EXPLOITATION OF ABIOTIC STRESS TOLERANT STRAINS OF *Trichoderma* spp. FOR THE MANAGEMENT OF SOIL BORNE FUNGAL PATHOGENS

By

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(2016-11-005)

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2

DECLARATION

I hereby declare that the thesis entitled **"Exploitation of abiotic stress tolerant strains of *Trichoderma* spp. for the management of soil borne fungal pathogens"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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CERTIFICATE

Certified that the thesis entitled **"Exploitation of abiotic stress tolerant strains of *Trichoderma* spp. for the management of soil borne fungal pathogens"** is a record of research work done independently by **Ms. Stella Doncy P.P.** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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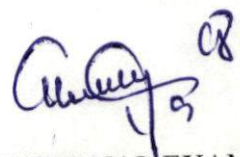Department of Plant Pathology
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CONTENTS

LIST OF TABLES

10

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

Appendix No.	Particulars
I	Composition of media used for serial dilution
II	Composition of media used for enzyme assay
III	Composition of media used for ACC deaminase assay

Introduction

1. INTRODUCTION

Soil borne phytopathogenic fungi pose serious threat to yield of many crops. Oerke (2006) reported that the yield loss caused by pathogens, animals and weeds altogether are responsible for 20 to 40 per cent in global agricultural productivity. To mitigate such crop ailments, farmers mostly depend on chemical methods of disease control such as fungicides and other pesticides. However, such methods will eventually lead to contamination of water, deterioration of environment and residual problems in the soil ecosystem. In addition, the unscientific use of pesticide will also pave way for the development of resistance or insensitivity of pathogens to these chemicals. Therefore, it is necessary to design and employ alternative methods for the management of crop diseases.

To date, biological control is an ecofriendly approach in the effective management of crop diseases. The fungi belonging to the genus *Trichoderma* are one among the most exploited biocontrol agents in the field of agriculture for the management of crop diseases. The antifungal properties of *Trichoderma* spp. are attributed to competition for nutrients (Elad, 2000), production of hydrolytic enzymes (Benitez *et al.*, 2004) and their ability to produce antibiotics (Vinale *et al.*, 2008). Moreover, *Trichoderma* species are known to promote plant growth and induce biotic and abiotic stress resistance in plants (Djonovic *et al.*, 2007). The genus *Trichoderma* is thus a rich source to explore potential bioagents for application across adverse climatic zones.

Nowadays, a major problem in rainfed agro ecosystems is predominance of abiotic stresses like high temperature, salinity, acidity and drought where the survival of bioinoculants is a problematic issue as the microbes are known to be affected by these conditions. Also, the variations in results from laboratory to field are more compounded due to various abiotic stresses that prevail under field conditions for a microbial inoculant to establish and to show the desired effect. Although numerous efficient biocontrol strains of *Trichoderma* spp. have already been identified, their efficiency under conditions of abiotic stress has not been adequately elucidated as they show poor inconsistent performance in fields which is attributed to their low osmotolerance and thermotolerance levels by varying

environmental conditions. Moreover, the formulations of *Trichoderma* spp. available in the market are suitable only for general conditions. Therefore, such problems can be managed by sound screening programme for efficient stress tolerant *Trichoderma* spp. for effective deployment of these strains to draw one or more beneficial effects that could sustain and survive the fluctuating temperature and which can tolerate salinity, acidity, pesticide and even drought conditions.

According to Marulanda *et al.* (2007), inoculation of plants with native *Trichoderma* spp. may increase drought tolerance of plants growing in arid or semiarid areas. Moreover, these microorganisms are known to enhance the tolerance of plants to salinity (Han and Lee 2005), chilling injury (Barka *et al.* 2006) and metal toxicity (Dell'Amico *et al.*, 2008). Likewise, these beneficial microorganisms can colonize the rhizosphere/ endorhizosphere of plants and promote growth of the plants through various direct and indirect mechanisms.

Hence, the present study addresses this issue and an attempt was made to isolate strains of *Trichoderma* spp. from abiotic stressed ecosystems of Kerala and to study the ability of these strains to withstand adverse environmental conditions such as high temperature, salinity, drought and acidity along with antagonistic activity. The study encompasses the following objectives:

- Collection of soil samples and isolation of native *Trichoderma* spp. from different stressed ecosystems
- *In vitro* screening of *Trichoderma* spp. for stress tolerance
- Biochemical characterization of stress tolerant isolates of *Trichoderma* spp.
- *In vitro* evaluation of the stress tolerant strains of *Trichoderma* spp. for their antagonistic potential
- *In vivo* evaluation of the promising stress tolerant strains for biocontrol efficacy and growth promotion
- Identification of promising isolates of *Trichoderma* spp.

