"STUDIES ON PRODUCTION TECHNOLOGY OF LIQUID FORMULATION OF *TRICHODERMA*''

A Thesis submitted to the

MAHATMA PHULE KRISHI VIDYAPEETH,
RAHURI - 413 722, DIST. AHMEDNAGAR,
MAHARASHTRA, INDIA

By

MISS. PREETI MAHADEO HIRALKAR
Reg. No. 10/198

In partial fulfilment of the requirements for the degree
Of

MASTER OF SCIENCE (AGRICULTURE)

In

PLANT PATHOLOGY

DEPARTMENT OF PLANT PATHOLOGY
AND AGRICULTURAL MICROBIOLOGY,
POST GRATUATE INSTUTUTE MAHATMA PHULE KRISHI VIDYAPEETH,
RAHURI-413722.DIST-AHMEDNAGAR,
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(2012)

CANDIDATE'S DECLARATION

I hereby declare that this thesis entitled **"STUDIES ON PRODUCTION TECHNOLOGY OF LIQUID FORMULATION OF *TRICHODERMA*"** or part thereof has not been previously submitted by me or any other person to any other University or Institute for a Degree or Diploma.

Place : Rahuri.

Date : / /2012 **(Preeti M. Hiralkar)**

CONTENTS

ABSTRACT

"STUDIES ON PRODUCTION TECHNOLOGY OF LIQUID FORMULATION OF *TRICHODERMA*"

By

MISS PREETI MAHADEO HIRALKAR

MASTER OF SCIENCE (AGRICULTURE)

Post Graduate Institute Rahuri

2012

Research Guide	**:**	**Dr. C. D. Deokar**
Department	**:**	**Plant Pathology.**

Trichoderma used as biocontrol agent because it controls the pathogenic organism by the mechanism like competition, mycoparasitism, and antagonism. It excretes the enzymes like viridin, and gleotoxin there by enhance the root growth. Hence it has got significant importance in ecofriendly disease management as biopesticide. The solid based formulation of *Trichoderma* is impractical for commercial production with shorter shelf life, whereas liquid *Trichoderma* is having better shelf life and does not losses its properties due to sub culturing. So the study was undertaken on "Production technology of liquid formulation of *Trichoderma*" during 2011-2012.

In laboratory studies the efficient strains of *Trichoderma harzianum* was identified by studying morphological, cultural character and antagonistic properties by using poison food technique. The result showed that *Trichoderma harzianum* is the most efficient species than *Trichoderma viride, Trichoderma hamatum and Trichoderma konigii* to control the disease pathogen viz. *Fusarium oxysporum.* Selection of medium was carried out by using the chemicals like (ammonium nitrate, $K_2H\ PO_4$, Magnesium sulphate, 7H2O, potassium chloride, glucose, ridomil, rose bengal, agar, chloramphenicol and distilled water) in the media by using boiled water and distilled water, with addition of agar and without agar. The result revealed that *Trichoderma* selective medium with distilled water and agar is found suitable for mass production of liquid *Trichoderma*. The medium is selected because the growth of *Trichoderma harzianum* is found superior than the medium with boiled water and without agar as well as the medium with distilled water without agar.

For mass production of liquid *Trichoderma* a procedure is standardized which includes grinding of thick mat of *Trichoderma* and keeping it for incubation at 25^0C and flow chart for mass production of liquid *Trichoderma* is prepared. For shelf life studies microbial count up to twelve month were recorded and evaluated.

From the studies it is observed that *Trichoderma* selective medium by using distilled water is found to be suitable for mass production of liquid formulation of *Trichoderma* because the *CFU* count is more in case of distilled water than the medium with boiled water.

This work endorse the claim that liquid formulation of *Trichoderma* with distilled water is promising technique for improving shelf life, viability, storage condition and to prevent from the effect of high temperature.

Liquid formulation of *Trichoderma* is more practical to apply as it does not loss its properties due to sub-culturing and it has better survival on seeds and soil. Hence it is better to produce liquid formulation of *Trichoderma* than solid form. This technique is definitely useful for mass production of liquid *Trichoderma* in future.

This technique is definately useful for mass production of liquid formulation of *Trichoderma* in future.

INTRODUCTION

In worldwide the pests, insects, disease causing pathogens, weeds and nematodes are estimated to cause an annual loss of about 33-35 percent of the potential food production. In India annual crop losses are around Rs. 290,000 million. In order to increase agricultural production "Green Revolution" have been launched in that the introduction of dwarf, high yielding cultivars, higher fertilizers doses, intensive cropping, monocropping, etc.are inevitable. Due to introduction of new inputs lead to serious pest problems.

A worldwide an estimated 67000 different pest species attack on agricultural crops, to protect them, many chemical pesticides have an unfavorable environmental impact, and there is pressure for decreased reliance on such agents and greater regulatory control of their use.

Estimated Crop losses due to pest and diseases

Crop	Percentage Losses	Rs. (Crore)
Rice	18.16	5,512
Wheat	11.4	1,415
Jowar	10.0	173
Pulses	7.0	484
Oilseeds	25.0	4,180
Cotton	22.0	2,000
Sugarcane	15.0	1,336

Moreover, many pests have acquired resistance to widely used chemical pesticides. The trend is towards integrated pest management (IPM) programmes that decrease the frequency and intensity of the genetic selection of resistant insect mutants by employing in concern different means of insect population control. This strategy was very effective, but not sustainable and many small farmers were not able to take the advantages of high yielding varieties. The sustainability on environmental quality as the key components for the growth of agriculture and the protection. A natural resource base for which the intensification of integrated pest management in crop protection is mandated. IPM is a proven technology that is economical, socially accepted and environmentally sound. The integration of cultural practices, host plant resistance, mechanical method and application of biological suppression methods often avoids the need for pesticide application.

Biocontrol agents and Biopesticide in Indian agriculture

In spite of increasing chemical consumption, the agricultural production in India is almost static since 1989 due to poor soil nutrition status and pests and diseases causing over Rs. 30,000 crore crop losses per annum. Indiscriminate use of chemical pesticides has resulted in resistance of pest, resurgence of minor pests and high level of pesticides residues, due to which more than Rs. 4,000 crore worth of Indian agricultural exports get rejected every year. High pesticidal residues in food chain cause pesticides poisoning cases and deaths through malfunctions, immune suppression, neurotoxicity, impairment of reproductive functions, carcinogenicity, paralysis, etc. and harm to beneficial fauna and flora.

As we approach the 21st century, there is an enlarging stress on organically produced food, conservation of biodiversity, unpolluted environment and sustainable agriculture. To compound these challenges, Biocontrol agents and biopesticides have emerged as viable alternative in pest and disease control. Biopesticides are advantageous in view of their ecosafety, specificity, reduced number of applications, no resistance in the pests, increased yield and quality improvement of crops, higher acceptability and higher value of produce for exports and suitability for rural masses.

Biocontrol agents and biopesticides take care of losses of crops, losses of exports losses of man-hours and lives and losses of beneficial, natural parasites and predators. In spite of thrust from Government of India and some State Governments, the adoption of bio-control agents and biopesticides by farmers is still in its infancy, only about 5,000 villages and around 1,00,000 farmers having reaped the knowledge and benefits.

The slow rate of adoption of bio-inputs is basically due to a preference for the procurement at lowest rates, giving no important to quality. The lack of feedback on the effectiveness of biopesticides supplied under the various scheme has also shifted progress. The literature provided is mainly concerned with chemical pesticides and does not emphasize biopesticides, thus continuing with the old practices, because presumably a change in practices to biological would require considerable extension effort. Very little has been done to quality assess biocontrol agents and biopesticides and in absence of adequate knowledge and updating of the state pesticide testing laboratories for biopesticides is still awaited.

The biopesticide industry has enlarged and a variety of products are now available across the country, but most of the entrepreneurs have set up units without much concern for quality. However the few of them seriously manufacture quality products, follow regulatory procedures contribute significantly to the proper extension down to end users. In absence of available local advice the farmers depend totally on dealers.

Trichoderma spp. are cosmopolitan and abundant fungi in soil in a wide range of ecosystem and climatic zones. They have been used as biocontrol agents to protect plants against soil- borne and foliar diseases in several crops. The most common method for growing *Trichoderma* is on solid media which is too expensive and impractical for commercial production. Whereas the liquid fermentation based formulation of *Trichoderma spp.* are vulnerable to desiccation compared to solid state fermentation based formulations.

