009 showed the lowest inhibition values (14.63) The inhibited percentage detected for other *Trichoderma* isolates ranged from 47.13 to 30.55%.

#### **2.4.3 Antagonistic efficacy of *Trichoderma asperellum* isolates against *Fusarium solani***

Delfina Popiel *et al.* (2008) were used dual culture bio assays to study the impact of 92 isolates (belonging to 29 fungal species) against three toxigenic species, i.e. *Fusarium avenaceum* (Corda) Saccardo, *F. culmorum* (W. G. Smith) Saccardo and *F. graminearum* Schwabe. Both *F. culmorum* and *F. graminearum* isolates produce trichothecene mycotoxins and mycohormone zearalenone and are considered to be the most important cereal pathogens worldwide. Isolates of *Trichoderma* sp. were found to be the most effective ones to control the growth of examined

*Fusarium* sp. The response of *Fusarium* isolates to antagonistic activity of *Trichoderma* isolates varied and also the isolates of *Trichoderma* differed in their antagonistic activity against *Fusarium* isolates. The production of MON by two isolates of *F. avenaceum* in dual culture on rice was reduced by 95% to 100% by *T. atroviride* isolate AN 35.

Siameto *et al.* (2010) selected sixteen isolates of *T. harzianum* from Embu, Kenya and tested for antagonism against five soil borne phytopathogenic fungi (*Rhizoctonia solani*, *Pythium* sp, *Fusarium graminearum*, *F. oxysporum* f. sp *phaseoli* and *F. oxysporum* f. sp *lycopersici*) using dual culture assay and through production of nonvolatile inhibitors. *T. harzianum* isolates had considerable antagonistic effect on mycelial growth of the pathogens in dual cultures compared to the controls. All isolates of *T. harzianum* tested inhibited the growth of *R. solani* with isolate 029E giving the highest percent inhibition (61.55%) while 063E the lowest (25.88%). The inhibition process was parasitic, the parasite penetrated the host cell wall directly without formation of appressorium-like structures suggesting mechanical activity.

Poornima Sharma (2011) were screened several *Trichoderma* isolates for their biocontrol activity against *Fusarium oxysporum* f.sp. *pisi*. Eighteen of these isolates (T1 to T18) showed considerable biocontrol potential and were taken further in the study. Initial counter inhibition was observed in all the eighteen dual culture sets where *Trichoderma* and *Fusarium* both posed varying degree of inhibition on each other. In Most of the *Trichoderma* isolates showed considerably good antibiosis and parasitism. Therefore, the primary criterion for selection of the best potential biocontrol agent was poised on the time taken by *Trichoderma* to complete the sequence of events until parasitism of target pathogen. Among the eighteen isolates of *Trichoderma*, *T. atroviride* (Isolate T1) was found to show best activity followed by two isolates of *Trichoderma harzianum* (Isolate T8 and T11) in terms of time taken to parasitize *Fusarium*. The present study stresses on quick control on plant disease as a desired quality for popularization of biocontrol agents.

Choudhari and Mohanka (2012) nineteen isolates of *Trichoderma* were isolated and these were ascribed to three species namely: *Trichoderma harzianum* (Th), *T. viride* (Tv) and *T. koningii* (Tk). Efficacy of these bio-antagonists were investigated in *in vitro* conditions by employing dual culture technique and liquid culture filtrate assay. The outcome of *in vitro* dual culture testing revealed that among the different isolates of *Trichoderma* isolate-5 and 7 of Th, 2 and 18 of Tv and isolate 9 of Tk were found to be more efficient amongst all, as they showed better antagonism against the tested phytopathogen. The isolate Th-5 caused maximum inhibition (82.8%) followed by Th-7 (82.3%), Tv-2(79.2%) Tv-18 (74.4%) and Tk-9 (71.0%).

Dolatabadi *et al.* (2012) Studied dual culture on the antagonistic fungi against F1 isolate of FOL indicated that at 2, 4 and 6 days after incubation, *T. harzianum* caused maximum growth inhibition on mycelium FOL, then *T. viride* had a marked significant inhibitory effect on the growth of the pathogens (Figure 1-a). The growth inhibition of F2 isolate of FOL by antagonistic fungi after 2 and 4 days past incubation revealed that *T. viride* and *T. harzianum* resulted in maximum growth inhibition. *P. indica* and *S. vermifera* were ineffective in reducing radial growth of FOL after 2 and 4 days of incubation. However, *P. indica* and *S. vermifera* were effective in radial growth inhibition of FOL after 6 days of incubation. *Trichoderma* species differentially limited the colonial growth of the pathogen, overgrew the pathogen colony and produced yellow pigment. Microscopically, the hyphal interaction indicated that it was antagonistic.

Matarese *et al.* (2012) were performed antagonistic tests to assess the ability of the ten *Trichoderma* isolates to inhibit the growth of *F. graminearum* 124 and *F. culmorum* 627 mycotoxigenic strains. Growth rates of both pathogens in the presence and absence of each antagonist were evaluated, all resulting in highly significant values ( $R^2 \geq 0.86$ ) when submitted to regression analysis. Among all the *Trichoderma*/ pathogen combinations, three of the ten antagonistic strains (*T. gamsii* 6085, *T. gamsii* 6317 and *T. velutinum* 4837) significantly reduced *F. graminearum* or *F. culmorum* growth in at least one of the two substrates.

Anupama Sonawane *et al.* (2015) isolated fungal pathogen from wilt infected Pomegranate plant. In primary screening, antifungal activity against for fungal isolates F1, F5, F6 which were obtained from rhizospheric soil of pomegranate plant. In dual culture method, fungal isolates F1, F5, F6 and *Trichoderma* showed inhibition of radial mycelial growth of *Fusarium*. The percent inhibition of radial growth (PIRG) values for all isolates ranged from 92.40 to 100. The highest PIRG values were observed with isolates F1 and F6 as 100. Colony overgrowth by fungal isolates F1, F5, F6 and *Trichoderma* were varied from 3 to 9 days. As compared to *Trichoderma* sp, where PIRG was found to be 96.38, 92.40, 95.13, for isolate F6 and F1 PIRG was found to be 100. This study demonstrated that isolate F6 showed 100% inhibition of *Fusarium* in 8 days. The growth rate of fungal isolate F6 was faster than other isolates i.e. it showed spore formation within 2 days.

Belete *et al.* (2015) Reported that all isolates of *Trichoderma* spp. had strong biological control activity against *F. solani* *in vitro* as well as *in vivo* pot experiment. Under dual culture, the percentage of mycelial growth inhibition of *F. solani* by the *Trichoderma* ranged from 33.9 to 67.0%. The highest (67.0%) inhibition was obtained from isolate TS036, while the lowest (33.9%) by isolate TS015. Pathogen-inoculated faba bean plants grown in pots that were treated with antagonists had taller plant heights and biomass than the *Trichoderma* untreated control inoculated with *F. solani*. The *Trichoderma* isolates significantly reduced black root rot severity on faba bean seedlings with disease reduction ranging from 64.4 to 74.6% over control. Use of *Trichoderma* species can be a potential source of biological control agents for the management of black root rot in faba beans grown in the region. Hence, the potential *Trichoderma* isolates under field condition might used as a components in the integrated management of *F. solani* that caused faba bean black root rot in the highlands of northeastern Ethiopia.

## CHAPTER III

### MATERIAL AND METHODS

The present investigation entitled “Assessment of different formulations of *Trichoderma* for their shelf life” was carried out in the laboratory at Department of Plant Pathology, Post Graduate Institute, Dr. Panjabrao Deshmukh Krishi vidyapeeth, Akola during the year 2017-2018.

#### 3.1 Material

##### 3.1.1 Glassware and plastics

Whenever required, the glasswares of Borosil and Schott make viz. petri plates, test tubes, conical flasks of different capacities (250, 500, 1000 and 2000 ml), beaker, glass pipette and measuring cylinder were used during the course of investigation. All the glasswares, polythene bags, carrier materials, equipments and chemicals were procured from the department of Plant Pathology, PGI, Dr. PDKV, Akola.

##### 3.1.2 Equipments

The laboratory equipments viz. autoclave (Equitron, India), BOD incubator (Sanco, India), laminar airflow work station (Klenzaid, India), refrigerator (L.G, India), Hot air oven (Bio-techniques, India), digital weighing balance (Anamed, India), Research microscope (Olympus, India), Bunsen burner, digital camera (Kotak, India), double distillation unit (JSGW, India) were used in present investigation.

##### 3.1.3 Chemicals

Analytical grade chemicals viz. sodium alginate, gelatin, hard gelatin coated capsule and others such as streptomycin sulphate and other chemicals procured from the department of Plant Pathology, PGI, Dr. PDKV, Akola.

##### 3.1.4 Carrier materials

Sorghum grains, talc powder, charcoal powder, lignite, fly ash, standard packaging material 75  $\mu$  HDPE were used.

### 3.1.5 Miscellaneous material

Non-absorbent cotton, polyethylene bags, cork borer (5 mm), micropipette, dissection needle, forceps, permanent marker, cello tapes, test tube stand, tray, wash bottle, potato, rubber band, scissors, empty capsules were used during the investigation.

## 3.2 Soil sampling

### 3.2.1 Selection of sites for sampling and soil sample collection

Soil samples were collected from different districts of Maharashtra. The approachable locations of different districts were selected and visited for soil sampling (Table No.1). Generally healthy plants were selected from standing crop of that location and rhizosphere soil was collected. For rhizosphere soil, plant was gently and carefully uprooted, soil tightly adhering the root was collected, such four samples were collected randomly from the crop field, mixed and 1/4th part was used as composite rhizosphere soil sample of the region.

**Table 1: Soil samples collected from different districts of Maharashtra**

Sr. No.	Location	Crop associated
1	Akola	soybean
2	Rahuri	Maize
3	Beed	sorghum
4	Ratnagiri	cowpea

## 3.3 Isolation of *Trichoderma* sp. from rhizosphere soil

### 3.3.1 Preparation of serial dilution

The *Trichoderma asperellum* was isolated from the soil collected from the different locations by serial dilution technique. The *Trichoderma* selective medium was used for the isolation of *Trichoderma asperellum*. One ml of soil suspension from dilutions ( $10^{-3}$  and  $10^{-4}$ ) was aseptically added to sterile petri plates containing twenty ml of *Trichoderma* selective medium and incubated 27°C for 3 days. After incubation, well

separated individual colonies with yellow green and whitish green pigments were marked. The individual colonies were picked up with sterile loop and transferred to Potato Dextrose agar media plates and the pure cultures so obtained were stored in a refrigerator for further use. (Arumugam K. *et al.* 2013).

### 3.3.2 Isolation of *Trichoderma asperellum*

*Trichoderma* selective medium (TSM) (Elad *et al.*, 1981: Mukherjee, 1991) was used for isolation of *Trichoderma asperellum*. The composition of TSM is given below.

### 3.3.3 Composition of *Trichoderma* Selective Medium (TSM)

Chemicals	Quantity
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.2 g
K <sub>2</sub> HPO <sub>4</sub>	0.9 g
KCl	0.15 g
NH <sub>4</sub> NO <sub>3</sub>	1.0 g
Glucose	3.0 g
Chloramphenicol	0.20 g
Apron 35SD	0.015 g
Captan	0.2 g
Rose Bengal	0.15 g
Agar-agar	20.0 g
Distilled water	To make volume 1 litre.

One ml soil suspension was taken with help of 5 ml pipette and poured in the Petri plate seeded with TSM. The entire process was done in an inoculation chamber under aseptic conditions. The plates were incubated at 27±2°C for 5 days. Observation on the appearance of the colonies was recorded from 3rd to 5th day. Individual colonies were picked up and maintained in pure culture for further study.

**Table 2: Location and code of *Trichoderma asperellum* isolates**

Sr. No.	Location	Crop associated	Code name
1	Akola	Soybean	Ta1
2	Rahuri	Maize	Ta2
3	Beed	Sorghum	Ta3
4	Ratnagiri	Cowpea	Ta4

### **3.3.4 Purification of *Trichoderma asperellum* cultures**

*Trichoderma asperellum* isolates were purified by single spore culture. The spores of the isolates were inoculated into a Petri dish seeded with PDA medium. Sub-culturing was done from the growing front of the single new colony. Small amount of spores was taken on the tip of a sterilized inoculating needle and placed on PDA poured petri dishes. This process was repeated by taking inoculum from edge of colonies growing in the freshly streaked petri plate and again streaking it on PDA plates. Colony arising from single spore was picked up and inoculated on a fresh plate. This culture was used for further studies after identification.

### **3.3.5 Identification of *T. asperellum***

The culture of *Trichoderma asperellum* is identified on the basis of their morphological characteristics and microscopic examination at department of Plant Pathology, PGI, Dr. PDKV, Akola in accordance to Bissett, 1984 and 1991.

### **3.4 Methods**

#### **3.4.1 Sterilization of glassware, media, water, blotter paper etc.**

Glasswares were cleaned with high quality liquid soap and rinsed with tap water. All these glasswares then placed in the chromic acid solution, cooled at room temperature and added with constant stirring to concretion  $H_2SO_4$  for overnight and rinsed with tap water followed by distilled water. then dried and sterilized in hot air oven at  $180^{\circ}C$  for one hour. Whereas the media, distilled water and blotting papers were sterilized in autoclave at 15 psi for 15 min. The sugars and other thermo labile compounds were sterilized by bacterial proof filters. The material viz. inoculating needle, forceps, scalpel were disinfected by dipping in 90% alcohol, sterilized over flame and then cooled at room temperature before use.

#### **3.4.2 Precautions to eliminate contamination**

All the isolation and inoculation work of microbial cultures was carried out under aseptic condition in laminar airflow. The working area of laminar airflow was sterilized by glowing ultraviolet light for half an hour prior to commencement of work. The working surface and side glasses of laminar airflow were sterilized with denatured spirit.

### **3.5 General procedure followed**

#### **3.5.1 Design**

Complete Randomized Block design (CRD) was used with three replications for this experiment.

#### **3.5.2 Medium**

*Trichoderma* selective media (TSM), Potato dextrose agar (PDA), potato dextrose broth (PDB) and sorghum grain media were used during laboratory studies of fungus.

#### **3.5.3 Preparation of PDA**

Washed 200 g potato, peeled off the skin and sliced them into pieces. Boil 200 g sliced peeled potatoes in 800 ml distilled water for 30 minutes in open vessel or pan. After boiling, collected the potato extract by

filter through muslin cloth or net filter. Then mix with dextrose (20 g), agar (20 g) and boil to dissolve and volume made up one litre. Autoclaved the media for 15 min. at 121.6°C. Final P<sup>H</sup> of the medium was 5.6 ± 0.2. streptomycine sulphate (30 mg) was supplemented in molten PDA medium in order to check the bacterial contamination prior to pouring in petridishes. A similar growth medium, potato dextrose broth (PDB) was formulated identical to PDA, omitting the agar.