Review of literature

2. REVIEW OF LITERATURE

Plant diseases caused by soil borne phytopathogenic fungi pose serious threat to the yield of several crops worldwide. Biological control, the use of specific micro organisms that interfere with plant pathogens and pests is an ecofriendly approach to overcome the problems caused by agrochemicals. *Trichoderma* spp. are one such bioagent that are present in nearly all agricultural soils and in other environments such as decaying wood. Major mechanisms involved in the biocontrol activity of *Trichoderma* species are competition for nutrients and space (Elad, 2000), production of hydrolytic enzymes like chitinase, cellulases and β-1,3- glucanases (Benitez *et al.*, 2004) and production of diffusible and/ or volatile antibiotics (Vinale *et al.*, 2008). These lytic enzymes partially degrade the pathogen cell wall leading to its parasitization. This process of mycoparasitism limits the growth and activity of plant pathogenic fungi.

Like crops getting affected by abiotic stresses, microbes are also known to be affected by these conditions. High temperature, soil salinity, low pH and metal toxicity have a significant impact in the performance of agriculturally important micro organisms such as *Trichoderma* spp. The selection and adoption of these organisms in stressed ecosystems therefore require a concerted research and technology development. In view of this, a study on abiotic stress tolerance of *Trichoderma* spp. and its bioefficacy under pot culture conditions were carried out.

2.1. ELECTRO CHEMICAL PROPERTIES OF STRESSED ECOSYSTEMS OF KERALA

Electrical conductivity (EC) and soil reaction (pH) of the soil reflects the type of soil microflora present in that particular ecosystem. A cross section through the electro-chemical properties of soils of Kerala is given below.

A study by Bastin *et al.* (2014) reported that soil samples collected from Palakkad district showed a pH ranging from 5.58 to 8.58 and 34.44 per cent of the samples analyzed were found to be acidic, 37. 78 per cent neutral and 27.78 per cent of the samples were alkaline. In contrast to this, 72.22 per cent of the soils collected from Alathur taluk were acidic in reaction. They also reported that, pH of the soil samples collected from different parts of Alappuzha showed pH raging from 3.91 to 7.50 with a majority of samples (97.22%) in the acidic range. The district also falls under non- saline category with an EC ranging from 0.02 to 1.60 dS m^{-1} with a mean value of 0.16 dS m^{-1}. In the same study, soil samples of Kumarakom were found acidic with a pH range of 5.0- 5.4 with very high values of EC (32.76 dS m^{-1}). pH of the surface soils collected from Wayanad district were also estimated and the data showed values ranging from 5.27 to 6.98. It was observed that 80.56 per cent of the analyzed samples were acidic and 19.44 per cent were neutral. The electrical conductivity of the soil samples ranged from 0.03 to 0.85 dS m^{-1} with a mean of 0.15 dS m^{-1} rendering them non- saline.

Bastin *et al.* (2014) also reported that in Thrissur district, 92.19 per cent of soil samples were categorized as acidic soils with a pH range of 3.5- 7.4. With regard to electrical conductivity, the whole area was found to be non- saline and EC value ranged from 0.01 to 0.46 dS m^{-1} with an average of 0.06 dS m^{-1}. In a similar study by Kavitha and Sujatha (2015) they noticed that, Thrissur soils showed pH ranging from 2.9 to 7.7 indicating extremely acidic to slightly alkaline soil reaction.

Pokkali soils of Kerala represent a periodically saline water inundated land, thus contributing to the major saline soil areas of the state. Due to the frequent ingression of tidal water from the sea, perennial problems such as salinity, high acidity and submergence prevail in this soil type. Most of the saline soils of Kerala are acidic with a pH ranging from 3.0 to 6.8 (Koruth *et al.*, 2014). Chemical analysis of Vytilla soil during mound preparation and harvest showed a pH ranging from 3.9 to 4.26 (Mohan and Sreelatha, 2016).

2.1.1. Isolation and enumeration *Trichoderma*spp.

Attempts have been made by several researchers to study the isolation of *Trichoderma* spp. from various sources as well as its enumeration, growth, morphology and sporulation in different media. *Trichoderma* species are heterotrophic fungi that can utilize a wide range of substrates for meeting their growth requirements.