Advantages of Liquid Biopesticide (*Trichoderma*):

1] Better shelf life
2] No effect of high temperature
3] Identified by typical fermented smell
4] No loss of properties due to sub-culturing
5] Better survival on seeds and soil

Voluminous work on the chemical pesticide has been reported by several workers. However, no systematic work has so far been undertaken on liquid *Trichoderma* about its isolation and identification of efficient strain, suitable media for production, standardize procedure for mass production, microbial population and self life or viability for six months, hence it is necessary to undertake studies on these topic

In view of the above circumstances, the present investigation was undertaken to study the "Production technology of liquid formulation of *Trichoderma* with following objectives

1. Identification of efficient strains of *Trichoderma*.

2. To identify suitable media for growth of efficient strains of liquid formulation of *Trichoderma*.

3. To standardize the procedure for mass production of liquid formulation of *Trichoderma*.

4. Shelf life or viability studies and microbial population up to twelve months.

REVIEW OF LITERATURE

The present study was undertaken to investigate the production technology of liquid formulation of *Trichoderma* in the laboratory of the Department of Plant Pathology and Agricultural Microbiology MPKV Rahuri during 2011-2012. Literatures pertaining to these aspects is reviewed in this chapter and represented accordingly.

2.1 *Trichoderma* as a Biocontrol agent:

Weindling (1932) reported that the potential of *Trichoderma spp*. as biocontrol agents of plant pathogens was first recognized in the early 1930s.

Dennis and Webstar (1971) reported that the competition for space, infestation sites are the possible mechanisms involved in the biocontrol activities of *Trichoderma spp*.

Papavizas (1985) reported that *Trichoderma spp*. acts as biocontrol agent.

Trosnmo(1986) reported that *Trichoderma spp*. are able to survive in the phylloplane for more than one year.

Rossman (1996) stated that *Trichoderma spp*. are economically important as they are primary decompers.

Kubicek and Penttila (1998) reported that *Trichoderma spp*. acts as biocontrol agents against a wide range of plant pathogens.

Klein and Eveleigh (1998) reported that *Trichoderma spp*. are cosmopolitan and abundant fungi in a wide range of ecosystem and climatic zones.

Tronsmo and Hjeljord (1998) reported that nutrient competition may be one of the mechanisms involved in the biocontrol activities of *Trichoderma spp*.

Harman *et al* (1998) studied on production of conidial biomass of *T.harzianum* for biological control.

Hermosa *et al* (2000) reported that *T. herzianum* has been widely used as biocontrol agents in the aerial environment.

Meyer and Roberts (2002) reported a strategy to enhance the effect of a biological wound treatment could be the use of a mixture of biocontrol strains, which may provide a greater protection under different environmental conditions than the application of individual biocontrol strains.

Howell (2003) stated that *Trichoderma spp*. was applied successfully as biocontrol agents against several plant diseases in commercial agriculture.

Zhang *et al* (2005) reported that *T.harzianum* is the dominant species among soil isolates.

Fravel (2005) reported that many commercial products of *Trichoderma spp*. are available for the management of soil borne plant diseases.

Woo *et al* (2006) suggested that Induced resistance is a phenomenon often suggested to be related to biocontrol by *Trichoderma spp*.

2.2 Media for Isolation:

Valerie *et al* (1981) compared the production of 6-pentyl-α-pyrone (6-PP) by *Trichoderma harzianum* in liquid and in solid state cultivation (LC and SSC). The maximum concentration of 6-PP produced by *T. harzianum* in SSC was 2.8 mg (g dry cell mass)$^{-1}$ equivalent to 0.9 g l^{-1} of impregnation medium, which is 17 times higher than that obtained in LC. The glucose consumed total yield 6-PP in SSC was 52 mg (glucose)$^{-1}$, eight times higher than that found in LC.

J.A. Lewis *et al* (1981) reported that isolates of *Trichoderma spp*. grew and produced chlamydospores as well as conidia in molasses-corn steep liquor (M-CSL), sucrose nitrate (SN), and glucose startrate (GT) media. In M-SCL, isolates of *T. hamatum*, *T. viride*, and *T. harzianum* formed 10.4, 5.9 and 1.11 × 10^8 chlamydospores g^{-1} dry weight of mycelium.

Gilly zimand *et al* (1994) worked on the use of RAPD procedure for the identification of *Trichoderma* strains.

Sivasithamparam (1998) reported that the compounds produced may be classified chemically as volatile, water-soluble or water-insoluble compounds.

Fravel *et al* (1998) reported that carriers of inocula are inert ingredients in the sense that they do not have disease control capacity.

Kredicts *et al* (2000) established that water activity in the production medium influenced the growth as well as extracellular enzyme activities of the biocontrol strains of *Trichoderma spp*.

Kulling *et al* (2001) reported the high degree of misidentification of *Trichoderma spp*.

Druzhinia *et al* (2005) reported that for identification of *Trichoderma* strains, Trichokey and Trichoblast are convenient tools available online that are based on the sequence comparisons of ITS or tef regions.

M. Anees *et al* (2010) reported that morphological characterization was based on microscopic measurements of mycellial fragments.

2.3 Media for liquid formulation:

Shields and Atwell (1963) reported that *Trichoderma spp*. had a limited effect on *polyporus adustus*.

Zuber *et al* (1981) Trans disclose micro cycle conidiation of *T. harzianum* in liquid cultures and several growth media are disclosed for this method.

Elad, Y., *et al*, (1982) Can. J. Microbial 28: 719-725; Vezina, C., *et al*. (1965) Mycologia 57: 722, Aube and Gagnon, (1969) Can. J. Microbiol., 15:703-705 studied the growth and sporulation of several isolates of *T. viride* in liquid cultures. The method involved growing the organisms for a week in the dark at 20° C. as a stationary culture.

Harman *et al* (1991) reported that liquid fermentation can facilitate abundant production of conidial biomass at shorter period.

Jin *et al* (1991) reported that the osmoticum of the production medium can be adjusted by the addition of poly – ethylene glycol or glycerol that can induce trehalose production and provide the desiccation tolerance.

Askew *et al* (1993) used *Trichoderma*-Selective Medium (TSM) in which three agar discs (5 mm) were removed from non sporulating regions and placed on *Trichoderma*-Selective Medium.

Connick *et al* (1996) reported that environmental condition during storage also affect the viability of the formulation derived from liquid fermentation.

Fravel *et al* (1998) reported that carriers of inocula can profoundly affect time of germination as well as viability of conidia.

Hjeljored *et al* (2001) showed that the addition of nutrients (C and N sources) resulted in an increase in germinations rate and conidia viability.

Hjeljored (2001) reported that the addition of glucose and urea caused faster colonization of the wood samples by *Trichoderma spp*. and in their presence the protective effect was increased.

Duffy (2003) reported that melanin is primary defence system in all organisms and that resistance of pathogenic fungi to microbial lysis is positively correlated with the melanin content in hyphae.

Chandra K., Shrivathsa R. S. H., Greep S., and Ingle S. R.(2004) found that liquid formulation contain stickers which improve adherence of an organism to foliage and persistence during wind and rain, hence liquid inoculums is always superior

Elzein *et al* (2004) reported that factors like medium and inoculum type affect the viability of the formulation derived from liquid fermentation.

Singh *et al* (2006) reported that wettable powder formulation is popular in India though they have shorter shelf life.

Friesen *et al* (2006) found that method of drying as well as the addition of protectants affect the viability of the formulation derived from liquid fermentation.

Kolombet *et al* (2008) recently developed a paste formulation of *Trichoderma* asperellum with the addition of glycerol in the formulation that had extended shelf life.

Gogoi *et al* (2008) reported that the addition of glycerol (2%) as carbon source promoted the biosynthesis of secondary metabolite by *Trichoderma spp*.

M. Schubert *et al* (2008) mentioned that conidia were enriched with nutrients showed an increased water requirement resulting in reduced germination due to lowered salt water potential.

Subbaraman Sriram *et al* (2011) reported that liquid fermentation based formulation of *Trichoderma spp*. are vulnerable to desiccation compared to solid state fermentation based formulation.

Maheshwarappa Jeyammasavitha *et al* (2011) reported that the addition of glycerol at 3to 6 percent extended the self life from 7 to 12 months respectively compared to 4 to 5 month self life in formulation derived without the addition of glycerol.

2.4 Antimicrobial properties of *Trichoderma*:

In Ann. Rev Microbiology (1981). It was found that *Trichoderma spp*. can effectively reduces diseases caused by some soil-borne plant pathogens.

Windels *et al* (1985) reported that colonization by the antagonistic candidate is a general requirement for biocontrol.

Chet (1987) reported that *Trichoderma spp*. develops a hook like structure known as appresoria.