#### **The composition of potato dextrose agar medium**

Sr. No.	Ingredient	Quantity
1	Peeled and sliced potato	200 g
2	Dextrose (C <sub>6</sub> H <sub>12</sub> O <sub>6</sub> )	20 g
3	Agar-agar	20 g
4	Distilled Water	1000 ml

#### **3.5.4 Preparation of mycelium mat**

To get enough mycelium, pure culture of *Trichoderma* isolates were mass multiplied by transferring mycelium tissue into 150 ml of PDB and kept for incubation in BOD incubator at 27 ± 2°C for seven days without disturbing the flasks. The mycelial mat from broth was separated and used for the preparation of tablets formulations.

#### **3.5.5 Preparation of sorghum grain medium**

Sorghum grain was used for solid state fermentation. 250 gram sorghum grains moistened and poured in 500 ml capacity conical flasks and sterilized in autoclave at 15 psi and 121.6°C for 15 minutes. Sterilized sorghum grains medium was used for mass multiplication of *T. asperellum* as solid state fermentation.

#### **3.5.6 Pouring of medium in petriplate**

Laminar air flow work station sterilized by UV light for 30 minutes followed by surface sterilization with 70% alcohol before starting work. Water bath at 100°C used to store conical flasks containing molten

PDA medium. Transferred conical flasks of PDA, sterilized Petri-dishes, inoculating needle and other materials on platform of laminar air flow work station. Approximately 15-20 ml molten PDA medium poured in 90 mm diameter petri-dishes in aseptic condition, gently rotated the dishes that the medium covers the base of the dish evenly. It is ensured that the base of the plate covered by medium not touching to lid of the plate and care should be taken that surface was smooth with no bubbles.

### **3.5.7 Sub culturing of *Trichoderma* sp.**

Sub culturing was done on PDA medium in petri plates and incubated at  $27 \pm 2^{\circ}\text{C}$  for seven days with periodic observation for the development of colonies of *T. asperellum*. The green colour colonies were identified by key based on branching conidiophores, shape of phialides, emergence of phialides and spore characters (Gams and Bisset, 1998). Periodically sub culturing was done by using PDA slants. Loopful growth of *T. asperellum* transferred in sterilized PDA slants and incubated for five days at  $27 \pm 2^{\circ}\text{C}$  in BOD incubator. After incubation the slants were stored in refrigerator at  $7-9^{\circ}\text{C}$  for further study.

### **3.5.8 Mass multiplication of *Trichoderma* sp.**

The 500 ml capacity conical flasks containing 150 ml sterilized potato dextrose broth were inoculated with 5 mm mycelial disc of 3 days old *T. asperellum*. A pinch of streptomycin was added in broth prior to pouring to check the bacterial contamination. The flasks plugged with cotton and incubated at  $27 \pm 2^{\circ}\text{C}$  for 7 days. Thick mycelial mat of *T. asperellum* was observed. The mycelial mat of *T. asperellum* was collected for further studies.

## **3.6 Preparation of formulations of *Trichoderma* sp.**

### **3.6.1 Solid state fermentation**

The isolated species of *Trichoderma asperellum* from Akola location was used. The isolate of *T. asperellum* incubated at  $27 \pm 2^{\circ}\text{C}$  on potato dextrose agar plates and shown to be sufficient for mycelial growth and sporulation.

Sorghum seeds were overnight soaked in water and next day drained out water. Pour 200 g soaked sorghum seeds in each 500 ml capacity conical flasks and sterilized by autoclaving it at 121.6°C for 15 min. (at 15 lbs/in<sup>2</sup>). After cooling the flasks, five mm disc of five days old culture of *T. asperellum* inoculated on sterilized sorghum grains medium and incubated for 12 to 15 days. The dark green spore coating were produced on sorghum grains by *Trichoderma*. These grains covered by fungal mycelium growth and spores. This pure culture of *Trichoderma* on sorghum grains were grind with the help of grinder and made a fine powder form and then packed in HDPE bags with completely sealed by electric sealer. These bags were stored at room temperature for further studies.

### **3.6.2 Liquid state fermentation**

#### **3.6.2.1 Tablet form**

*Trichoderma* grown in broth medium was used for preparation of tablet form *Trichoderma* culture. Four different carriers viz. talc powder, charcoal powder, lignite and fly ash were used in addition with 1% CMC as sticking agent. The liquid *Trichoderma* culture blended together with different carriers @ 1:2 ratio. The mixture was dried under shade and prepared 1000 mg round/ spherical tablet. These tablets stored in room temperature for further study.

#### **3.6.2.2 Capsule form**

In present study hard gelatin coated capsules of size 20 mm length, 08 mm diameter and radius 04 mm were used. The volume of cylindrical capsule was 1.0048 cm<sup>3</sup>. It was made up of two shells, the capsule body and shorter cap supplied by M/s. Kumar Rushabh medical, Gandhi road, Akola. The cap fits highly over open end of the capsule body, these hard gelatin capsules were made from mixture of gelatin, sugar and water. The capsule body color was purple with black cap and another was with yellow body and brown colored cap having the body shell with the cylindrical shape.

*Trichoderma* grown on PDA plates was used for filling capsule. Seven days old culture showing mycelial mat and abundant

sporulation mixed with different carriers viz. talc, gelatin, alginate, flyash and charcoal separately as well as in combination of carrier @1:1 ratio and were dried in oven for 48 hrs at 40°C. simultaneously pure frozen mycelial mat along with spores was transferred in test tube and crushed with sterilized glass rod in aseptic condition and made powder form. All these dry solid *Trichoderma* culture was filled in each @ 1000 mg in empty capsule. Accordingly capsule was made and stored at room temperature for further study.

### **3.7 Delivery system and application technology**

Tablet and capsule form of *Trichoderma* culture were used to test to improve application technology. Six capsule form viz. talc, gelatin, alginate, alginate + charcoal (1:1), gelatin + fly ash (1:1) and pure *T. asperellum* frozen mycelia with spores capsule and four tablet form viz. talc, lignite, charcoal, fly ash were used for present study.

Sterilized 1000 ml capacity conical flask contained 500 ml distilled water enriched with 10 g jaggery used for this study. After cooling each conical flask inoculated with one tablet/capsule separately and incubated at room temperature, 30°C for 12 h. The initial population of *T. asperellum* from each formulation was assessed by serial dilution technique. After 12 h of incubation period, the culture was serially diluted to obtain  $10^{-7}$  concentration and 1 ml from  $10^{-7}$  dilution factor was poured in sterilized petri-dish. Thereafter, molten PDA medium was poured @ 20 ml/plate rotated horizontally for uniform distribution of inoculum and incubated at  $27 \pm 2^\circ\text{C}$  for 48 hours and observation was taken for the development of colonies of *T. asperellum*. Three replications were maintained for each treatment. Colony forming units (CFU) of *T. asperellum* was calculated by the formula derived by Schmidt & Caldwell, 1967.

### **3.8 Viability of *T. asperellum* in different formulations under *in vitro* condition**

Eleven carrier based formulations of *Trichoderma asperellum* were evaluated for viability from date of manufacturing upto 270 days and observed at 30 days interval by adopting the following method.

One tablet/capsule was drawn from each formulation and transferred in 9 ml sterilized water in test tube and shaken thoroughly for two minutes to make  $10^{-1}$  dilution factor. One ml suspension of stock solution was transferred in next test tube containing 9 ml distilled water by using sterilized pipette and shaken to make  $10^{-2}$  dilution and seven test tube to make up  $10^{-7}$  dilution. One ml of suspension was taken from the dilution of  $10^{-7}$  and transferred in petri plates containing 20 ml sterilized PDA and gently shaken to spread evenly. These petri plates were incubated at  $27 \pm 2^{\circ}\text{C}$  for 72 h and periodic observations were taken for the development of colonies of *T. asperellum*.

Observations for colony forming units (CFU) were taken by using formula. (Schmidt, and Caldwell, 1967):

$$\text{CFU per gram} = \frac{\text{CFU per plate} \times \text{dilution factor}}{\text{Weight of substrate (g)} \times \text{amount plated (ml)}}$$

### **3.9 Collection of pure cultures of soil inhibiting plant pathogens**

Pure cultures of soil born plant pathogen viz., *Fusarium solani*, *Sclerotium rolfii* and *Rhizoctonia bataticola*, were collected from Department of Plant Pathology, PGI, Dr. PDKV, Akola, which previously known pathogenic nature.

#### **Details of dual culture method-**

The Dual culture Technique was conducted in the laboratory with following details.

Design – Completely Randomized Design (CRD)  
 Treatment - 5  
 Replication - 3

**Treatment detail:**

**Table 3.9.1 Antagonistic efficacy of *Trichoderma asperellum* isolates against *Sclerotium rolfsii* in dual culture technique**

<b>Treatment No.</b>	<b>Code name</b>	<b>Description</b>
T <sub>1</sub>	Ta1	<i>Trichoderma asperellum</i> + <i>sclerotium rolfsii</i>
T <sub>2</sub>	Ta2	<i>Trichoderma asperellum</i> + <i>sclerotium rolfsii</i>
T <sub>3</sub>	Ta3	<i>Trichoderma asperellum</i> + <i>sclerotium rolfsii</i>
T <sub>4</sub>	Ta4	<i>Trichoderma asperellum</i> + <i>sclerotium rolfsii</i>
T <sub>5</sub>	control	<i>Sclerotium rolfsii</i>

**Table 3.9.2 Antagonistic efficacy of *Trichoderma asperellum* isolates against *Rhizoctonia bataticola* in dual culture technique**

<b>Treatment No</b>	<b>Code name</b>	<b>Description</b>
T <sub>1</sub>	Ta1	<i>Trichoderma asperellum</i> + <i>R. bataticola</i>
T <sub>2</sub>	Ta2	<i>Trichoderma asperellum</i> + <i>R. bataticola</i>
T <sub>3</sub>	Ta3	<i>Trichoderma asperellum</i> + <i>R. bataticola</i>
T <sub>4</sub>	Ta4	<i>Trichoderma asperellum</i> + <i>R. bataticola</i>
T <sub>5</sub>	control	<i>Rhizoctonia bataticola</i>

**Table 3.9.3 Antagonistic efficacy of *Trichoderma asperellum* isolates against *Fusarium solani* in dual culture technique**

<b>Treatment No</b>	<b>Code name</b>	<b>Description</b>
T <sub>1</sub>	Ta1	<i>Trichoderma asperellum</i> (TA1) + <i>F. solani</i>
T <sub>2</sub>	Ta2	<i>Trichoderma asperellum</i> (TA2) + <i>F. solani</i>
T <sub>3</sub>	Ta3	<i>Trichoderma asperellum</i> (TA3) + <i>F. solani</i>
T <sub>4</sub>	Ta4	<i>Trichoderma asperellum</i> (TA4) + <i>F. solani</i>
T <sub>5</sub>	control	<i>Fusarium solani</i>

### 3.9.1 Dual culture Technique

Antagonistic activity of *Trichoderma* isolats were assayed against *Rhizoctonia bataticola*, *Sclerotium rolfsii* and *Fusarium oxysporum* f. sp *solani* by using dual culture inoculation technique described by Vincent, (1927), Mandal *et al.*, (1999) in Petri plates. Five mm disc from the periphery of actively growing pathogen on PDA was placed in centre of 90 mm diameter petri plates containing PDA. Five mm disc of three days and actively growing isolates of *Trichoderma asperellum* were placed at equidistance on all four sides 30 mm apart from centre disc of pathogenic fungus. The plates were incubated at ambient condition under alternate dark and light cycle up to 7 days. Simultaneously the pathogenic fungus disc (5 mm) was incubated on PDA Petri plates alone and incubated under similar condition for same period. Plates were observed every day for the behaviour at the point of intermating of two cultures under stereoscopic microscope. On seventh days after incubation, the growth of pathogenic test fungus was measured and per cent growth inhibition was calculated using the following formula.

$$\text{Per cent Growth inhibition} = \frac{C - T}{C} \times 100$$

Where,

C = Mycelial growth (mm) in control plate.

T = Mycelial growth (mm) in treatment plate.

## CHAPTER IV

### RESULTS AND DISCUSSION

The present investigation on “Assessment of different formulations of *Trichoderma* for their shelf life” was conducted at Department of Plant Pathology, PGI, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola during 2017-2018. Data generated during the period of investigation is presented in this chapter.

#### 4.1 Collection of soil samples

The soil samples were collected from different locations of Maharashtra viz, Akola, Rahuri, Beed, Ratnagiri for the isolation of *Trichoderma asperellum* on *Trichoderma* Selective Media (TSM).

**Table 3: Soil samples collected from different Locations of Maharashtra.**

Sr. No.	Locations for sample collection	Crop associated
1	Akola	soybean
2	Rahuri	maize
3	Beed	sorghum
4	Ratnagiri	cowpea

#### 4.2 Isolation of *Trichoderma asperellum* from soil samples of different Districts

Total four isolates of *Trichoderma asperellum* were isolated from different districts of Maharashtra. Isolation was done by employing serial dilution technique (Arumugam K. *et al.*, 2013). The isolates were purified by single spore culture. Whitish green to green pigmented fungal colonies identical to *Trichoderma* were maintained on PDA and used for present investigation.

Rahman *et al.*, (2011) collected the native population of *Trichoderma* sp. from different habitats was quantified and characterized.

### 4.3 Confirmation of *Trichoderma asperellum* isolates

*T. asperellum* isolates identification was confirmed from its growth characteristics and morphological characters. Microscopic examination was carried out (Table no 4).

The colonies become whitish turn green to dark green with maturity. The older colonies emit coconut odour. The mycelium was hyaline, septate and branched. Conidiophores were branched and arise in a compact form on the aerial hyphae, rarely irregularly dispersed on the surface of the colony. The main conidiophores produce smaller side branches called phialides. Phialides were sigmoid, formed in a false whorl. Conidia were green, globose to obvoid (Chandra sekhar *et al.* 2017).

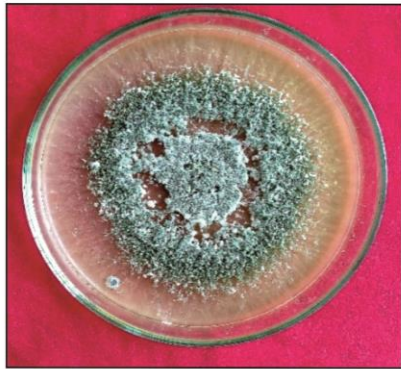
**Table 4: Details of *T. asperellum* isolates**

<b>Sr. No.</b>	<b>Locations for sample collection</b>	<b>Crop associated</b>	<b>Abbreviation</b>
<b>1</b>	<b>Akola</b>	<b>soybean</b>	<b>Ta1</b>
<b>2</b>	<b>Rahuri</b>	<b>maize</b>	<b>Ta2</b>
<b>3</b>	<b>Beed</b>	<b>sorghum</b>	<b>Ta3</b>
<b>4</b>	<b>Ratnagiri</b>	<b>cowpea</b>	<b>Ta4</b>

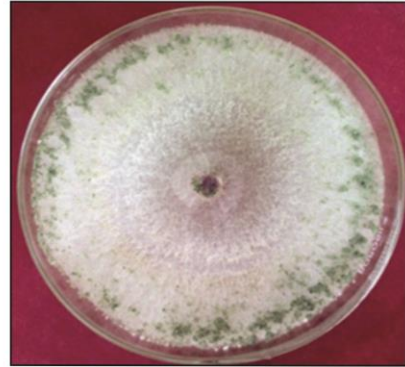
### 4.4 Evaluation of different *Trichoderma asperellum* isolates on colony character and sporulation

Four different isolates of *Trichoderma asperellum* were tested for their mycelial growth, colony characters and sporulation. Colony characters in terms of radial growth in mm, growth type, color and sporulation was recorded and found that isolate Ta2 was superior (90 mm) than other isolates at 7 DAI followed by Ta4, Ta1, however minimum growth was recorded in isolate Ta3 (87.50 mm).