Papavizas (1981) described significantly more colonies when 1:1000 soil dilutions were inoculated on *Trichoderma* medium E (TME) (9.8 X 10^3) rather than on Rose Bengal Agar medium (RBA) (5.6 X 10^3). He also observed that, bacterial contamination was less in TME medium. However, contamination with *Rhizopus* sp. and *Mucor* sp. was a problem which was solved by the addition of pentachloronitrobenzene (PCNB) to the TME medium. According to Papavizas (1985), disaccharides like sucrose and monosaccharides like dextrose can satisfy the carbon and energy requirement of *Trichoderma* spp.

Elad *et al.* (1981) also isolated *Trichoderma* quantitativelyusing *Trichoderma* selective medium (TSM), by employing serial dilution technique from soil. Selectivity of the medium was achieved by the addition of chloramphenicol as a bacterial growth arrester and PCNB and Rose Bengal as fungal growth inhibitors.

The variations in growth and morphology of *Trichoderma* spp. was studied by Harman *et al.* (1991) on different selective media such as Corn Meal agar, Oat Meal Agar, Potato Dextrose Agar,Czapek's Dox agar and Special Nutrient Media.

Likewise, Kucuk and Kivank (2004) also evaluated the difference in growth of isolates of *Trichoderma* spp. on different growth media like potato dextrose agar (PDA,), malt extract agar (MEA), Rose Bengal agar (RBA) and oat flour agar which were incubated at 28^0C for 5 days.

Narayanan *et al.* (2006) made morphological observations from *Trichoderma* cultures grown at 28^0C for about one week on potato dextrose agar (PDA), corn meal dextrose agar (CMA), oat meal agar (OMA), special nutrient agar (SNA) and *Trichoderma* selective medium (TSM).

Mustafa *et al.* (2009) studied the growth, morphological characters and sporulation of *Trichoderma* isolates in different media such as corn meal agar media, oatmeal agar, potato dextrose agar (PDA), Czapek's Dox agar and special nutrient media. Out of all this, PDA was found to be the ideal one with highest mycelial growth (mm/ day), number of spores per 100 ml of culture medium and biomass yield (mg ml^{-1} of culture medium).

Savitha and Sriram (2015) used serial dilution method for the isolation and enumeration of *Trichoderma* and recorded 3×10^{14} to 16×10^{14} cfu g^{-1} of the soil sample.

A glimpse through the literature showed several reports of *Trichoderma* spp. isolated from various stress prone areas. Rahman *et al.* (2011) quantified and characterized *Trichoderma* spp. collected from soil by employing serial dilution technique where one ml 10^{-3} dilution of kitchen waste extract was pipetted on to Rose Bengal Agar medium which was incubated for one week at 28^0C. The highest populationof *Trichoderma* spp. ($36.67 \pm 3.66 \times 10^3$ cfu g^{-1}) was obtained from compost. According to the study, *T. harzianum* was found most common in all the habitats.

Rashmi *et al.* (2017) isolated and identified altogether seven *Trichoderma* isolates from peanut growing regions in Manipur, where they observed two isolates of *T. viride* and five of *T. harzianum*.

A study by Vithya (2017) revealed a total of ten isolates of *Trichoderma* spp. from rhizosphere soil of black pepper among different parts of Thrissur district, where the highest population of the fungus was noticed in Mupliyam (96.5×10^3) followed by Kodakara (74×10^3).

2.2. PURIFICATION AND PRESERVATION OF *Trichoderma* spp.

Several workers have conducted studies on preservation of fungal cultures using different methods. Skim milk method of preservation was developed by Perkins (1963) for *Neurospora* spp. Other methods include preservation in soil, mineral oil or water covered agar slants, cryopreservation using liquid nitrogen or at low temperature ($-20\,^0C$ and $-80\,^0C$) (Catcheside and Catcheside, 1979; Kolmark, 1979), and lyophilization (Butt *et al.*, 2001).

Nuzum (1989) evaluated the efficacy of vacuum drying method for the preservation of different fungi. Fungi such as *Pythium*, *Rhizoctonia*, and some basidiomycetes species retained their viability for 18 months under vacuum drying, whereas, ascomycetes and mitosporic fungi survived as long as two years. Pounder and Bowman (1999) explored the scope of preservation of aconidial *Neurospora* strains through freezing ofmycelia..Kitamoto *et al.* (2002) suggested that preservation of fungal cultures is a cumbersome process and it should be done with continuous subculturing and storage at $4\,^0C$.