Liese *et al* (1988) recorded and measured callus growth, discoloration in areas of the wounds and the effectiveness of the antagonist i.e. *Trichoderma*.

Simon *et al* (1988) observed the inhibition of *Trichoderma spp*. by different bacteria in dual culture tests.

Lorito *et al* (1998) reported that *Trichoderma* directly attacks the pathogen by excreating the lytic enzymes such as chitinases and proteases.

Naar and Kecskes (1998) demonstrated that in several *in vitro* tests the bacteria clavibacter michigens and pseudomonas syringae caused significant inhibition of *Trichoderma spp*. by the production and excretion of antibiotic substances.

Hjeljord and Tronsmo (1998) reported that *Trichoderma spp*. are producers of antibiotic as well as enzymes.

Howell (1998) reported that *Trichoderma spp*. inhibit plant pathogenic fungi through mycoparasitism as well as competition.

Elad (2000) stated that apart from the antagonistic activity, effective biological control also involves the ability to survive in the habitat where the agent is applied.

Hermosa *et al* (2000) reported that *T. herzianum* has been widely reported as biological agents in the aerial environment.

Kulling *et al* (2000) reported that *Trichoderma* produces diffusible hydrolytic enzymes and inhibits *R. Solani* when fungi are grown together.

Kubicek *et al* (2001) reported that appresoria is coupled with production of lytic enzymes and then penetrates the pathogen hyphae.

Lutz *et al* (2003) observed a negative influence and a reduction of antagonistic activity by the fungi *Fusarium culmorum* and *Fusarium graminearum.*

Perello *et al* (2003) stated that some form of competition could be the mode of action of the *Trichoderma* strains, since pertidishes were quickly colonized.

Howell (2003) reported that coiling of the plant pathogenic fungal hyphae by *Trichoderma spp*. one parameter used to characterize the mycoparasitism.through mycoparasitism as well as competition.

Grosch *et al* (2006) reported that sclerotia of *Rhizoctonia* on potatoes have been shown to be a source of beneficial antagonistic micro-organism including *Trichoderma spp*.

Woo *et al* (2006) suggested that Induced resistance is a phenomenon often suggested to be related to biocontrol by *Trichoderma spp*.

Almeida *et al* (2007) reported that no correlation between coiling frequencies and cell wall degrading enzymes was found.

Verma *et al* (2007) reported that *Trichoderma spp*. may inhibit plant pathogenic fungi either by inducing resistances and plant defence reaction.

Mghalu *et al* (2007) compared the structure of fungal communities in healthy and diseased areas in fields affected by *R. solani* which showed the accumulation of *Trichoderma spp*. in latter stage.

Vinale *et al* (2008) reported that *Trichoderma spp*. have also been reported to produce a plethora of secondary metabolites showing antimicrobial activity.

Anees *et al* (2010) reported that in the fields affected *R.Solani* – *Trichoderma spp*. accumulated were proposed to be responsible for the increased suppresiveness.

T. Pramod kumar *et al* (2009) reported that significantly highest population of antagonistic *Trichoderma harzianum* was recorded in molasses yeast medium both at 15 and 30 days.

Anees *et al* (2010) reported that *Trichoderma* may be responsible for the increased disease suppression inside the patches.

MATERIAL AND METHODS

The present study was carried out in laboratory of the Department of Plant pathology and Agricultural microbiology, Mahatma Phule Krishi Vidyapeeth, Rahuri - 413 722 (M.S.) India.

The details of material required and methods followed during the period of investigation are described briefly in succeeding paragraph.

3.1 Material

3.1.1 Places for soil sample:

The soil samples were collected from the fields of cotton, chilli, tomato, brinjal and betelvine at central campus, MPKV, Rahuri.

3.1.2 Microbial inoculants:

Trichoderma **inoculant**

The commercial based (talcum powder) inoculant packet (250g) of *Trichoderma* was obtained from the Biofertilizer production unit, Department of Plant Pathology and Agricultural microbiology MPKV Rahuri for comparative studies.

3.1.3 Glassware

The various types of glassware of corning and borosil brand like petriplates, test tubes, conical flasks, beakers, pipettes, measuring cylinder, volumetric flasks, funnel, burettes, glass rods, round bottom flask, digestion tubes and slides etc were used.

3.1.4 Equipments

Where and whenever required the common branded laboratory equipments like autoclave, hot air oven, BOD incubator, isolation chamber, refrigerator, electronic balance, grinder, digestion and distillation unit, cover slips spirit lamp etc. were used.

3.1.5 Miscellaneous Material:

Blotting papers, forceps, labels, sieve, cotton plug, sterile water, muslin cloth, scalpel, test tube stands, chemicals, Whatman No 40 filter paper available in the laboratory were used.

3.1.6 Suitable Media:

3.1.6.1 For isolation of *Trichoderma*:

Trichoderma Selective Medium (TSM) was used for isolation of *Trichoderma*

Table No. 3.1Composition of TSM

Sr. No.	Name of Chemical	Quantity
1	Magnesium sulphate	0.20 g
2	K_2HPO_4	0.90 g
3	Ammonium Nitrate	1.00 g
4	Potassium Chloride	0.15 g
5	Glucose	3.00 g
6	Agar	20.00 g
7	Ridomil/Mancozeb	0.15 g
8	Rose Bengal	0.15 g
9	Chloramphenicol	0.25 g
10	Distilled Water	1000 ml

3.1.6.2 For maintenance of fungal isolates:

Potato dextrose agar (PDA) medium was used.

Table No. 3.2 Potato dextrose agar (PDA) medium

Sr. No.	Component	Quantity
1	Potato	200 g
2	Dextrose	20 g
3	Agar	20 g

3.2 Method:

3.2.1 Isolation of *Trichoderma spp.*:

One gm of collected soil was taken and serially diluted up to 10^5 and plated on *Trichoderma* selective medium and Potato Dextrose Agar medium. The plates were incubated at 28± 2^0 C for 4 to 5 days. Further, purification of the colonies was done on Potato dextrose agar medium.

3.2.2 Characterization of *Trichoderma*:

Morphology and Cultural characters:

The morphological and cultural characters were studied for identification and characterization of *Trichoderma spp.* on plates of the selective medium.

3.2.3 Evaluation of *Trichoderma spp.* (*in vitro*)

3.2.3.1 *In vitro* effect of *Trichoderma spp.*

Antagonistic potential of four species of *Trichoderma* viz.,*T harzianum*,*T.viride*, *T.konigii*, *T.hamatum* was assessed against *Fusarium oxysporum f.sp. lycopersici* by dual inoculation technique.(Upadyay and

Rai,1987) Four treatments one control and three replications were carried out. The treatment details of bioagents was as per Table 3.3

Mycellium disc of 5 mm diameter was cut from the margin of 7 days old culture of test pathogen and antagonistic agent respectively and placed opposite to each other on PDA in 3 petriplates having diameter of 90 mm. The discs were placed 30 mm away from each other. The petriplates inoculated with disc of *Fusarium oxysporum* alone served as control.

The inoculated plates were incubated in inverted position at 27 ± 1^{0} C in BOD for 7 days. The radial growth of *F. oxysporum* was measured to assess the antagonistic potential of *Trichoderma spp*. against pathogen. The percent growth inhibition of pathogen colonies was calculated as per following formula.

Percent growth inhibition = $$\frac{D1 - D2}{D1} \times 100$$

Where,

D1 = Diameter of pathogen colony in control.

D2 = Diameter of pathogen colony in treatment.

Table No.3.3 *Trichoderma* tested against pathogen *F. oxysporum*.

<table>
<tr><th>Sr. No.</th><th>Bio Agent Used</th><th>Source</th></tr>
<tr><td>1</td><td>Trichoderma harzianum</td><td>Collected from soil sample.</td></tr>
<tr><td>2</td><td>Trichoderma viride</td><td rowspan="3">Department of Plant pathology and Agril Microbiology. M.P.K.V. Rahuri</td></tr>
<tr><td>3</td><td>Trichoderma hamatum</td></tr>
<tr><td>4</td><td>Trichoderma konigii</td></tr>
</table>

3.2.4 Procedure:

After isolation of *Trichoderma spp*. the strains were incubated until the thick mat was produced. The mat was then removed from the flask and grinded into minute pieces.

Following chemicals are added in above mixture.

1. Groundnut oil - 2.0 %
2. Tween-80 - 0.5 %
3. Streptocyclin - 0.2 %

The above solution was prepared in two flasks containing distilled water and boiled water respectively. After preparing the two separate flasks, the following chemicals were added to both the flasks.