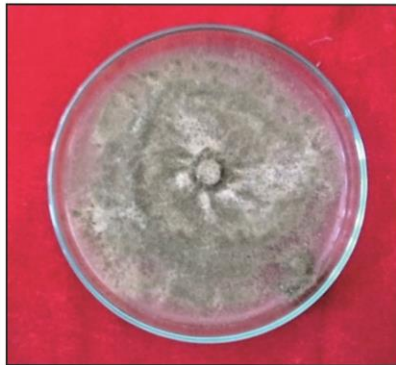
Among the four isolates of *T. asperellum*, the isolate no. Ta1, Ta3, Ta4 showed sub aerial and disperse growth type, however disperse and spherical growth type was observed in Ta2. It is also clear from the



Akola (Ta1)



Rahuri (Ta2)



Beed (Ta3)



Ratnagiri (Ta4)

Plate 1 : Pure culture of *Trichoderma asperellum* isolates.

data presented in table no. 4 that milky white and dark green color colonies were observed in isolate Ta1, Ta2 and Ta3, however milky white to grayish green color colony was recorded in Ta4. Excellent sporulation was recorded in isolate Ta1, Ta2 and Ta4 and medium sporulation was observed in isolate Ta3. Same type of result was also reported by Chandra sekhar *et al.* (2017) isolated ten isolates of *Trichoderma* sp. and considerable variations were observed regarding radial growth, growth rate and also revealed that colony showed dark green to dark bluish green sporulation, colony reverse was amber or uncolored.

All four *T. asperellum* cultures were maintained on PDA slants and used for further studies. Details of four isolates of *T. asperellum* is as follows.

**Table 5: Evaluation of different *T. asperellum* isolates on colony character and sporulation**

Sr. No.	Isolates	Radial growth (mm) at 7 DAI	Colony characters		Sporulation
			Colony growth type	Colony colour	
1.	Ta1	87.90	Sub aerial and disperse	Milky white to dark green	+++
2.	Ta2	90.00	Disperse and superficial	Milky white to dark green	+++
3.	Ta3	87.50	Sub aerial and disperse	Milky white to dark green	++
4.	Ta4	89.33	Sub aerial and disperse	Milky white to grayish green	+++

- +++ - Excellent sporulation
- ++ - Medium sporulation
- + - Fair sporulation
- - No sporulation

#### **4.5 Mass multiplication of *T. asperellum***

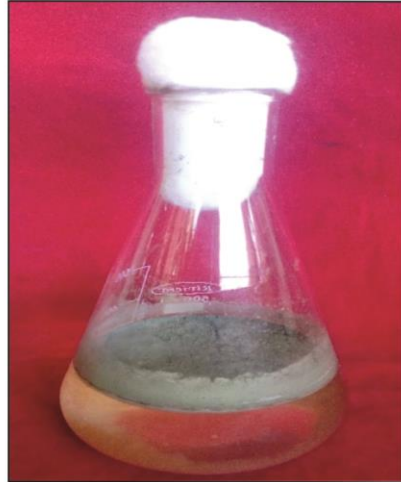
For mass multiplication of the culture, liquid state fermentation using potato dextrose broth and for solid state fermentation moistened sterilized sorghum grains was followed. Various formulations for testing viability were prepared using the mass culture produced as and where necessary showed in plate no. 2 to 7. Whereas, in *Trichoderma* treatment T<sub>5</sub> to T<sub>10</sub> culture discs from PDA plates was used for capsule formulations. All these formulations were tested for cfu up to 270 days of storage. The details are furnished in Table 6.

#### **4.6 Evaluation of viability of *Trichoderma asperellum* in different formulations**

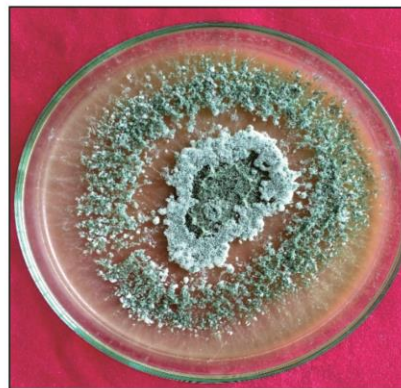
Eleven formulations and delivery systems were evaluated in the present study. Shelf life of all eleven formulations was studied up to 270 days and the data is presented in Table 6. The study revealed that, more than desired minimum count i.e.  $1 \times 10^7$  was noticed in all the formulations. Reduction in cfu was noticed in all the formulations during 270 days storage at ambient conditions.

Survival rate estimated as cfu shall be enough even after storage of nine months for marketing and application at farm level with easy in distribution.

It is evident from the data that among the eleven formulations of *T. asperellum*, the maximum cfu/g ( $42.00 \times 10^7$ , plate no.17) was obtained in *Trichoderma* treatment T<sub>10</sub> i.e. frozen culture mass along with medium disc capsule even after 270 days of storage followed by T<sub>11</sub>, sorghum grains ( $30.67 \times 10^7$  cfu/g, plate no.18 ), T<sub>6</sub>, *T. asperellum* alginate capsule ( $29.67 \times 10^7$  cfu/g, plate no.13), T<sub>5</sub>, *T. asperellum* gelatin capsule ( $28.67 \times 10^7$  cfu/g, plate no.12), T<sub>8</sub>, *T. asperellum* gelatin + flyash capsule ( $21.00 \times 10^7$  cfu/g, plate no.15), T<sub>7</sub>, *T. asperellum* talc capsule ( $20.00 \times 10^7$  cfu/g, plate no.14), T<sub>9</sub>, *T. asperellum* alginate + charcoal capsule ( $19.33 \times 10^7$  cfu/g, plate no.16), T<sub>4</sub>, *T. asperellum* flyash tablets ( $18.00 \times 10^7$  cfu/g, plate no.11), T<sub>2</sub>, *T. asperellum* charcoal tablet ( $17.67 \times 10^7$  cfu/g, plate no.9), T<sub>1</sub>, *T. asperellum* talc tablet ( $12.33 \times 10^7$  cfu/g, plate no.8) and T<sub>3</sub> *T.*



*T. asperellum* broth culture



Pure *T. asperellum* culture

Plate 2: Pure culture



**Talc Powder**



**Charcoal Powder**



**Lignite Powder**



**Fly ash Powder**

**Plate 3: Carrier materials**



*T. asperellum* talc Tablets



*T. asperellum* charcoal Tablets



*T. asperellum* lignite Tablets



*T. asperellum* flyash Tablets

Plate 4 :Tablets Formulation



*T. asperellum* gelatin capsules



*T. asperellum* alginate capsules



*T. asperellum* talc capsules



*T. asperellum* gelatin + flyash capsules



*T. asperellum* alginate + charcoal capsules



Pure *T. asperellum* capsules

Plate 5 : Capsules Formulation



Plate 6 : Sorghum grains covered with *T. asperellum*



Plate 7 : Sorghum grains filled in milky white bags

*asperellum* lignite tablet ( $11.33 \times 10^7$  cfu/g, plate no.10) after 270 days of storage.

It is apparent that charcoal, talc and lignite as carrier either in gelatin capsule or tablet form are also supporting the cfu upto 270 days of storage however as compared with other reduced cfu was recorded in these formulations. The least cfu was recorded in T<sub>3</sub> *T. asperellum* lignite tablet ( $11.33 \times 10^7$  cfu/g, plate no.10) and T<sub>1</sub>, *T. asperellum* talc tablet ( $12.33 \times 10^7$  cfu/g, plate no.8) after 270 days of storage followed by T<sub>2</sub>, *T. asperellum* charcoal tablet ( $17.67 \times 10^7$  cfu/g, plate no.9), T<sub>4</sub>, *T. asperellum* flyash tablets ( $18.00 \times 10^7$  cfu/g, plate no.11).

Viability were observed in all formulations till 270 days of storage and maintained cfu more than the standard. The minimum cfu i.e ( $11.33 \times 10^7$  cfu/g, plate no.10) was observed in T<sub>3</sub>, *T. asperellum* lignite tablet followed by T<sub>1</sub>, *T. asperellum* talc tablet ( $12.33 \times 10^7$  cfu/g, plate no.8), T<sub>2</sub>, *T. asperellum* charcoal tablet ( $17.67 \times 10^7$  cfu/g, plate no.9) and T<sub>4</sub>, *T. asperellum* flyash tablets ( $18.00 \times 10^7$  cfu/g, plate no.11) in tablet formulation. The minimum cfu ( $19.33 \times 10^7$  cfu/g, plate no.16) was recorded T<sub>9</sub> capsule formulation as compared to other capsule formulations i.e T<sub>7</sub>, T<sub>8</sub>, T<sub>5</sub>, T<sub>6</sub>, however maximum cfu was found in T<sub>10</sub> ( $42.00 \times 10^7$ , plate no.17) as compared with different formulations. Over all more viability were noticed in all capsule formulations when compared with tablet and solid state fermentation (sorghum grain formulation). This may be due to hard coated gelatin capsule which is a mixture of peptides and protein produced by partial hydrolysis of collagen extracted from the skin, bones and connective tissues of animals, chicken and fish (Ward, 1977 and Stegemamn, 2002).

Whereas agar consists of mixture of two polysaccharides; agarose upto 70% and rest with agaropectin. Agarose is a linear polymer, made up of repeating units of agarobiose, a disaccharides made up of D-galactose and 3,6- anhydro-L-galatopyranose (Rafael and Fernando, 1987).

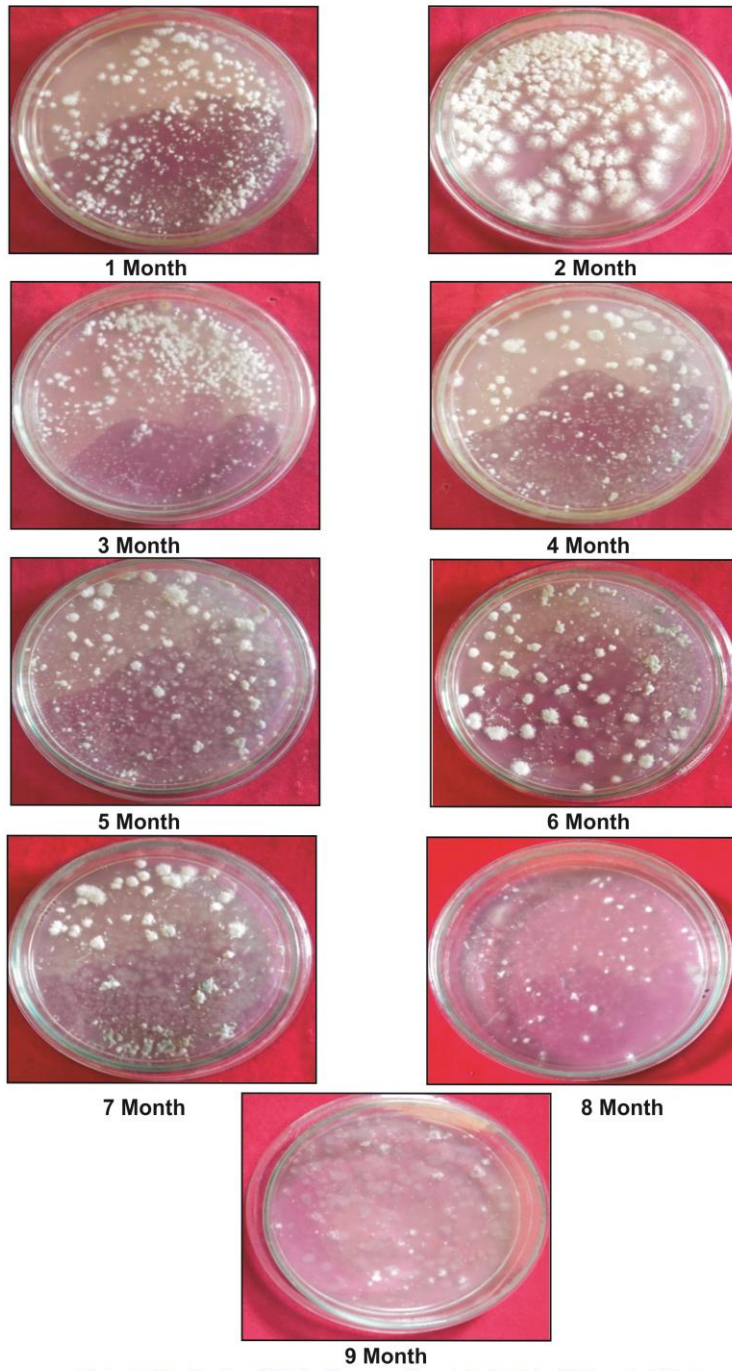


Plate 8 :Monthwise CFU in *T. asperellum* talc Tablets (T<sub>1</sub>) upto 270 days.

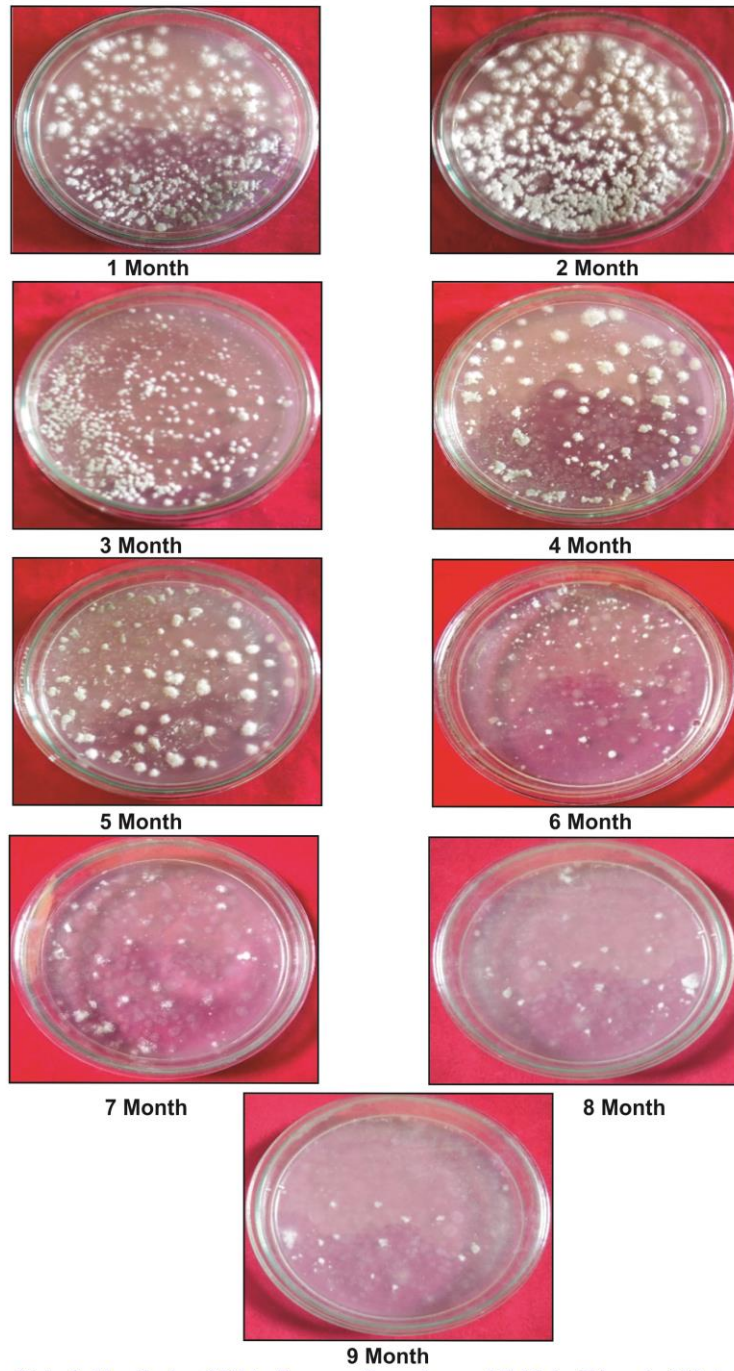


Plate 9 :Monthwise CFU in *T. asperellum* charcoal Tablets (T<sub>2</sub>) upto 270 days.

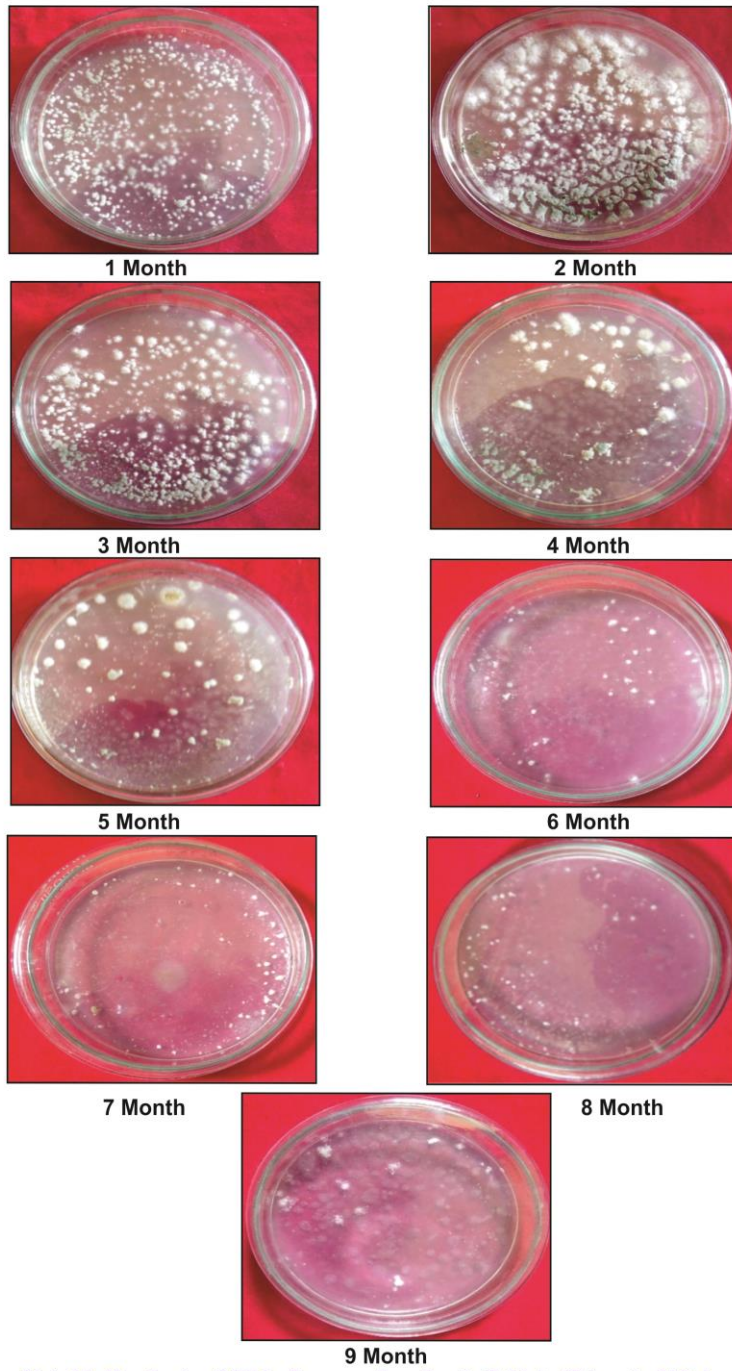


Plate10 :Monthwise CFU in *T. asperellum* lignite Tablets (T<sub>3</sub>) upto 270 days.

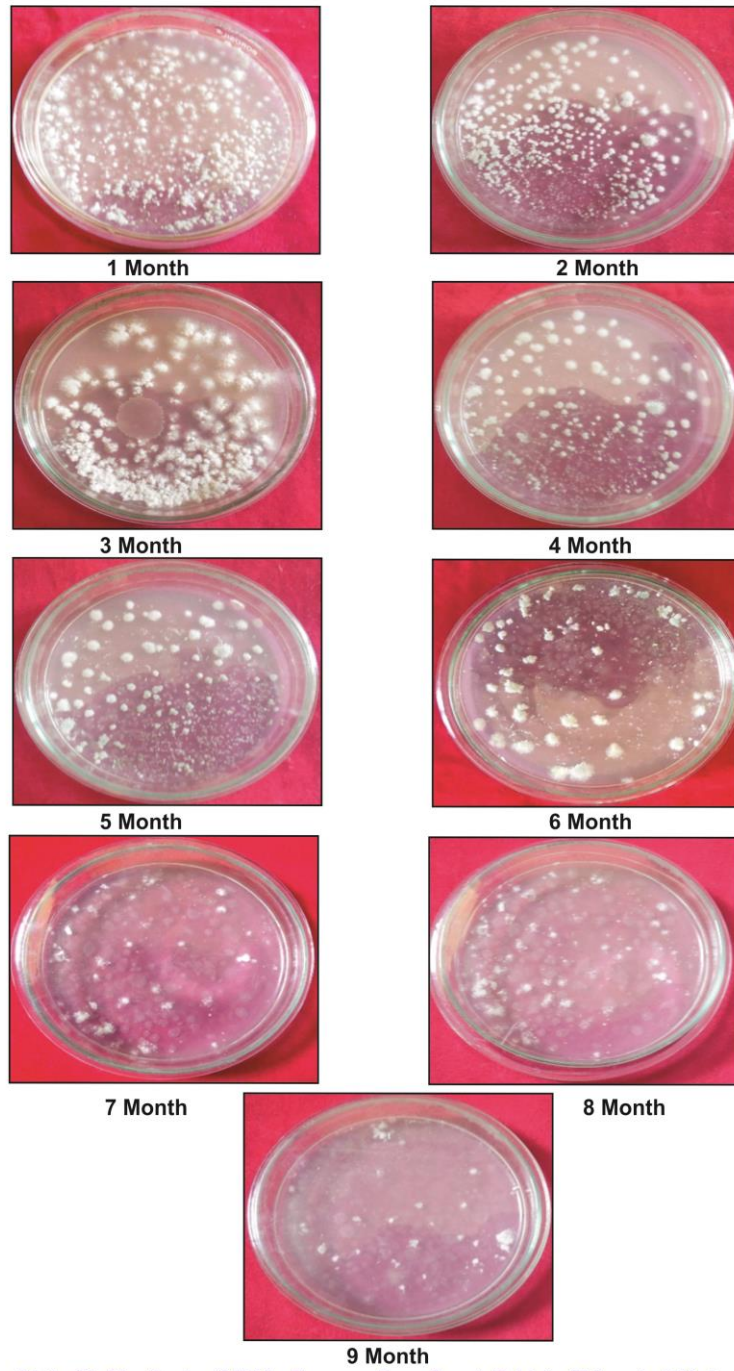


Plate 11 :Monthwise CFU in *T. asperellum* flyash Tablets (T4) upto 270 days.

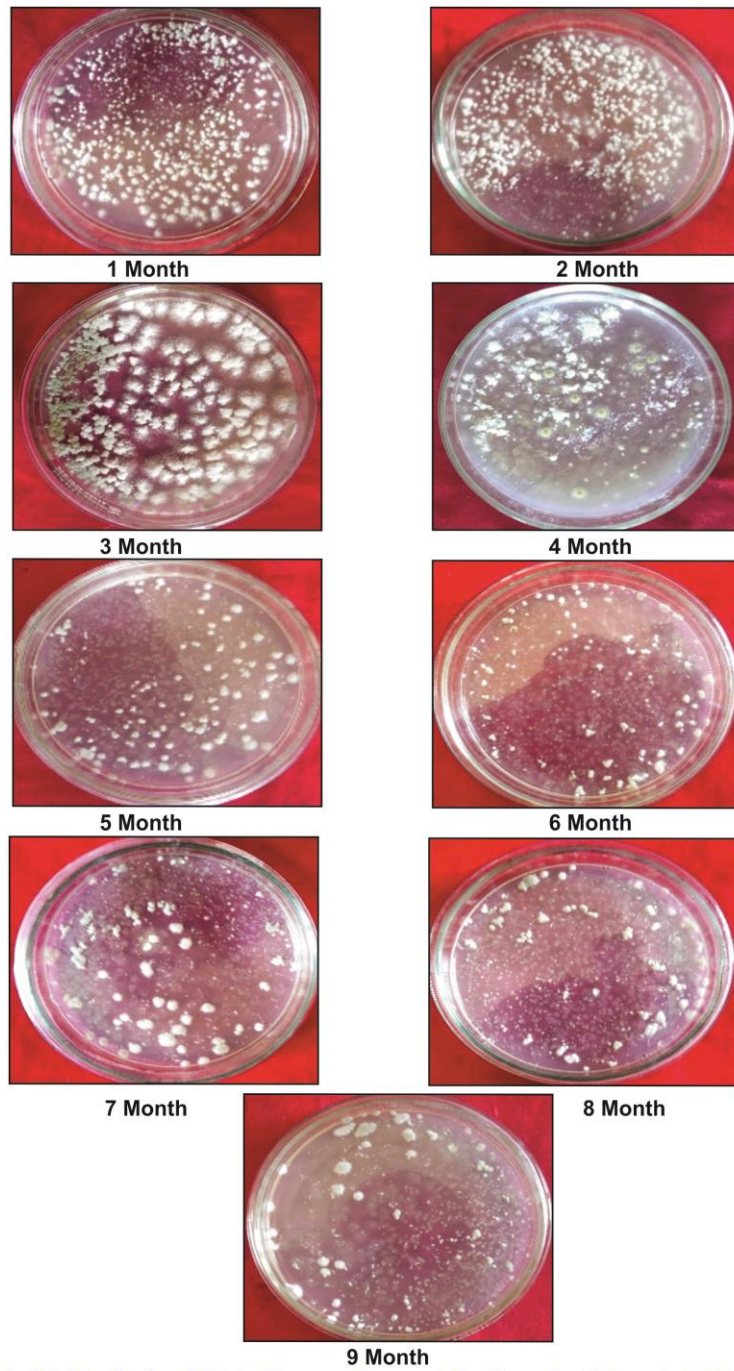


Plate 12 :Monthwise CFU in *T. asperellum* gelatin Capsules (T<sub>5</sub>) upto 270 days.

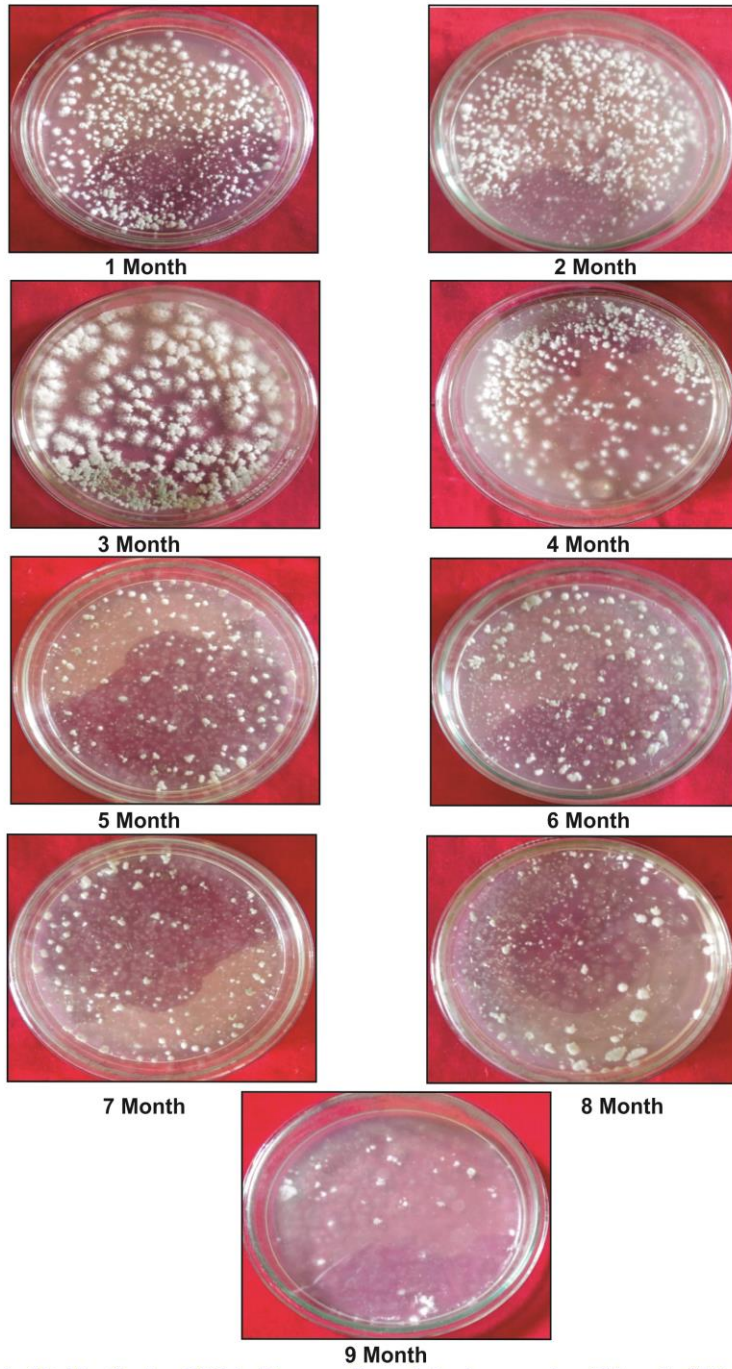


Plate 13 :Monthwise CFU in *T. asperillum* alginate capsules ( $T_6$ ) upto 270 days