1. Polyvynyl acetate -2%
2. Glycerol -3 to 5%
3. Malic acid -0.1%

The broths of both distilled and boiled water were kept for incubation at 25 to 28^0C. After one month they were again distributed in six flasks of 250 ml.

1. Distilled water 250 ml
2. Boiled water 250 ml

The solution was then filtered through Whatsman filter paper no.40 in 250 ml bottle each. Now from two bottles i.e. distilled and boiled water, 1 ml culture was taken and serial dilution was carried out from 10^{-7} to 10^{-9}.

The *CFU* counts are taken for,

10^{-7} -3 plates
10^{-8} -3 plates
10^{-9} -3 plates

The above colony counts were taken for both boiled and distilled water culture. The counts were taken with the interval of one month upto twelve

.

RESULT

A laboratory experiment was conducted at Department of Plant Pathology and Agril. Microbiology to study the production technology of liquid formulations of *Trichoderma*. The results obtained from the experiment are presented in this chapter as

4.1 Isolation of *Trichoderma spp*. from the rhizosphere:

An isolate of *Trichoderma* was isolated from rhizosphere soil of tomato plant of central campus of M. P. K. V. Rahuri on TSM It was found that the soil sample contained is of harzinum spp.whereas the other species of *Trichoderma* viz. *T. hamatum, T. viride, and T. koligii* were obtained from the Dept. of plant Pathology and Agril.microbiology. The fungal colonies which appeared after 4-5 days of incubation were transferred to PDA medium followed by PDA slant and the morphological characters as well as cultural characters were recorded.

4.1.2 Characterization of *Trichoderma*:

The morphological characteristics (sterile hairs, conidia) and cultural characters (colony characters) were studied and presented in Table No.1

Based on various morphological and cultural characters the *Trichoderma* isolates were identified.

Table No. 4.1: Characterization of *Trichoderma*

Sr. No.	*Trichoderma* Isolates	Morphological Characteristics		Cultural characteristics [Growth of *Trichoderma* isolate (in mm) on PDA]			
		Sterile Hairs	Conidia	20^0C	25^0C	30^0C	35^0C
1	*T. harzianum*	Absent	Globose to subglobose	44.67	59	68	40
2	*T. hamatum*	Straight	Ellipsoidal	41.7	53	35	23
3	*T. viride*	Absent	Globose to subglobose	45.0	55	40	0.7
4	*T. koligii*	Absent	Ellipsoidal to oblong	40.3	54	52	2.3

The measurements of cultures on PDA media represent the mean of three independent values measured after 72 hrs. in the dark. All the isolates showed green pigmentation of spores. No growth was observed at 40^0 C on media and pustules formation on PDA.

In the above species *T. harzianum* was most dominant species and it had highest growth than other species at 30^0C. Hence *T. harzianum* was selected for the research work.

4.2 Evaluation of *Trichoderma spp*.:

The inhibitory growth study of the test fungus *Fusarium oxysporum* by using the antagonistic *Trichoderma* was carried out in *in-vitro* condition. The result of inhibition of *Fusarium oxysporum* is given in table no 4.6.

Table No. 4.2 Evaluation of *Trichoderma spp*.

Sr. No.	Biological Agent	Mean colony diameter (mm)* after 7 days	Percent inhibition
1	*T. harzianum*	13.00	83.75
2	*T. viride*	23.00	71.25
3	*T. hamatum*	26.30	67.05
4	*T. koningii*	29.00	63.75
5	Control(*Fusarium oxysporum*)	80.00	-

*** = Average Colony Diameter**

The result revealed that, all the antagonists showed inhibitory effect on growth of the test fungus and were effective in controlling the growth of pathogen. Among these 4 species of *Trichoderma,* the *Trichoderma harzianum* was found to be most effective in inhibiting the growth of *Fusarium oxysporum* of about 83.75% growth of test fungus, followed by *Trichoderma viride* inhibited 71.25%, *Trichoderma hamatum* inhabited 67.05% and *Trichoderma koningii* inhibited 63.75% growth of test fungus which was less effective than above species.

4.3 Chemical composition of media identified for production of liquid *Trichoderma*

From the laboratory studies on identification of suitable media for procedure of liquid formulation of *Trichoderma* the results were presented in table no. 4.2.

Table No. 4.3: Chemical composition of media identified for production of liquid *Trichoderma*.

Sr. No.	Name of component	Quantity
1	Magnesium sulphate	0.20 g
2	K_2HPO_4	0.90 g
3	Ammonium nitrate	1.00g
4	Potassium chloride	0.15 g
5	Glucose	3.00 g
6	Agar	20 g
7	Ridomil	0.15 g
8	Rose Bengal	0.15 g
9	Chloremphenicol	0.25 g
10	Distilled Water	1.00 lit
11	Ground nut oil	2%
12	Twin 80	0.5%
13	Streptocyclin	0.2%
14	Polyvynyl Acetate	2.0%
15	Glycerol	5%
16	Malic Acid	0.1%

In the medium 0.5 percent Twin 80 acted as a preservative whereas the addition of 3 to 5 percent glycerol extended the shelf life of liquid *Trichoderma* from 7 to 12 months.

4.4 Procedure for mass production of liquid *Trichoderma*

From the studies under taken on mass production of liquid formulation of *Trichoderma* the following procedure was found suitable for the mass production of liquid *Trichoderma*.

Trichoderma spp. was isolated from the rhizosphere soil sample taken from tomato plant at central campus of M.P.K.V. Rahuri. The strains isolated were observed for morphological and cultural characteristics and the suitable strain i.e. *T. harzianum* based on studies undertaken was selected for research.

The strain was incubated on the media containing with addition of agar and distilled water, without addition of agar and distilled water, with addition of agar and boiled water, without addition of agar and boiled water in respective four flasks.

The strain was incubated at 28^0 C temperature until the thick mat is formed. The thick mat was formed in the flask containing agar and distilled water and boiled water. When the mat was formed, an upper layer of the mat was removed carefully, so that the material other than *Trichoderma* could not mix with the mat. The mat was grinded.

After grinding the mat the chemicals Twin 80 0.5% was added which acted as preservative. At the same time ground nut oil 2% and streptocyclin 0.2% was added.

The formed solution was divided into two different flasks- one is containing boiled water whereas the other containing distilled water. Thereafter in both the flasks, PVA- 2%, Glycerol- 5% and Malic acid - 0.1% were added.

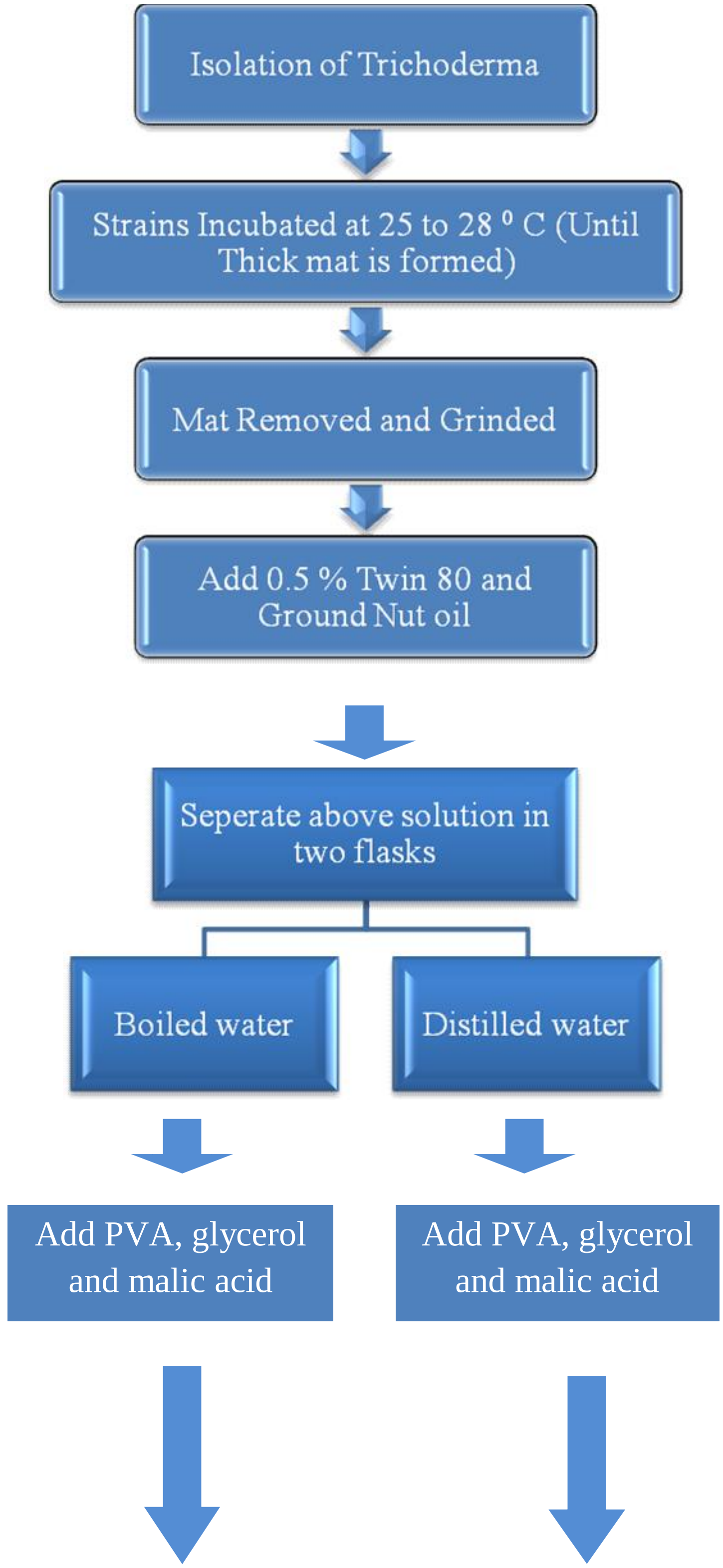
After adding the above chemicals both the flasks were incubated at 25 to 28^0C temperature for one month.