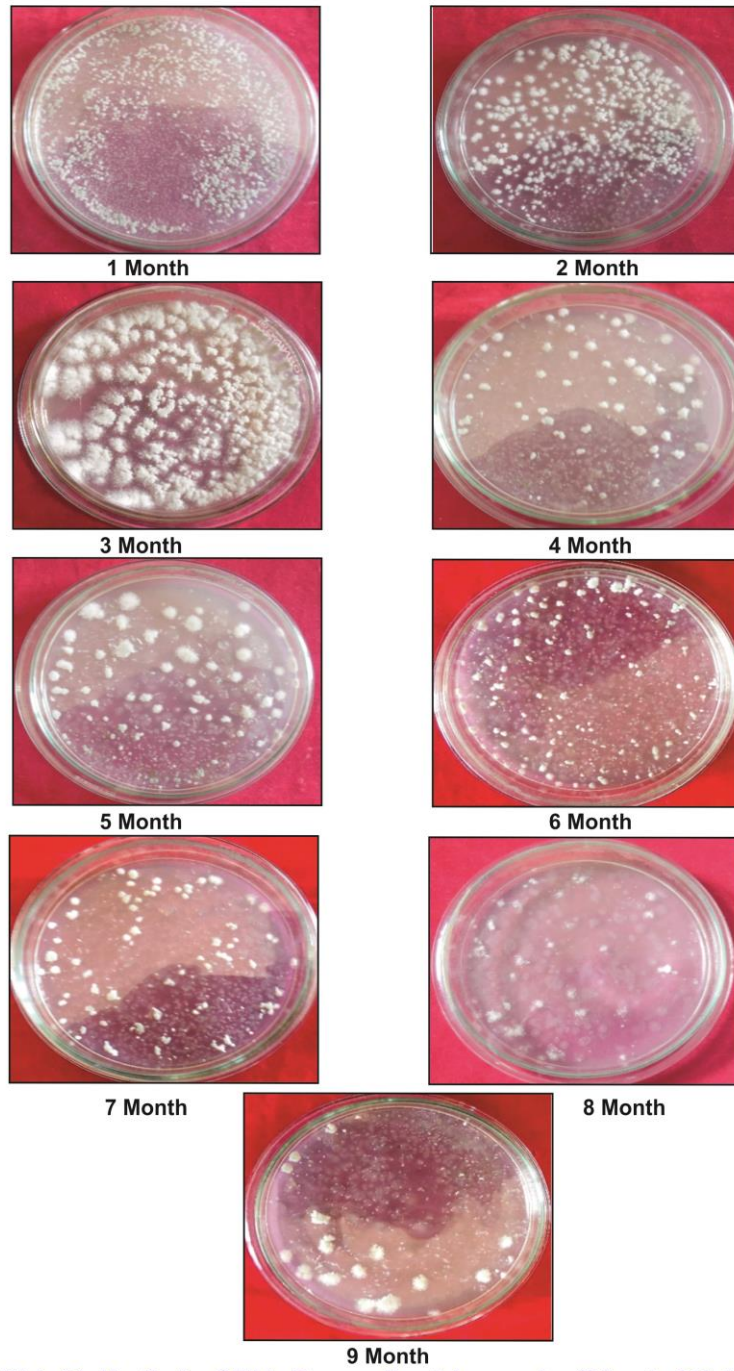


Plate 14 :Monthwise CFU in *T. asperillum* talc capsules (T<sub>7</sub>) upto 270 days.

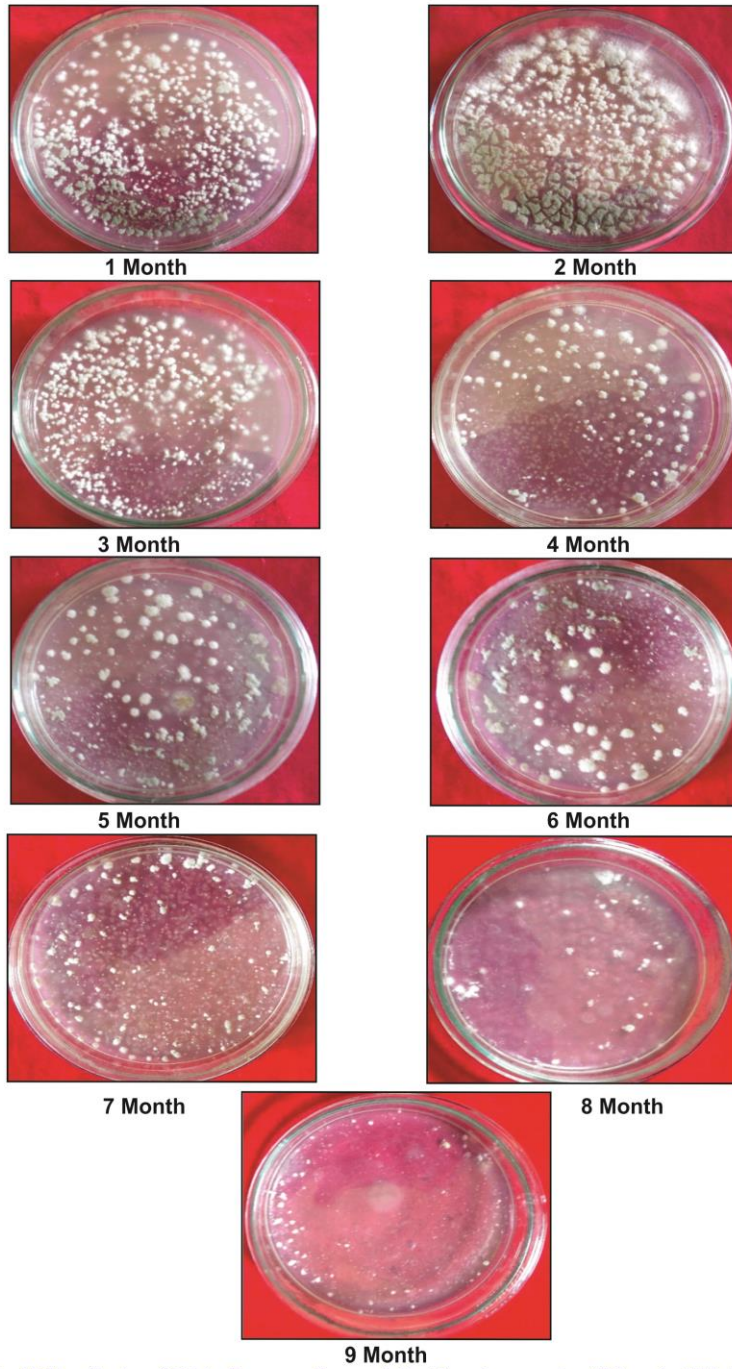


Plate 15: Monthwise CFU in *T. asperellum* gelatin+flyash capsules ( $T_8$ ) upto 270 days

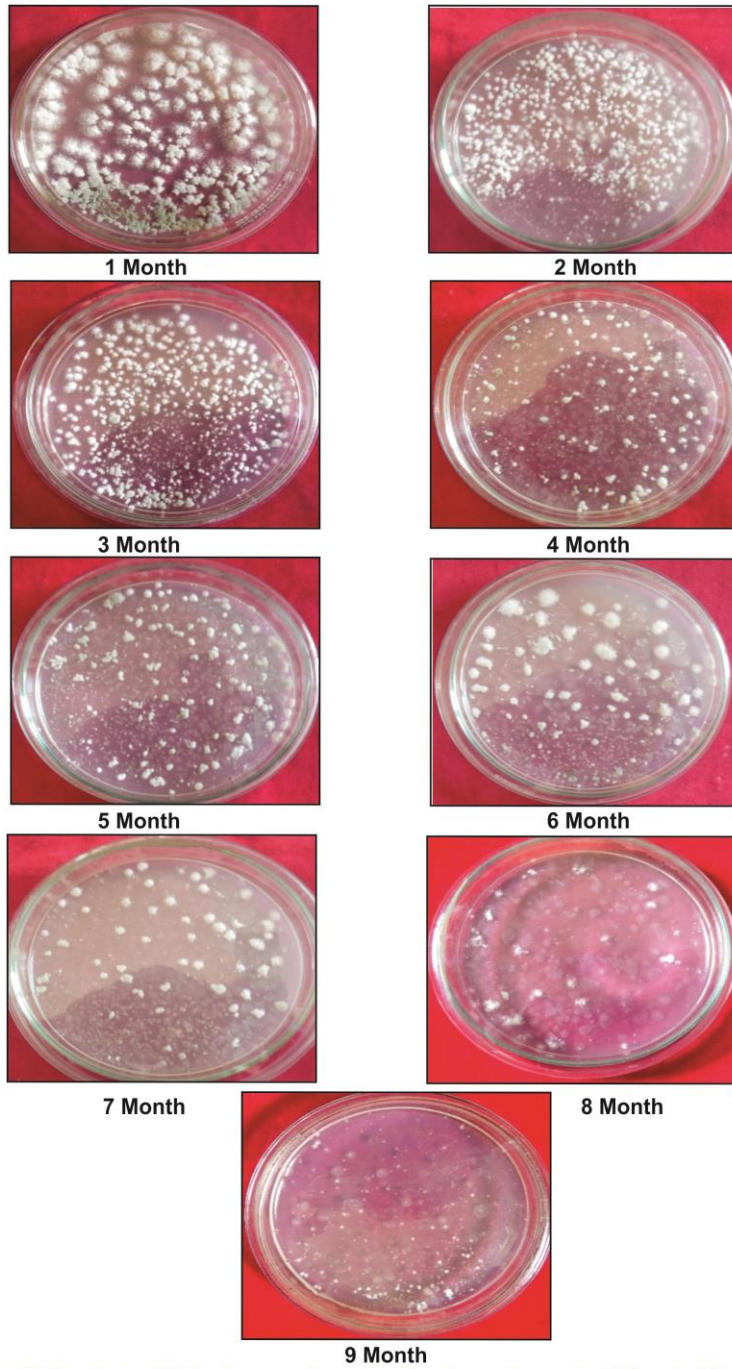


Plate 16 :Monthwise CFU in *T. asperellum* alginate+charcoal capsules (T<sub>9</sub>) upto 270 days

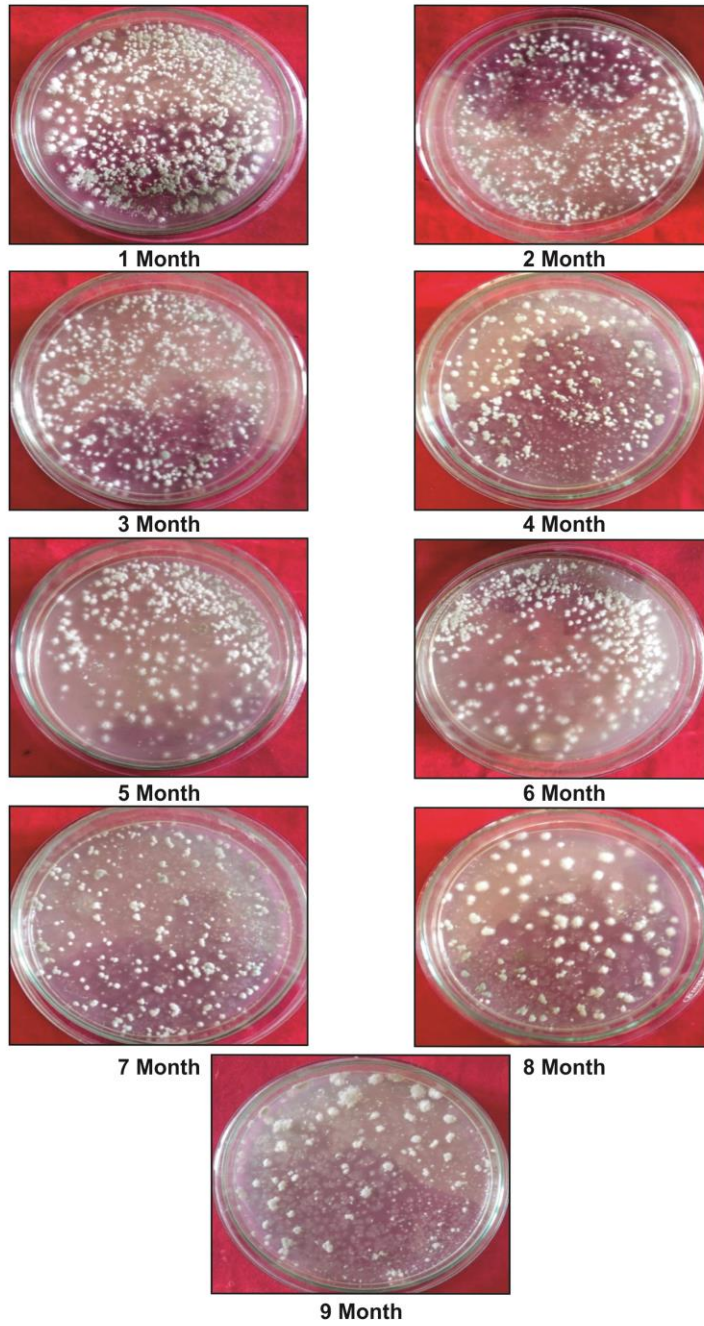


Plate 17 : Monthwise CFU in pure *T. asperellum* (Frozen mycelia along with spores) Capsules (T<sub>10</sub>) upto 270 days

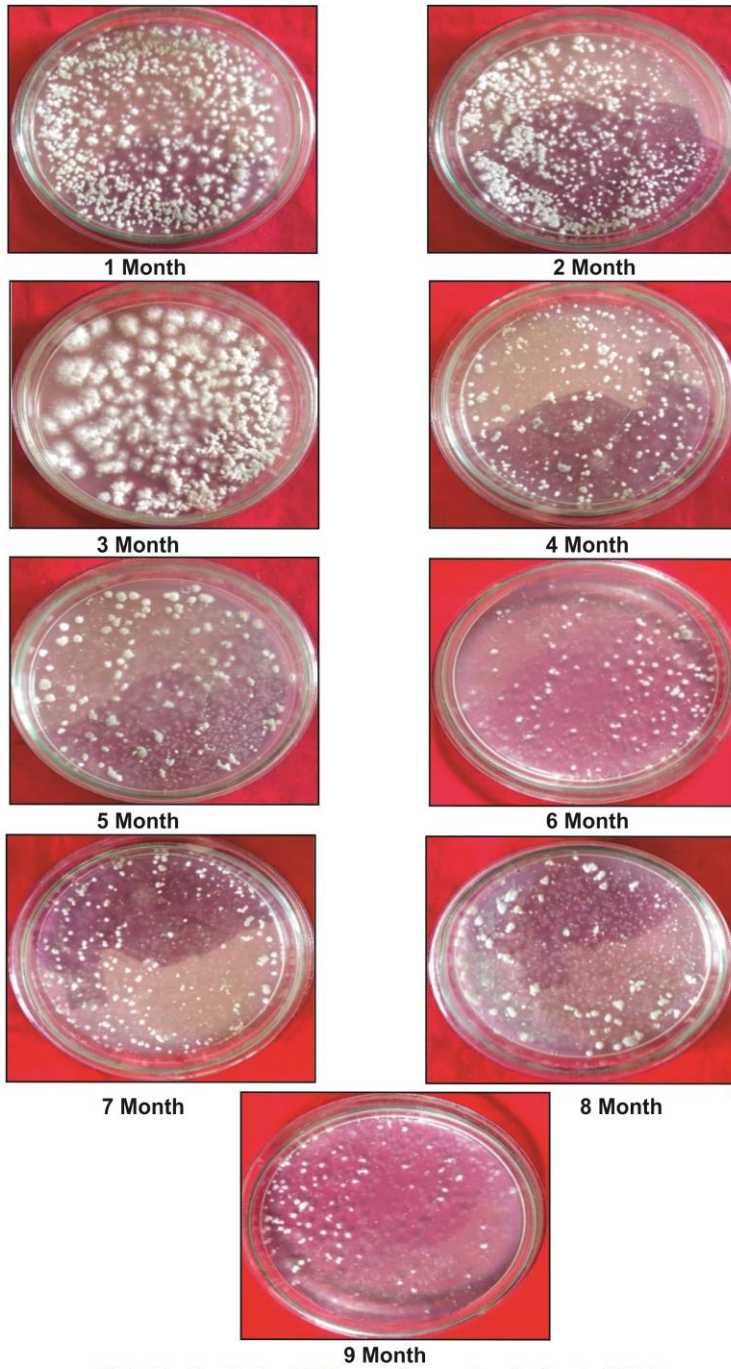
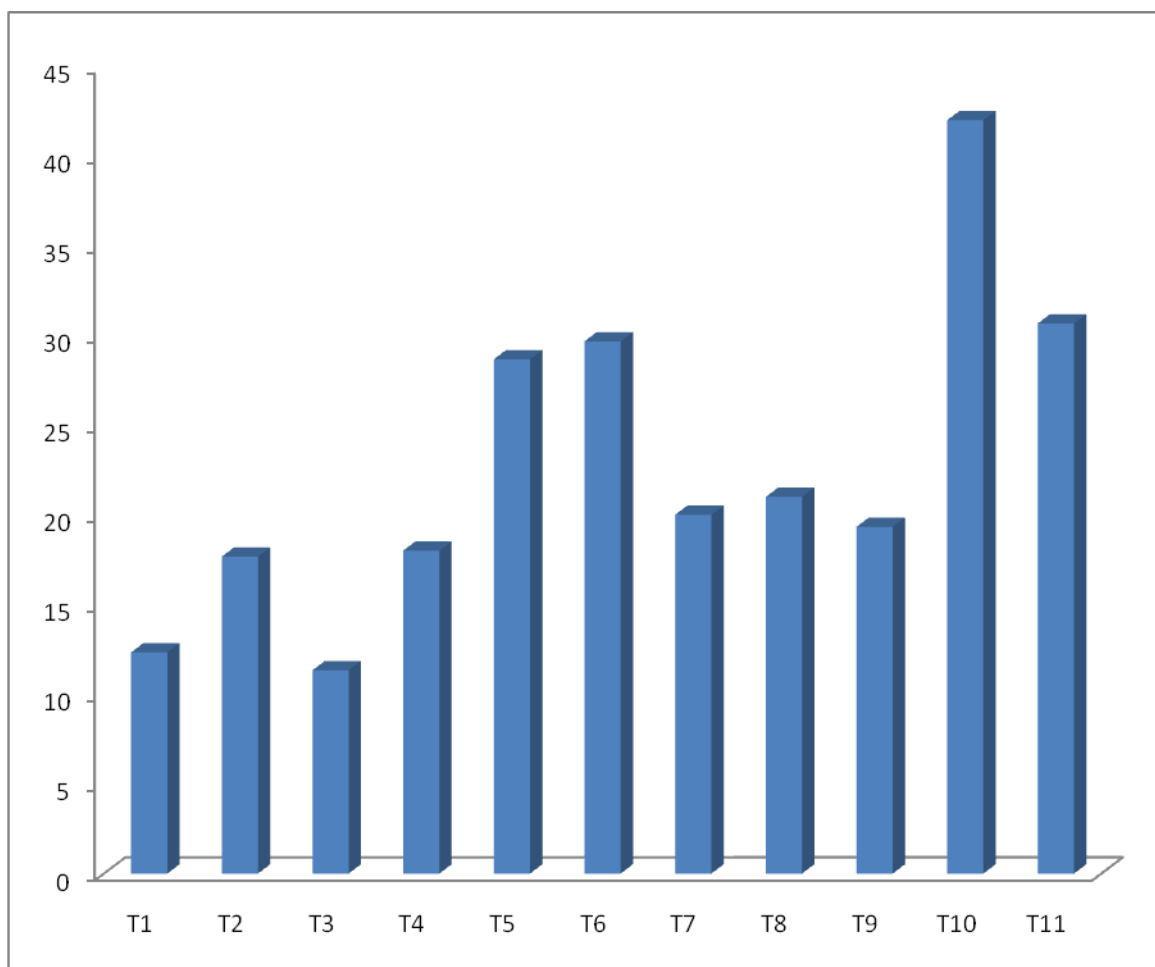


Plate 18 : Monthwise CFU in sorghum grains (T<sub>11</sub>) upto 270 days

**Table 6: Evaluation of shelf life of *T. asperellum* in different formulations**

Tre. no.	Formulations	10 <sup>7</sup> CFU/g 30 days after storage	10 <sup>7</sup> CFU/g 60 days after storage	10 <sup>7</sup> CFU/g 90 days after storage	10 <sup>7</sup> CFU/g 120 days after storage	10 <sup>7</sup> CFU/g 150 days after storage	10 <sup>7</sup> CFU/g 180 days after storage	10 <sup>7</sup> CFU/g 210 days after storage	10 <sup>7</sup> CFU/g 240 days after storage	10 <sup>7</sup> CFU/g 270 days after storage
T <sub>1</sub>	<i>T. asperellum</i> talc tablets	107.33	91.33	88.00	76.67	43.33	32.67	28.33	20.00	12.33
T <sub>2</sub>	<i>T. asperellum</i> charcoal tablets	95.00	83.33	82.33	60.33	56.67	38.67	35.00	24.67	17.67
T <sub>3</sub>	<i>T. asperellum</i> lignite tablets	104.33	85.67	81.33	36.67	33.33	28.33	22.00	15.00	11.33
T <sub>4</sub>	<i>T. asperellum</i> flyash tablets	105.33	92.67	77.00	68.33	63.33	47.33	41.00	29.67	18.00
T <sub>5</sub>	<i>T. asperellum</i> gelatin capsules	115.67	110.67	97.33	78.67	42.67	40.33	42.67	33.00	28.67
T <sub>6</sub>	<i>T. asperellum</i> alginate capsule	121.33	130.67	85.67	78.00	76.67	63.00	59.33	43.67	29.67
T <sub>7</sub>	<i>T. asperellum</i> talc capsules	135.33	83.67	75.33	66.67	40.00	35.00	34.67	21.33	20.00
T <sub>8</sub>	<i>T. asperellum</i> gelatin + flyash capsules	95.67	82.33	95.67	85.67	87.33	42.00	37.33	23.33	21.00
T <sub>9</sub>	<i>T. asperellum</i> alginate + charcoal capsules	84.33	83.67	83.33	45.33	31.67	27.67	24.33	15.33	19.33
T <sub>10</sub>	<i>T. asperellum</i> frozen culture mass along with disc capsules	205.00	176.67	179.67	125.00	90.33	80.33	75.33	68.67	42.00
T <sub>11</sub>	Sorghum grains	221.33	187.00	147.33	110.00	82.00	78.67	74.67	58.67	30.67
	S.E (M)±	1.206	1.307	1.030	0.810	0.835	0.882	1.124	0.980	0.785
	CD (P=0.01)	4.807	5.208	4.105	3.230	3.327	3.515	4.479	3.904	3.129



**Fig. 1 Evaluation of shelf life of *T. asperellum* in different formulations**

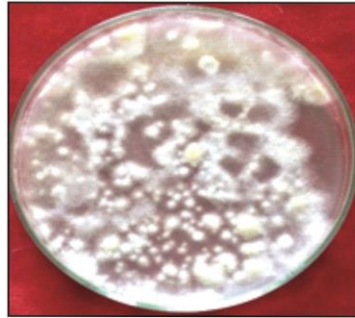
T <sub>1</sub>	<i>T. asperellum</i> talc tablets
T <sub>2</sub>	<i>T. asperellum</i> charcoal tablets
T <sub>3</sub>	<i>T. asperellum</i> lignite tablets
T <sub>4</sub>	<i>T. asperellum</i> flyash tablets
T <sub>5</sub>	<i>T. asperellum</i> gelatin capsules
T <sub>6</sub>	<i>T. asperellum</i> alginate capsule
T <sub>7</sub>	<i>T. asperellum</i> talc capsules
T <sub>8</sub>	<i>T. asperellum</i> gelatin + flyash capsules
T <sub>9</sub>	<i>T. asperellum</i> alginate + charcoal capsules
T <sub>10</sub>	<i>T. asperellum</i> frozen culture mass along with disc capsules
T <sub>11</sub>	Sorghum grains

The present findings are matched with the results of Baghel *et al.* (2014), studied various formulation viz. capsules and tablet with various carrier upto 260 days. *T. asperellum* Lignite tablets gave minimum cfu counts at 270 days of storage i.e.  $11.33 \times 10^7$  cfu/g. Kumar *et al.* (2013) reported that sorghum grains charcoal based formulation of *T. asperellum* was better up to 120 days of storage. In the present study too sorghum grains based formulation was next to agar based capsule. Charcoal based sorghum formulation was not included in the present study. Shafa khan *et al.* (2011) obtained good result with talc and gypsum based formulation upto 195 - 250 days. However, in the present study gypsum based formulation was not tried but talc based tablet have shown inferiority over talc based capsule. Shafa khan *et al.* (2011) and Kumar *et al.* (2013) tried carrier based formulations. However, in the present study the viability testing and delivery system was included so as to facilitate distribution at the farm gate.

#### **4.7 Innovative delivery system and application technology**

*T. harzianum* and *T. asperellum* are the widely used species and have been exploited on about 87 different crops and about 70 soil borne & 18 foliar pathogens (Sharma *et al.* 2014). Success of these depends on understanding and use of delivery system. The research of bioagents on delivery system is well below that of chemical pesticides. The effort has been made to improve the awareness and application technology which can improve the performance of antagonist. In the present study tablet and capsule delivery system of various formulations was tried.

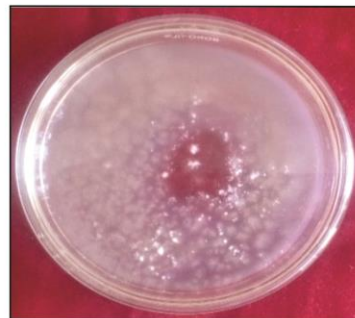
Six types of capsules viz. T<sub>5</sub> (*T. asperellum* gelatin capsule), T<sub>6</sub> (*T. asperellum* alginate capsule), T<sub>7</sub> (*T. asperellum* talc capsule), T<sub>8</sub> (*T. asperellum* gelatin + flyash capsule), T<sub>9</sub> (*T. asperellum* alginate + charcoal capsule), T<sub>10</sub> (*T. asperellum* Frozen culture mass along with disc capsule), and four types of tablets viz. T<sub>1</sub> (*T. asperellum* talc tablet), T<sub>2</sub> (*T. asperellum* charcoal tablet), T<sub>3</sub> (*T. asperellum* lignite tablet), T<sub>4</sub> (*T. asperellum* flyash tablet), and compared with each other.



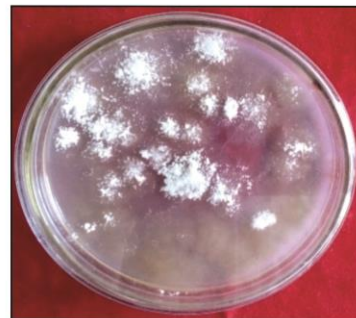
Pure *T. asperellum* capsules



*T. asperellum* alginate capsules



*T. asperellum* alginate + charcoal capsules



*T. asperellum* gelatin capsules

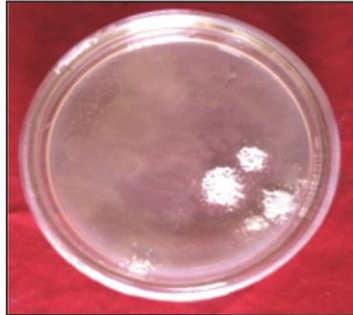


*T. asperellum* Gelatin + flyash capsules

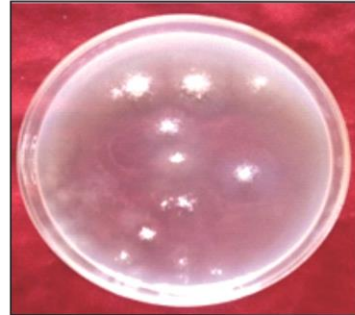


*T. asperellum* talc capsules

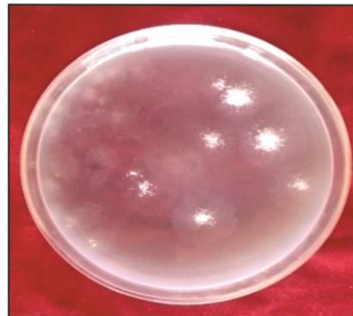
Plate 19 : CFU count after 270 days by using jaggery treatment in different capsule formulation in 48 hrs.



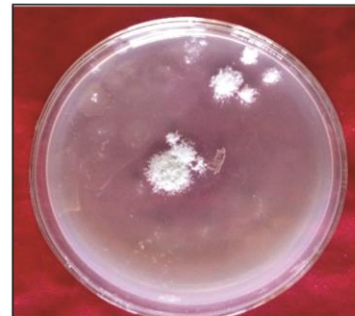
*T. asperellum* flyash Tablets



*T. asperellum* charcoal Tablets



*T. asperellum* talc Tablets



*T. asperellum* lignite Tablets

Plate 20 : CFU count after 270 days by using jaggery treatment in different Tablet formulation in 48 hrs.

#### 4.7.1 Application method

One capsule/tablet from each formulations was dispersed separately in 500 ml of sterilized water enriched with 10 g jaggery and incubated for 12 h. It is revealed from data presented in Table 7 that the population of *T. asperellum* gradually improved within 12 h of incubation. One gram of capsulated or tablet form of *T. asperellum* culture retained more than sufficient population. It is clear from data that maximum population of *T. asperellum* obtained ( $45.00 \times 10^7$  cfu/ml) in T<sub>10</sub>, *T. asperellum* Frozen culture mass along with medium disc capsule followed by ( $31.00 \times 10^7$  cfu/ml) in T<sub>6</sub>, *T. asperellum* alginate capsule, ( $21.33 \times 10^7$ cfu/ml) in T<sub>9</sub>, *T. asperellum* alginate + charcoal capsule, ( $20.66 \times 10^7$ cfu/ml) in T<sub>7</sub>, *T. asperellum* talc capsule, ( $20.33 \times 10^7$  cfu/ml) in T<sub>5</sub>, *T. asperellum* gelatin capsule, ( $15.00 \times 10^7$ cfu/ml) in T<sub>4</sub>, *T. asperellum* fly ash tablet, ( $14.33 \times 10^7$ cfu/ml) in T<sub>8</sub>, *T. asperellum* gelatin + flyash capsule, T<sub>2</sub>, *T. asperellum* charcoal tablets, ( $14.00 \times 10^7$ cfu/ml).