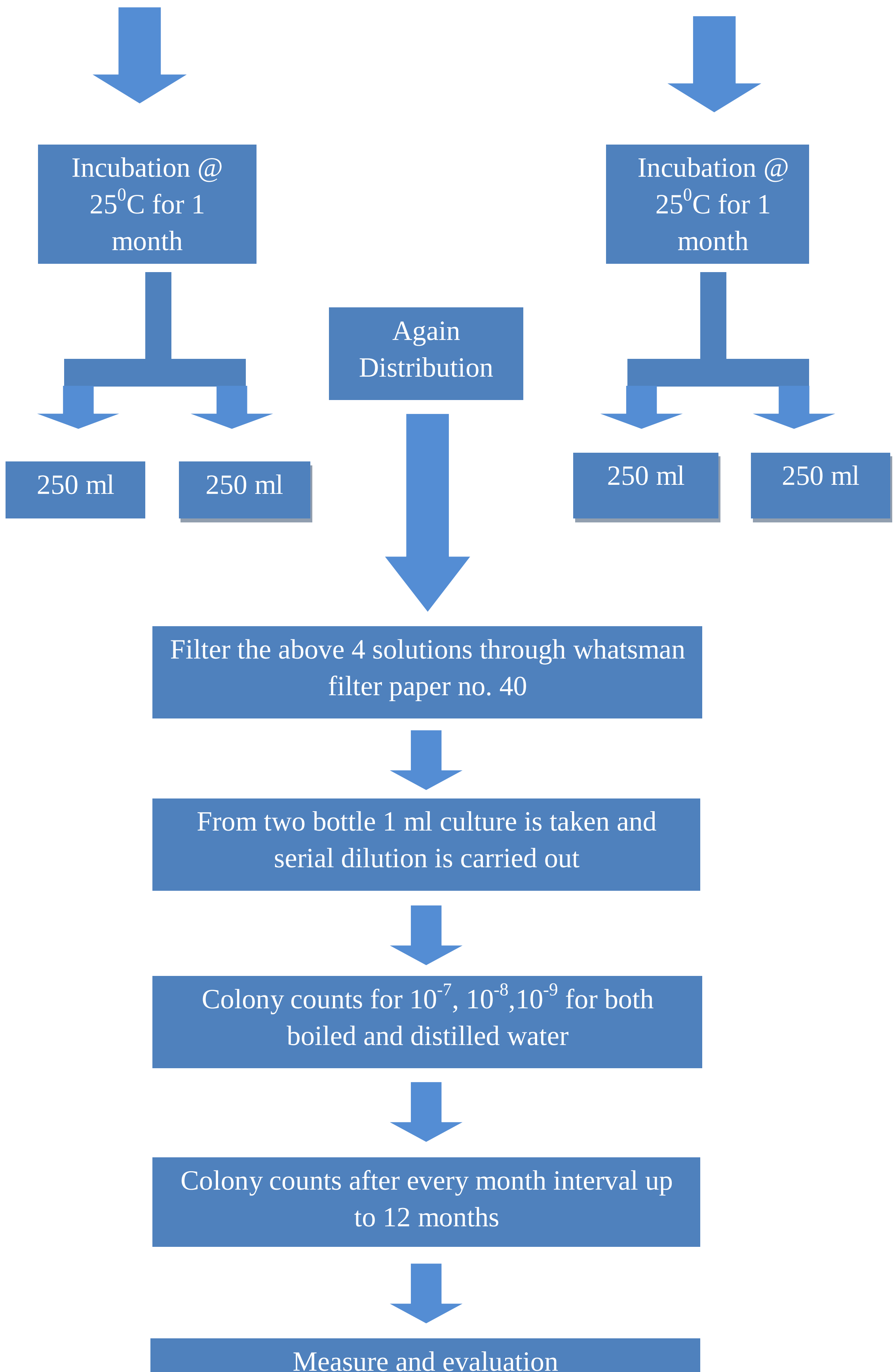
After one month the two flasks were again distributed in 250 ml flasks and the total no. of flasks were four. All these four solutions were passed through whatsman filter paper no.40.The four solutions were kept in sterilized 250 ml polypropylene bottles.

One ml culture was taken from two bottles i.e. distilled and boiled water respectively and serial dilution was carried out. Colony forming units were counted for 10^{-7}, 10^{-8} and 10^{-9} for both boiled water and distilled water.

Colony forming units/ml was taken after every month interval up to twelve months. Evaluation was carried based on the presence of spores at 10^{-7}, 10^{-8} and 10^{-9} dilution.

Flow chart of Procedure for Production of liquid *Trichoderma*




Incubation @ 25⁰C for 1 month
Incubation @ 25⁰C for 1 month
Again Distribution
250 ml
250 ml
250 ml
250 ml
Filter the above 4 solutions through whatsman filter paper no. 40
From two bottle 1 ml culture is taken and serial dilution is carried out
Colony counts for 10^{-7}, 10^{-8},10^{-9} for both boiled and distilled water
Colony counts after every month interval up to 12 months
Measure and evaluation

4.5 Shelf life or Viability studies and Microbial Population up to twelve month:

To study the effectiveness of liquid *Trichoderma* from first month (March 2011) to twelve month (February 2012), microbial population was counted up to twelve months. The following results were obtained.

4.5.1 Colony forming unit of *Trichoderma* at 10^{-7} for both boiled water and distilled water

The *CFU* was counted from March 2011 to Feb 2012 with respect to boiled water and distilled water. It was observed that in the first month, reading of *CFU*/ml was 88×10^{-7} and 93×10^{-7} of boiled water and distilled water respectively.

The counts were reduced as the monthly interval passed. In the month of May 2011 it was observed that *CFU* was came down to 81×10^{-7} in case of distilled water whereas in case of boiled water it reduced to 71×10^{-7}

After six months the *CFU* count for Aug 2011 was 74×10^{-7} and 23×10^{-7} for both distilled water and boiled water respectively. The counts were again came down to in the month of November 2011.They were recorded as 53×10^{-7} and 10×10^{-7} in case of distilled water boiled water respectively

In the last month i.e. in the month of Feb. 2012 the *CFU* count/ml was 13×10^{-7} and 7×10^{-7} for both distilled water and boiled water respectively.

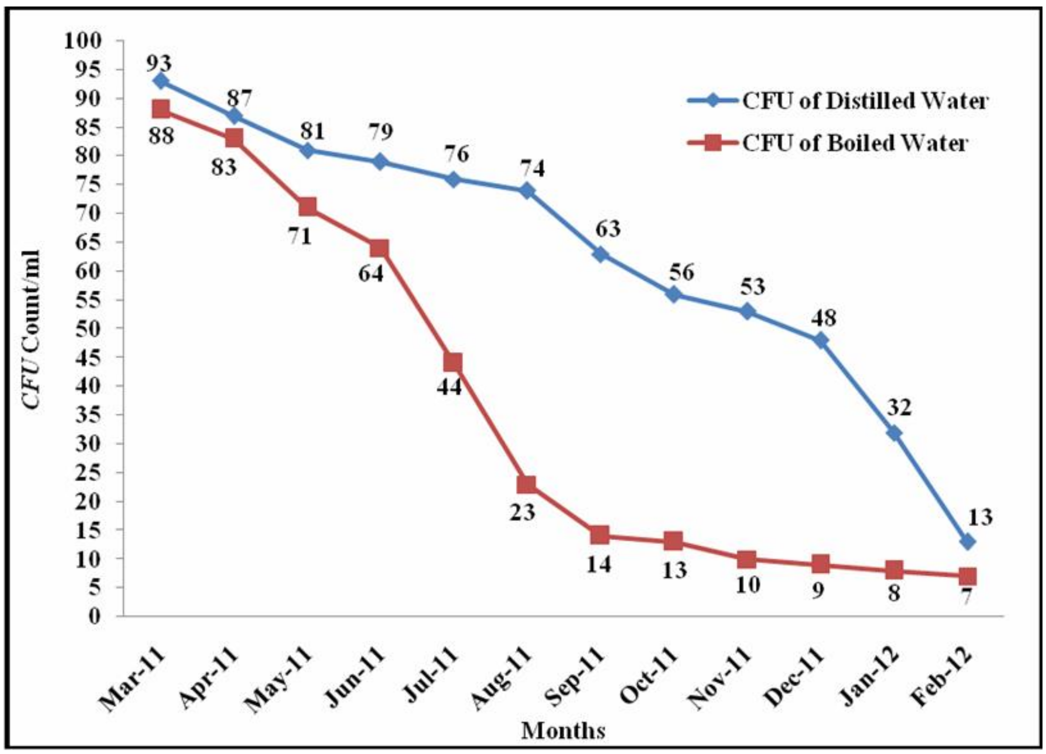
From the results it is observed that the *CFU* count of distilled water was more than that of boiled water up to 12 months. The results of *CFU* from March 2011 to February 2012 (Twelve month) are presented in Table4.3 and graphical presentation of *CFU* count is given in Table no. 4.3.1

In graphical presentation in case of boiled water a straight line was observed from the month of June 2011 to August 2011 i.e. from 64×10^{-7} to 23×10^{-7} and in case of distilled water a complete reduction in the *CFU* count was observed from the month of December 2011 to February 2012 i.e.48×10^{-7}to13×10^{-7}.

Table No. 4.5 Colony forming unit of *Trichoderma* at 10^{-7} for both boiled and distilled water.

Sr. No	Month	*CFU* of Distilled Water	*CFU* of Boiled Water
1	Mar-11	93×10^{-7}	88×10^{-7}
2	Apr-11	87×10^{-7}	83×10^{-7}
3	May-11	81×10^{-7}	71×10^{-7}
4	Jun-11	79×10^{-7}	64×10^{-7}
5	Jul-11	76×10^{-7}	44×10^{-7}
6	Aug-11	74×10^{-7}	23×10^{-7}
7	Sep-11	63×10^{-7}	14×10^{-7}
8	Oct-11	56×10^{-7}	13×10^{-7}
9	Nov-11	53×10^{-7}	10×10^{-7}
10	Dec-11	48×10^{-7}	9×10^{-7}
11	Jan-12	32×10^{-7}	8×10^{-7}
12	Feb-12	13×10^{-7}	7×10^{-7}

Fig. No.1 Graphical presentation of *CFU* at 10^{-7} for both boiled and distilled water.



4.5.2 Colony forming unit of *Trichoderma* at 10^{-8} for both boiled water and distilled water

The *CFU* was counted from March 2011 to Feb 2012 with respect to boiled water and distilled water. It was observed that in the first month of March 2011 the reading of *CFU*/ml was 72×10^{-8} and 86×10^{-8} for boiled water and distilled water respectively.

As the monthly interval passed the *CFU* count came down. In the month of June 2011 the *CFU* count was came down to 54×10^{-8} in case of distilled water whereas 52×10^{-8} in case of boiled water.

After six months in the month of September.2011 the *CFU* count was came down to 16×10^{-8} and 24×10^{-8} for boiled water and distilled water respectively.

The counts were reduced in the month of September 2011 to 24×10^{-8} in case of distilled water and it was 16×10^{-8} in case of boiled water.

In the last month i.e. in the month of Feb. 2012 the *CFU*/ml was 5×10^{-8} and 6×10^{-8} for boiled water and distilled water respectively.

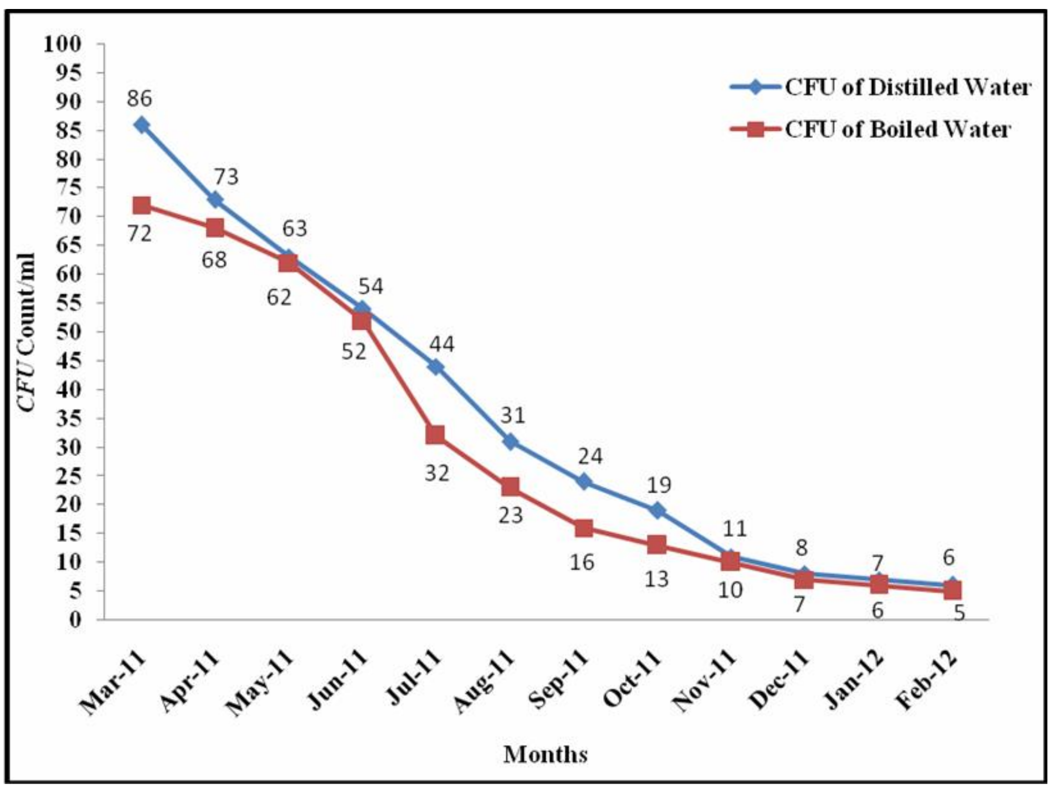
Here the *CFU* of distilled water was more than that of boiled water since March 2011 to Feb.2012 (i.e.12 months). The detail information of counts at 10^{-8} for 12 months is presented in Table no.4.4 and graphical presentation in Table no.4.4.1.

In graphical presentation of *CFU* at 10^{-8} a sharp reduction was observed in the counts from the month of July 2011 to October 2011 i.e. the counts were reduced from 44×10^{-8} to 19×10^{-8} in case of distilled water. Whereas in case of boiled water a *CFU* reading made a straight line from the month of June 2011 to July 2011 i.e. the *CFU* count is 52×10^{-8} and 32×10^{-8} of the June and July month respectively.

Table No. 4.6 Colony forming unit of *Trichoderma* at 10^{-8} for both boiled and distilled water:

Sr. No.	Month	*CFU* of Distilled Water	*CFU* of Boiled Water
1	Mar-11	86×10^{-8}	72×10^{-8}
2	Apr-11	73×10^{-8}	68×10^{-8}
3	May-11	63×10^{-8}	62×10^{-8}
4	Jun-11	54×10^{-8}	52×10^{-8}
5	Jul-11	44×10^{-8}	32×10^{-8}
6	Aug-11	31×10^{-8}	23×10^{-8}
7	Sep-11	24×10^{-8}	16×10^{-8}
8	Oct-11	19×10^{-8}	13×10^{-8}
9	Nov-11	11×10^{-8}	8×10^{-8}
10	Dec-11	8×10^{-8}	7×10^{-8}
11	Jan-12	7×10^{-8}	6×10^{-8}
12	Feb-12	6×10^{-8}	5×10^{-8}

Fig. No.2 Graphical presentation of *CFU* at 10^{-8} for both boiled water and distilled water:



4.5.3 Colony forming unit of *Trichoderma* at 10^{-9} for both boiled and distilled water:

The *CFU* was counted from March 2011 to Feb 2012 with respect to boiled water and distilled water. It was observed that in the first month, reading of *CFU*/ml was 39×10^{-9} and 66×10^{-9} of boiled water and distilled water respectively.