However least cfu was recorded in T<sub>3</sub>, *T. asperellum* lignite tablet ( $8.33 \times 10^7$ cfu/ml), T<sub>1</sub>, *T. asperellum* talc tablet ( $8.66 \times 10^7$ cfu/ml).

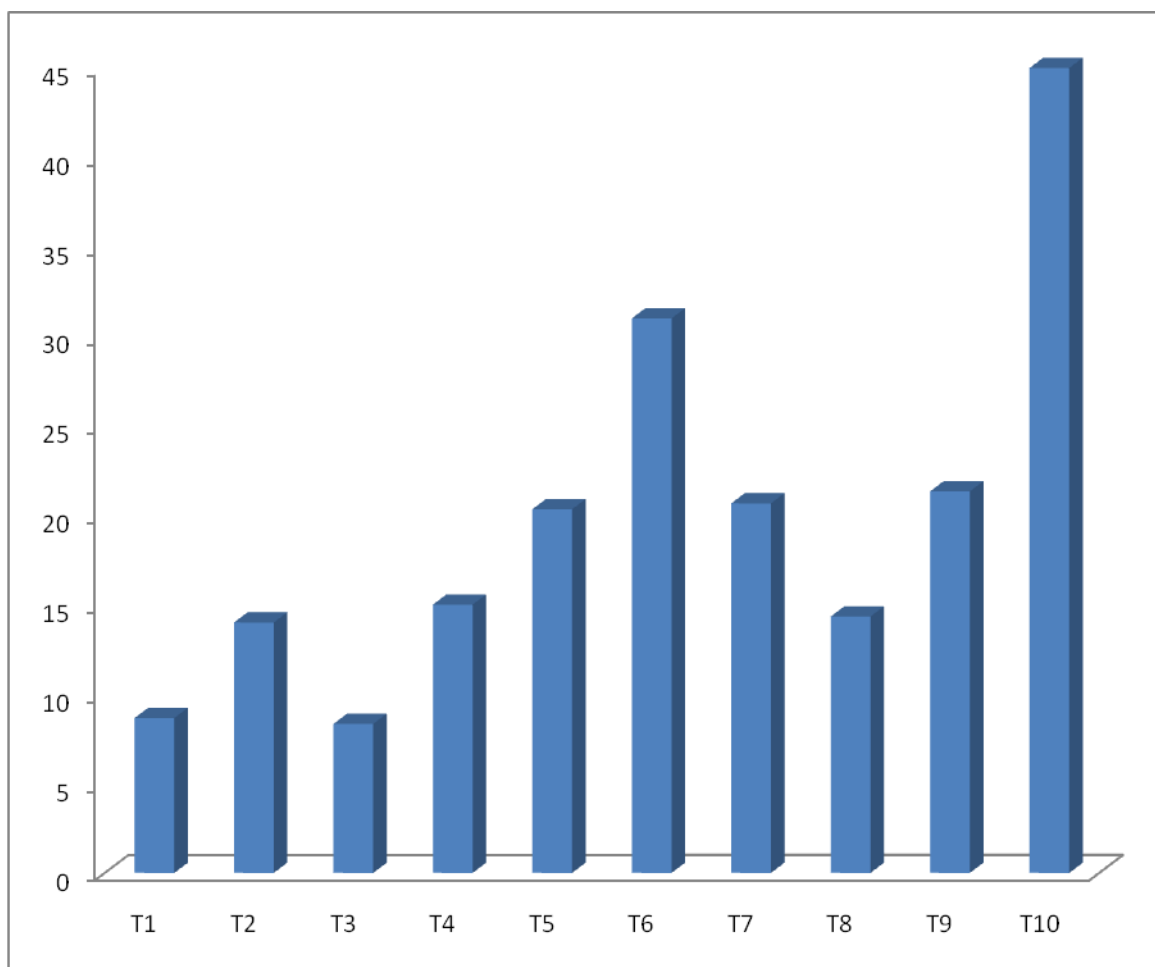
The present findings are in accordance with the studies of Kose and Totawar (2017) tried six types of capsules of *T. asperellum* viz. talc, gelatin, alginate, alginate + charcoal, gelatin + flyash and frozen mass culture along with medium disc capsules and four types of tablets of *T. asperellum* viz. talc, charcoal, lignite and flyash for delivery system and application and revealed that when one capsule/tablet from each formulation dispersed separately in 500 ml capacity conical flask containing 250 ml sterilized water enriched with 10 g jaggery and incubated for 12 h given maximum population of *T. asperellum*. Also they recorded the increased cell count by this technology was due to composition of hard gelatin capsule. In hypothesis one gram of capsule form of *T. asperellum* of treatment T<sub>10</sub> ( $45.00 \times 10^7$  cfu/ml) which is sufficient as compared with the available formulations in the market. Some strains of *Trichoderma* are nice lingocellulolytic degraders and are also included in decomposting cultures. Hence the better delivery system and formulate can be made applicable for supply of decomposting cultures and there is still a scope for exploring

more applicability. Saili *et al.* (2014), reported that soil prevalent fungus *Trichoderma* produces lignocellulolytic enzymes that assist the degradation of woody lignocellulose materials. *Trichoderma* isolates (5D, 10L<sub>2</sub>, 10P, 5E, 10X and 10E<sub>2</sub>) were found to be potential lignocellulolytic agents based on their diameter of halo zone formed on amended media.

The composition of hard gelatin capsule is a mixture of peptides and proteins produced by partial hydrolysis of collagen extracted from the skin, bones and connective tissues of animals such as domesticated cattle, chicken, pigs and fish (Stegemann, 2002). It is revealed from literature that agar consists of a mixture of two polysaccharides: agarose and agarpectin (Rafael and Fernando, 1987).

**Table 7: Evaluation of *T. asperellum* population for innovative delivery system and application technology**

Treat. no.	Formulation	10 <sup>7</sup> cfu/ml R <sub>1</sub>	10 <sup>7</sup> cfu/ml R <sub>2</sub>	10 <sup>7</sup> cfu/ml R <sub>3</sub>	Mean
T <sub>1</sub>	<i>T. asperellum</i> talc tablets	9	9	8	8.66
T <sub>2</sub>	<i>T. asperellum</i> charcoal tablets	14	13	15	14.00
T <sub>3</sub>	<i>T. asperellum</i> lignite tablets	9	8	8	8.33
T <sub>4</sub>	<i>T. asperellum</i> flyash tablets	16	15	14	15.00
T <sub>5</sub>	<i>T. asperellum</i> gelatin capsules	21	20	20	20.33
T <sub>6</sub>	<i>T. asperellum</i> alginate capsule	33	30	30	31.00
T <sub>7</sub>	<i>T. asperellum</i> talc capsules	22	21	19	20.66
T <sub>8</sub>	<i>T. asperellum</i> gelatin + flyash capsules	13	14	16	14.33
T <sub>9</sub>	<i>T. asperellum</i> alginate + charcoal capsules	20	21	23	21.33
T <sub>10</sub>	<i>T. asperellum</i> frozen culture mass along with disc capsules	44	45	46	45.00
	S.E (M)±	-	-	-	0.591
	CD (P=0.01)	-	-	-	2.017



**Fig. 2 Evaluation of *T. asperellum* population for innovative delivery system and application technology**

T <sub>1</sub>	<i>T. asperellum</i> talc tablets
T <sub>2</sub>	<i>T. asperellum</i> charcoal tablets
T <sub>3</sub>	<i>T. asperellum</i> lignite tablets
T <sub>4</sub>	<i>T. asperellum</i> flyash tablets
T <sub>5</sub>	<i>T. asperellum</i> gelatin capsules
T <sub>6</sub>	<i>T. asperellum</i> alginate capsule
T <sub>7</sub>	<i>T. asperellum</i> talc capsules
T <sub>8</sub>	<i>T. asperellum</i> gelatin + flyash capsules
T <sub>9</sub>	<i>T. asperellum</i> alginate + charcoal capsules
T <sub>10</sub>	<i>T. asperellum</i> frozen culture mass along with disc capsules

In present investigation so as to develop the innovative delivery system of *T. asperellum*, maximum population i.e.  $45.00 \times 10^7$  cfu/ml was observed when one capsule that has  $42.00 \times 10^7$  cfu/g population even after 270 days of storage dispersed in 500 ml sterilized water amended with 10 g jaggery solution and incubated for 12 h i.e. 1000 mg capsulated *T. asperellum* (frozen culture mass along with medium disc capsule) gives  $45.00 \times 10^7$  cfu/ml in 500 ml volume. It is revealed from present investigation that in treatment T<sub>10</sub> of *T. asperellum* capsule, some nutrition comes along with mycelial mat and spores, there is possibility that some of the nutrient media along with mycelial mat and spores would have supported the further growth and reproduction. More over jaggery added to solution also serves as source of minerals and carbohydrates to enhance populations. Certainly the pure frozen mycelial mat along with medium disc gelatin capsule in treatment T<sub>10</sub> supported the fungus for maximum cfu by providing the essential nutrients, proteins and carbohydrates during 12 h suspension in sterile water. Tablet of lignite, flyash, charcoal and talc based were comparatively less effective as compared to capsule.

More exploration to be required in this regard to make convenient delivery system of bioagents like *Trichoderma* and also would possible to entomopathogenic fungi.

However, it is also needed to work out with less quantum of sterile water.

#### **4.8 Antagonistic efficacy of *Trichoderma asperellum* isolates against *Sclerotium rolfsii*, *Rhizoctonia bataticola* and *Fusarium solani* (per cent growth inhibition) at 7 DAI**

In *Sclerotium rolfsii*, the minimum radial growth (18.40 mm) with maximum per cent inhibition (79.5%) was recorded in isolate Ta2 which was at par with Ta4 (79.41%) and Ta3 (78.8%). However, the isolate Ta1 (77.92%) showed minimum per cent inhibition over control (Table 8).

The present findings are in conformity with Bagwan N. B. (2011), who tested *Trichoderma* sp. Isolates against *Sclerotium rolfsii* in which, isolate T043 showed maximum (98.70%) growth inhibition. Rao and

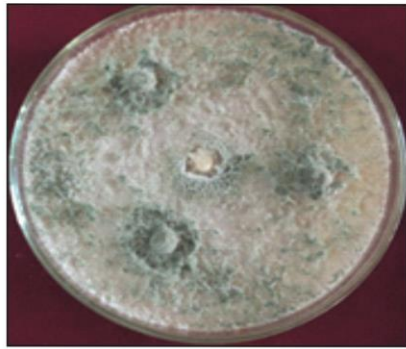
Kulkarni (2003) also reported the maximum (58.50%) growth inhibition of *Sclerotium rolfsii* by *Trichoderma asperellum*. Shrinivasulu (2005) also reported that *Trichoderma asperellum* was very effective in reducing the radial growth of *Sclerotium rolfsii*.

The per cent growth inhibition of *Rhizoctonia bataticola* by *Trichoderma asperellum* isolates was shown in Table 8. Antagonism between *Trichoderma asperellum* and test pathogen *Rhizoctonia bataticola* indicated that, the test pathogen stops growing upon contact with the antagonist *Trichoderma asperellum*. The maximum growth inhibition (79.7%) of *Rhizoctonia bataticola* was exerted by Ta4 isolate with minimum radial growth (18.20 mm) which was at par with Ta2 isolate (79.47%).

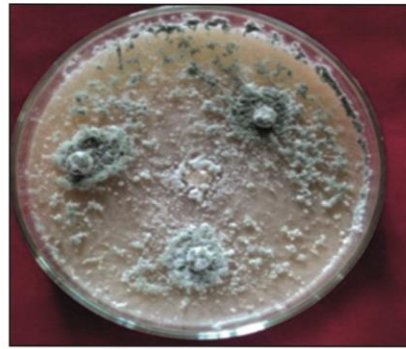
This study is further supported by Shalini and Kotasthane (2000), Sara mayo *et al.* (2015) who studied *in vitro* antifungal ability of the different *Trichoderma* isolates based on the ability to produce metabolites that may inhibit the growth of *R. solani*. *Trichoderma* isolates T003, T004, T006, T020, T022, T012, T013, T025, T016, T007, T024, T005, and T010 inhibited *R. solani* growth by more than 75%, with the highest inhibition produced by T003, T004, T006, T020 and T022 (86.70%). T019, T008, T002, T021, T001, T018, and T023 showed are markable inhibition (75–40%). Finally T015, T014, T017, T011, and T009 inhibited *R. solani* growth by less than 40% and T009 showed the lowest percentage (15.82%).

The maximum per cent inhibition (82.58%) and minimum radial growth (15.67 mm) of *Fusarium solani* was observed in isolate Ta2 which was at par with isolate Ta4 i.e 79.96% inhibition with 18.03 mm radial growth, whereas minimum per cent inhibition (73.66%) was recorded in Ta3 isolate (Table 8).

The results are in conformity with the findings of Belete *et al.* (2015) and Anupama sonawane *et al.* (2015) who found that the isolate *Trichoderma asperellum* showed the maximum (96.00%) per cent inhibition of *Fusarium*. In this preview *Trichoderma asperellum* has been investigated as an important antagonistic soil fungus having the ability to reduce the soil borne disease incidence.