The counts were reduced as the monthly interval passed. In the month of June 2011 the count was highly reduced from 39×10^{-9} to 21×10^{-9} of boiled water and in case of distilled water it was reduce from 66×10^{-9} to 24×10^{-9}.

In the month of November 2011 the *CFU* counts were highly reduced and they came down to 9×10^{-9}. In case of distilled water whereas in case of boiled water the count came down to 6×10^{-9}.

In the last month i.e. in the month of Feb. 2012the *CFU* count/ml was 2×10^{-9} and 3×10^{-9} for both boiled water and distilled water respectively.

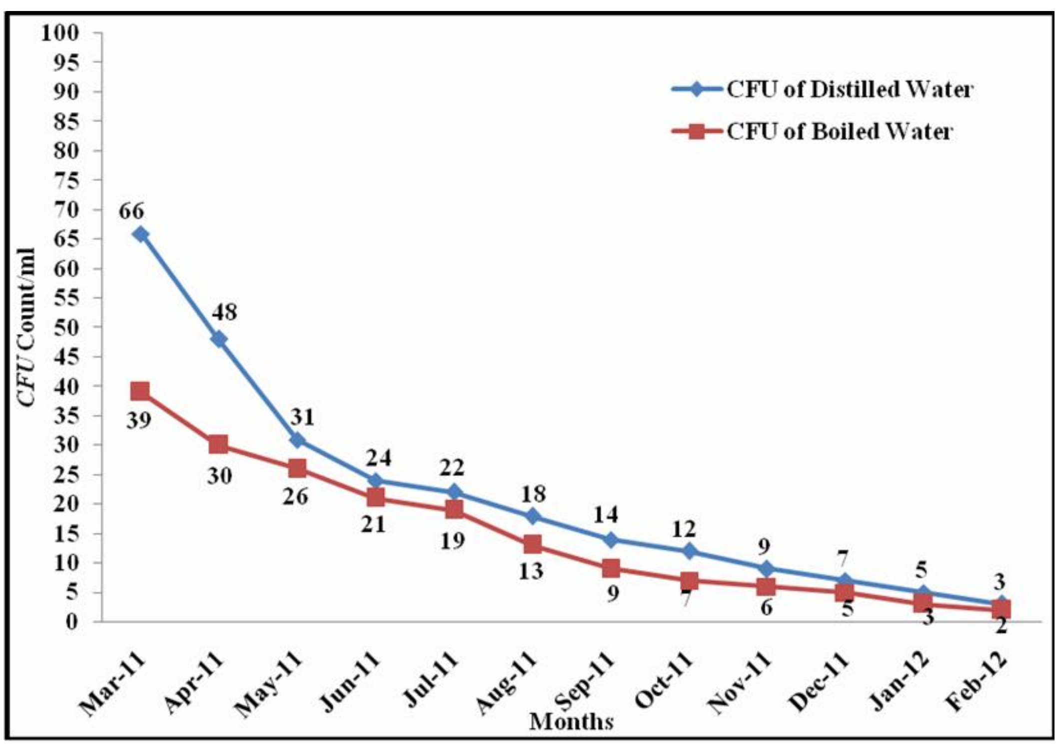
It is observed that the *CFU* of distilled water was more than that of boiled water at 10^{-9} from March 2011 to February 2012(i.e. for 12 months) The results of *CFU* count from March 2011 to February 2012 were presented in Table no. 4.5 and graphical presentation in Table no.4.5.1.

In graphical presentation in case of distilled water *CFU* count has become straight line from the month of March 2011 to May 2011 i.e. from 66×10^{-9} reduced to 31×10^{-9} whereas in case of boiled water no such straight line was observed but a noticeable difference was seen from 19×10^{-9} to 13×10^{-9} in the month of July 2011 to August 2011.

Table No. 4.7 Colony forming unit of *Trichoderma* at 10^{-9} for both boiled and distilled water.

Sr. No.	Month	*CFU* of Distilled Water	*CFU* of Boiled Water
1	Mar-11	66×10^{-9}	39×10^{-9}
2	Apr-11	48×10^{-9}	30×10^{-9}
3	May-11	31×10^{-9}	26×10^{-9}
4	Jun-11	24×10^{-9}	21×10^{-9}
5	Jul-11	22×10^{-9}	19×10^{-9}
6	Aug-11	18×10^{-9}	13×10^{-9}
7	Sep-11	14×10^{-9}	9×10^{-9}
8	Oct-11	12×10^{-9}	7×10^{-9}
9	Nov-11	9×10^{-9}	6×10^{-9}
10	Dec-11	7×10^{-9}	5×10^{-9}
11	Jan-12	5×10^{-9}	3×10^{-9}
12	Feb-12	3×10^{-9}	2×10^{-9}

Fig. No.3 Graphical presentation of *CFU* at 10^{-9} for both boiled water and distilled water:



It is seen from Table no 3, Table no 4 and table no 5 the *CFU*/ml count was more in distilled water than boiled water at 10^{-7}, 10^{-8} and 10^{-9} dilution.

It is concluded that the liquid formulation of *Trichoderma* with distilled water is more suitable than boiled water with respect of shelf life and viability.

Storage of Liquid *Trichoderma*

It was found that for mass production of liquid *Trichoderma* polypropylene bottles of 100 micron thickness having milky white colour were suitable for long term storage.as they do not react with the content polypropylene bottles could be used efficiently.

DISCUSSION

The present research was undertaken to study the production technology of liquid formulation of *Trichoderma*.

The purpose of producing liquid *Trichoderma* is for long term use. The common method for growing *Trichoderma* on solid media is laborious for commercial production, whereas liquid *Trichoderma* is very practical for commercial production as it is identified by typical fermented smell and there is no loss of properties due to sub culturing.

The present study was planned to study the identification of efficient strain of *Trichoderma,* to identify the suitable media for production of liquid *Trichoderma*, to standardize the procedure for mass production and shelf life or viability studies and microbial population up to twelve month. The results obtained on various attributes have been discussed below.

5.1 Antimicrobial properties of *Trichoderma*

To identify efficient strain of *Trichoderma*, food poisoning technique was carried out. As *Trichoderma* is antagonistic in nature it was found that *Trichoderma* resist the growth of other microbes where it grows.

From the species *T. harzianum* was found most efficient. *Trichoderma* produces antibiotics like viridin, gleoxin which controls the growth of diseases. The fact matched with Lorito *et al* (1998) who reported that *Trichoderma* directly attacks the pathogen by ecreting the lytic enzymes such as chitinases and proteases.

It was found that *Trichoderma* quickly colonise. This fact is supported by Windels *et al* (2005) as he reported that colonization by antagonistic candidate is general requirement for biocontrol.

5.2 To identify suitable media of efficient strain of liquid *Trichoderma*:

Trichoderma selective medium (TSM) was found suitable for production of liquid *Trichoderma*. This result corroborates the finding of Askew and Laing (1993) who used *Trichoderma* selective medium in which three agar discs (5mm) were removed from non sporulating regions and placed on *Trichoderma* selective medium.

It was found that the addition of glycerol at 3 to 6 % extended the shelf life from 7 to 12 months. Similar result was reported by Kolombet *et al* (2008) who developed a paste formulation of *Trichoderma* with the addition of glycerol in the formulation that has extended shelf life.

In TSM medium groundnut oil 2 % acted as a sticker. This found true as Chandra k *et al* (2005) reported that liquid formulation contain stickers which improve adherence of an organism to foliage and persistence during wind and rain.

5.3 To standardize the procedure for mass production of liquid *Trichoderma*:

While preparing liquid *Trichoderma* formulation, the thick mat which was removed from the TSM medium was grinded and after adding preservative i.e. 0.5% Twin 80 it was again incubated at 28^0C temperature to see the further growth similar result are reported by S. Sriram *et al* (2011) who inoculated the broth culture with *T. harzianum* and incubated at 28^0C.

The initial population and the population of viable conidia after one month of storage were enumerated in these formulations by placing of sample serially diluted to 10^{-7} dilution using pour plate method using a *Trichoderma* selective medium.

The above fact is similar with the findings of Edal *et al* (1981) who used the same method for the incubation of broth culture.