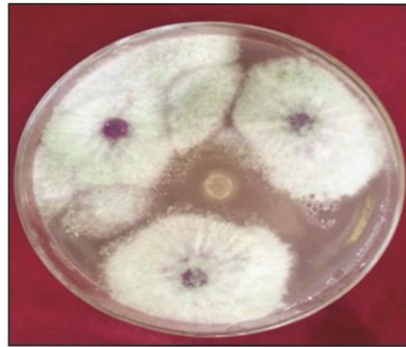
TA1



TA2



TA3

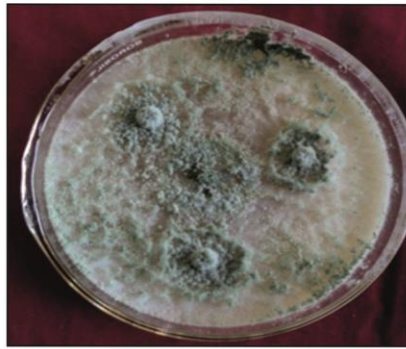


TA4

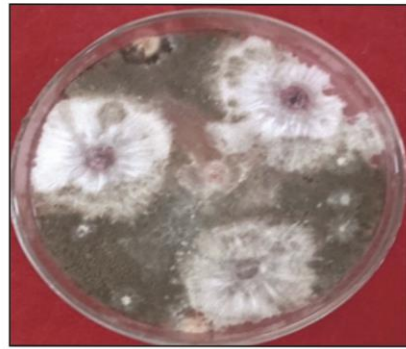


Control

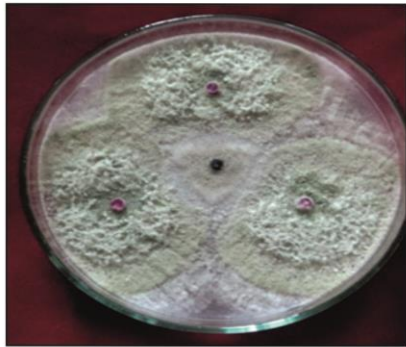
Plate 21 : Efficacy of *Trichoderma asperellum* isolates against *sclerotium rolfsii* (per cent growth inhibition) at 7 DA



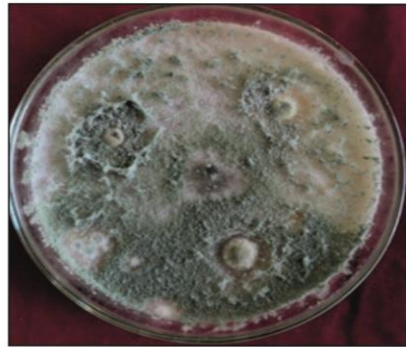
TA1



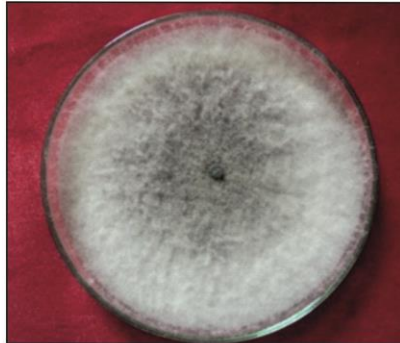
TA2



TA3

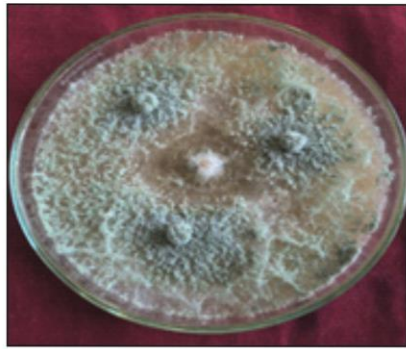


TA4

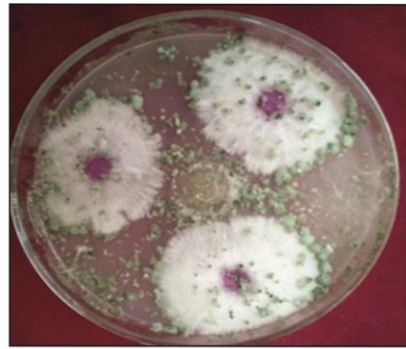


Control

Plate 22 : Efficacy of *Trichoderma asperellum* isolates against *Rhizoctonia bataticola* (per cent growth inhibition) at 7 DAI



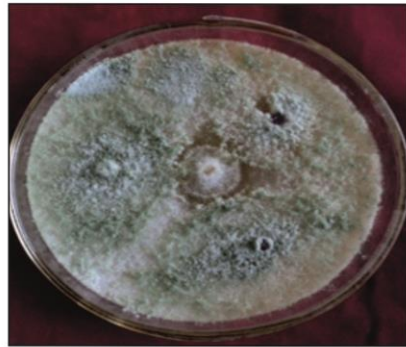
TA1



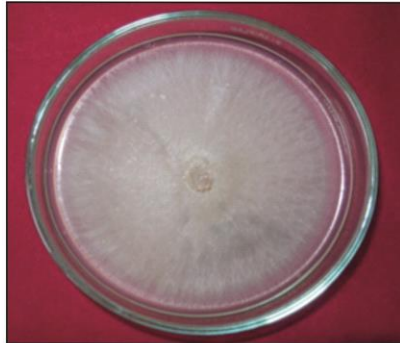
TA2



TA3



TA4

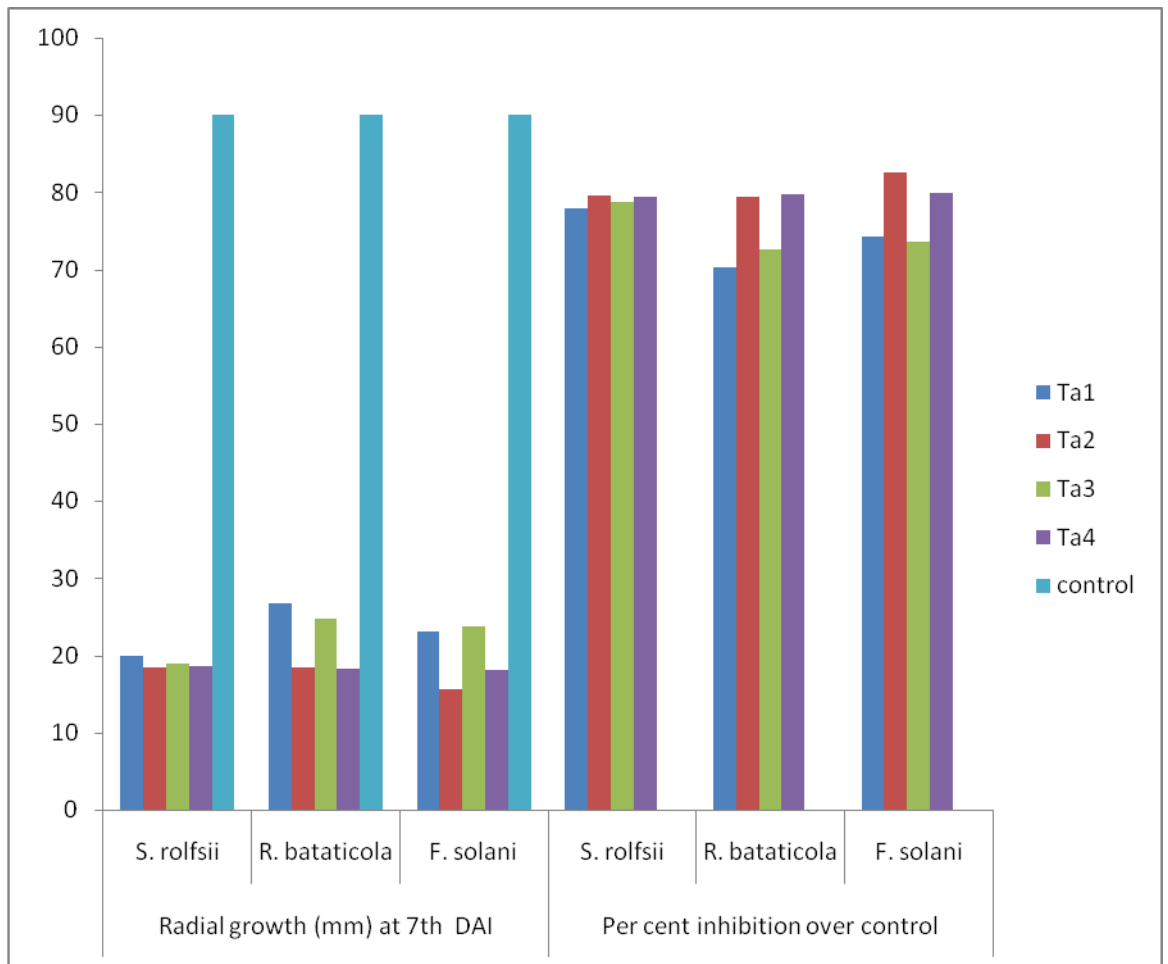


Control

Plate 23: Efficacy of *Trichoderma asperellum* isolates against *Fusarium solani* (per cent growth inhibition) at 7 DAI

**Table 8. Antagonistic efficacy of *Trichoderma asperellum* isolates against *Sclerotium rolfsii*, *Rhizoctonia bataticola* and *Fusarium solani* (per cent growth inhibition) at 7 DAI**

Sr. No.	Isolates	Radial growth (mm) at 7 <sup>th</sup> DAI			Per cent inhibition over control		
		<i>S. rolfsii</i>	<i>R. bataticola</i>	<i>F. solani</i>	<i>S. rolfsii</i>	<i>R. bataticola</i>	<i>F. solani</i>
1.	Ta1	19.87	26.73	23.10	77.92	70.3	74.3
2.	Ta2	18.40	18.47	15.67	79.5	79.47	82.58
3.	Ta3	19.00	24.67	23.70	78.8	72.58	73.66
4.	Ta4	18.53	18.20	18.03	79.41	79.7	79.96
5.	control	90.00	90.00	90.00	–	–	–
	F test	Sig	Sig	Sig.	–	–	–
	S.E (M)±	0.43	0.23	0.44	–	–	–
	C.D. at (p= 0.01)	1.94	1.04	2.00	–	–	–



**Fig. 3 Antagonistic efficacy of *Trichoderma asperellum* isolates against *Sclerotium rolfsii*, *Rhizoctonia bataticola* and *Fusarium solani* (per cent growth inhibition) at 7 DAI**

## CHAPTER V

### SUMMARY AND CONCLUSIONS

An experiment entitled “Assessment of different formulations of *Trichoderma* for their shelf life. ” was undertaken at Department of Plant Pathology, PGI, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola during 2017 – 2018.

Rhizosphere soil samples were collected from different districts of Maharashtra viz. Akola, Rahuri, Beed, Ratnagiri for the isolation of *Trichoderma asperellum* on *Trichoderma* Selective Medium (TSM) by serial dilution and pour plate technique. Purified cultures were maintained on PDA plates. Four different isolates were isolated and abbreviated as Ta1, Ta2, Ta3 and Ta4.

Eleven formulations of *Trichoderma asperellum* were evaluated in present investigation, among these four types of tablets formulation viz. talc tablets, charcoal tablets, lignite tablets, fly ash tablets, six capsules formulations viz. talc capsule, alginate capsules, gelatin capsules, alginate + charcoal capsules, gelatin + fly ash capsules, *T. asperellum* frozen culture mass along with medium disc capsules and one sorghum grains formulation were used and observation on shelf life up to 270 days from date of manufacturing at regular intervals of 30 days were taken.

maximum shelf life ( $42.00 \times 10^7$  cfu/g) was obtained in treatment T<sub>10</sub> i.e. frozen culture mass along with medium disc even after 270 days of storage followed by T<sub>11</sub>, sorghum grains ( $30.67 \times 10^7$  cfu/g), T<sub>6</sub>, *T. asperellum* alginate capsule ( $29.67 \times 10^7$  cfu/g), T<sub>5</sub>, *T. asperellum* gelatin capsule ( $28.67 \times 10^7$  cfu/g), T<sub>8</sub>, *T. asperellum* gelatin + flyash capsule ( $21.00 \times 10^7$  cfu/g), T<sub>7</sub>, *T. asperellum* talc capsule ( $20.00 \times 10^7$  cfu/g), T<sub>9</sub>, *T. asperellum* alginate + charcoal capsule ( $19.33 \times 10^7$  cfu/g), T<sub>4</sub>, *T. asperellum* flyash tablets ( $18.00 \times 10^7$  cfu/g), T<sub>2</sub>, *T. asperellum* charcoal tablet ( $17.67 \times 10^7$  cfu/g), T<sub>1</sub>, *T. asperellum* talc tablet ( $12.33 \times 10^7$

cfu/g) and T<sub>3</sub>, *T. asperellum* lignite tablet ( $11.33 \times 10^7$  cfu/g) after 270 days of storage.

One capsule/tablet from each formulations was when dispersed separately in 500 ml of sterilized water enriched with 10 g jaggery and incubated for 12 h. The population of *T. asperellum* gradually improved within 12 h of incubation. One gram of capsulated or tablet form of *T. asperellum* culture retained more than sufficient population. Maximum population of *T. asperellum* obtained ( $45.00 \times 10^7$  cfu/ml) in T<sub>10</sub>, *T. asperellum* Frozen culture mass along with medium disc capsule followed by ( $31.00 \times 10^7$  cfu/ml) in T<sub>6</sub>, *T. asperellum* alginate capsule, ( $21.33 \times 10^7$ cfu/ml) in T<sub>9</sub>, *T. asperellum* alginate + charcoal capsule, ( $20.66 \times 10^7$ cfu/ml) in T<sub>7</sub>, *T. asperellum* talc capsule, ( $20.33 \times 10^7$  cfu/ml) in T<sub>5</sub>, *T. asperellum* gelatin capsule, ( $15.00 \times 10^7$ cfu/ml) in T<sub>4</sub>, *T. asperellum* fly ash tablet, ( $14.33 \times 10^7$ cfu/ml) in T<sub>8</sub>, *T. asperellum* gelatin + flyash capsule, T<sub>2</sub>, *T. asperellum* charcoal tablets, ( $14.00 \times 10^7$ cfu/ml). However least cfu was recorded in T<sub>3</sub>, *T. asperellum* lignite tablet ( $8.33 \times 10^7$ cfu/ml), T<sub>1</sub>, *T. asperellum* talc tablet ( $8.66 \times 10^7$ cfu/ml).

The antagonistic efficacy of *Trichoderma asperellum* against the soil born plant pathogens viz., *Sclerotium rolfsii*, *Rhizoctonia bataticola* and *Fusarium solani* were tested. The maximum per cent inhibition (79.5%) and minimum radial growth (18.40 mm) of *sclerotium rolfsii* was observed in isolate Ta2.

In case of *Rhizoctonia bataticola* the minimum radial growth (18.20 mm) with maximum per cent inhibition (79.7%) was recorded in isolate Ta4 collected from Ratnagiri district. Antagonism between *Trichoderma asperellum* and test pathogen *Fusarium solani* showed maximum growth inhibition (82.58%) in Ta2 isolate.

## CONCLUSION

The collection of soil samples from different locations of Maharashtra was done for the isolation of *Trichoderma asperellum*.

Among the eleven formulations i.e tablet (04) and capsule (06) and one solid state fermentation tested, more viability was recorded in all capsule formulation than tablet and solid state fermentation especially in frozen mycelial mat along with disc capsule, showed maximum cfu ( $42.00 \times 10^7 \text{ g}^{-1}$ ). It is therefor to be used for delivery system as to facilitate distribution at the farm gate.

It is concluded that *T. asperellum* agar base gelatin capsule of frozen culture mass along with medium disc capsule dispersed in 500 ml sterilized water amended with 10 g jaggery solution and incubated for 12 h which gave maximum cfu count, to be used as an innovative delivery system.

The antagonistic efficacy test of *Trichoderma asperellum* was studied against the soil born pathogens viz. *Fusarium solani*, *Sclerotium rolfsii* and *Rhizoctonia bataticola* and found as a best bio-agent for the management of these pathogens up to 82.58% may be due to the reasons: rapid growth rate, antibiosis, antibiotic production and competition for nutrition.

The isolate Ta2 showed highest per cent growth inhibition (82.58%) against *Fusarium solani* and *Sclerotium rolfsii* (79.5%) in *Rhizoctonia bataticola* (79.7%) by Ta4 isolate.

## CHAPTER VI

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