In the research the formulations were stored in closed stored in polypropylene bottles at ambient temperature in closed storage cabinets and viability in terms of *CFU*s were enumerated as described earlier at monthly interval. This procedure corroborates the findings of Elzein *et al* (2004).

5.4 Shelf life or viability studies and microbial population up to twelve month

It was found that with increase in the concentration of glycerol added to the production medium, there was increased retension of moisture in the formulation during shelf- life.

The regression analysis of the moisture content in relation to shelf life revealed that the rate of reduction in moisture content in formulation was inversely proportional to the concentration of glycerol added in the production medium.

The above result matched with the findings of Hallsworth *et al* (1995) who reported that manipulation of intracellular glycerol enhances germination of conidia at low water availability.

SUMMARY AND CONCLUSION

The present investigation entitled “Studies on production technology of liquid formulation of *Trichoderma*” was undertaken in the Department of Plant Pathology and Agriculture microbiology, Mahatma Phule Krishi Vidyapeeth Rahuri during February 2011 to February 2012.

The objective of the research were isolation and identification of efficient strains of *Trichoderma*, To identify suitable media of efficient strains of liquid *Trichoderma* and shelf life or viability studies and microbial population up to twelve month.

6.1 Summary:

The important findings of the present investigation have been summarized as follows.

1] Isolates of *Trichoderma* were isolated from rhizosphere area of tomato plant and they were identified and found to be of *Trichoderma harzianum*.

2] The strain was inoculated on the TSM media and the media containing agar with distilled water was found to be superior over media containing agar with boiled water.

3] At the same time it was observed that addition of glycerol at 5% extended the shelf life from 7 to 12 months respectively compared to 4 to 5 months shelf life in formulation derived without the addition of glycerol.

4] As groundnut oil was added to the media it acted as stickers which improves adherence of spores to foliage and persistence during wind and rain.

5] The result in general revealed that liquid formulation of *Trichoderma* with distilled water had longer shelf life than that of liquid formulation of *Trichoderma* with boiled water.

6] The growth of *Trichoderma* in the form of thick mat was observed in case of TSM medium with agar having distilled water.

7] In the shelf life studies it was found that microbial population of *Trichoderma* in distilled water was greater than the microbial population of *Trichoderma* in boiled water.

8] The above results were obtained because distilled water is having low salt content, low electrical conductivity, as well as low pH.which favousrs the growth of *Trichoderma* as it favours minimum pH i.e. 6 to 7.

6.2 Conclusion:

This work endorses the claim that liquid formulation of *Trichoderma* with distilled water is promising technique for improving shelf life, viability, storage condition and to prevent from adverse effect of high temperature.

Liquid formulation of *Trichoderma* is more practical to apply as it does not losses its properties due to sub-culturing and it has better survival on seeds and soil. Hence it is better to produce liquid formulation of *Trichoderma* than solid form. This technique is definitely useful for mass production of liquid *Trichoderma* in future.

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*** Original are not seen**

APPENDIX

Trichoderma selective medium (TSM)

Sr. No.	**Component**	**Quantity**
1	Ammonium nitrate	1.0 g
2	$K_2H\ PO_4$	0.90 g
3	$MgSO_4.\ 7H_2O$	0.20 g
4	KCl	0.15 g
5	Glucose	3 g
6	Ridomil/Mancozeb	0.15 g
7	Rose Bengal	0.15 g
8	Agar	20.0 g
9	Chloramphenicol	0.25 g
10	Distilled water	1000 ml

VITA

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Of

MASTER OF SCIENCE (AGRICULTURE)

In

PLANT PATHOLOGY

2012

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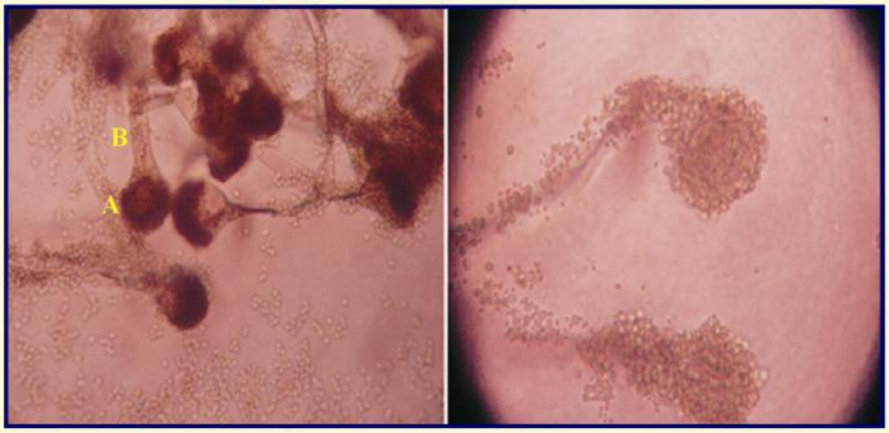
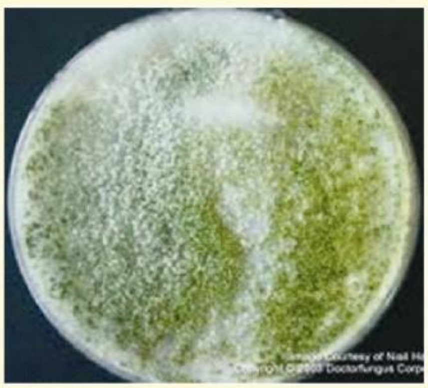
Plate 1

: 1.1 Morphological characteristics of *Trichoderma*

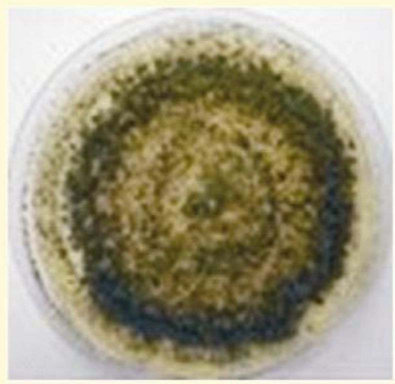


A-Conidia B-Conidiophore **Spores**

1.2 Cultural characteristics of *Trichoderma*

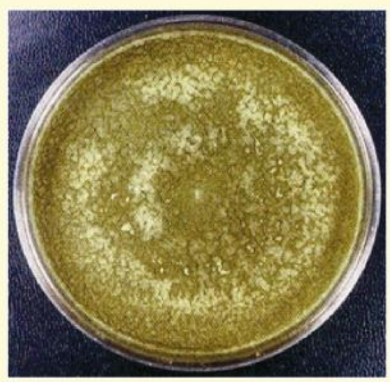


Trichoderma Viride



Trichoderma Konigii

Trichoderma hamatum



Trichoderma harzianum

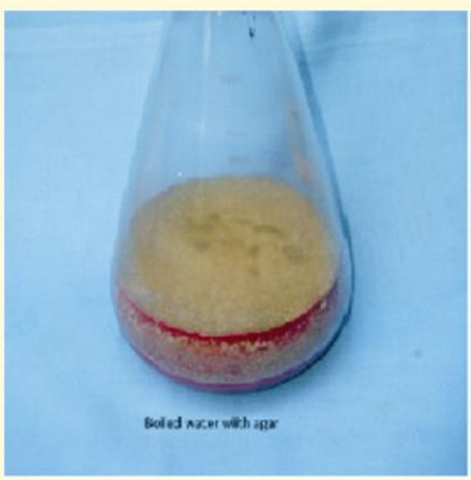
Plate 3 :Comparison of Media

3.1. Distilled water with agar

3.2 Distilled water without agar

Growth of *Trichoderma harzianum*

3.3 Boiled water with agar



3.4 Boiled water without agar

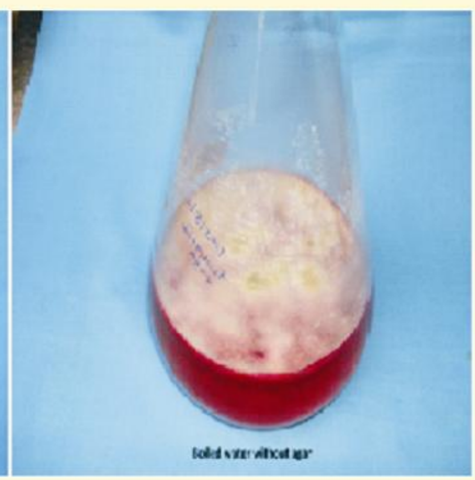


Plate 2 : Antagonistic activity of *Trichoderma* against *fusarium oxysporum*

Plate 4
Mass production of liquid *Trichoderma*

Storage of Liquid *Trichoderma*