Stocco *et al.* (2010) confirmed that *Trichodema* isolates survived up to 24 months of storage with preservation methods such as distilled water, mineral oil and cellulose filter paper.Paul *et al.* (2015) reported that 15 per cent glycerol was used for long time preservation of fungi and was viable upto 24 months at $4\,^0C$ for future studies. Iqbal (2017) detailed the preservation techniques of *Trichoderma viride* and according to them, fully grown cultures grown on PDA slants can be stored at refrigerated conditions ($4\,^0C$) for further use. Moreover, these fungal cultures can also be preserved in distilled water at room temperature by two ways *viz.,* storage of spore suspension and also by inoculation of mycelial discs in distilled water. Alsothey preserved *Trichoderma* isolates in 10 per cent glycerol solution in one ml eppendorf tubes which were stored in liquid nitrogen for more than two years. They also observed that, dry methods or high temperature preservation was found to be best as it is easy, convenient and economical.

2.2.1. Characterization of *Trichoderma*cultures

Many researchers have conducted several studies on the cultural and morphological characters of *Trichoderma* spp. Pioneers in research of *Trichoderma* like Rifai (1969) and Bisset (1991) noticed certain cultural characters which could be used for the purpose of identification of the fugus *viz.*, tuft of hyphae on the substrate, branched and unbranched hyphae bearing phialides laterally or terminally, spores and sterile hyphae, indefinite conidiophores.

Domsch *et al.* (1980) elucidated the micro morphological characters of *Trichoderma* spp. and they also observed that the conidiophores of *Trichoderma* spp. end in phialides which are borne on lateral branches in some species. Conidia may be green or hyaline with smooth or rough wall and chlamydospores are seen in older cultures. Similarly, Bisset (1991) also reported that phialides of *Trichoderma* spp. were ampulliform to lageniform which are often constricted at the base, comparatively swollen at the middle and with a sub cylindrical neck. They may be arranged on lateral branches of conidiophores or ending at the terminal position, seen singly or in whorls. Likewise, Lieckfeldt *et al.* (1999) also detailed the micro morphological characters of *Trichoderma* spp. and they observed that the phialides of *T. viride* was 9µm long, while those of *T. atroviride* and *T. koningii* were found to be shorter, with a mean length of less than 8.5µm.

Sutton *et al.* (1998) noticed complete growth of *Trichoderma* spp. within five days on potato dextrose agar at 25^{0}C and the colony appeared wooly and later turned compact. Colour of the fungal colony was white with bluish green conidia which formed concentric circular pattern.

De Hoog *et al.* (2000) studied about the cultural characters of *Trichoderma* spp. and they observed that the colony completes its growth within five days at 25^{0}C. Colony appeared white in colour and scattered blue- green conidia were formed in circular pattern.

Park *et al.* (2005) described the cultural characters of different isolates

ofTrichoderma spp. They noticed that conidia of *T. harzianum* were uniformly distributed throughout the plate as aggregated pustules, whereas, conidia of *T. atroviride* were more or less restricted to concentric rings.

Kumar *et al.* (2012) studied about the *Trichoderma* isolates obtained from soil samples collected from South Andaman and Andaman and Nicobar islands. They reported that *T. asperellum* possesses conidiophores terminating with two or more phialides. The primary branches usually arise from nearly 90^0 to the main axis. In *T. longibrachiatum*, conidiophores were much longer, paired and with secondary branches. Phialides typically arose from these secondary branches and they were not verticillated.

Sharma and Singh (2014) reported that, *T. virens* isolates showed profuse conidia formation without formation of any pustule. Some isolates produced yellow pigment on PDA. Conidial colour change was observed from white to varying shades of green. In most isolates conidia were formed by 48 h and turned green within 72 h. In some isolates conidiophores were characterized by highly branched and divergent conidiophores with longest branches formed near the base of the hyphae and nearest towards the main axis.

Shah *et al.* (2012) reported cultural characters of various isolates of *Trichoderma* on different culture media. *T. harzianum* showed a concentric growth pattern with one to two rings of green conidia. There was a denser conidia production in the centre than towards the margin whereas, *T. viride* was granular in appearance on PDA medium with a uniform distribution of green conidia. *T. pseudokoningii* appeared white in colour without the formation of conidia. They also reported the conidial characters of various *Trichoderma* isolates. *T. harzianum* possessed globose to subglobose conidia with dimensions 2.8 x 2.6 µm. The conidia of *T. viride* measured 3.0 x 2.8 µm with a globose structure. However, there was no conidia formation in five day old culture of *T. pseudokoningii.* Conidia was observed only after the seventh day of inoculation and they were smaller (2.4 x 2.6 µm) when compared to other species.

Detailed studies on the cultural and morphological characters of

Trichoderma isolates were carried out by Sekhar *et al.* (2017). The culture of *T. viride* showed dark green to dark- bluish green sporulation with an amber coloured reverse side. Conidiophores were found to be long, less branched and with verticillate conidiophores. Phialides appeared paired, lageniform, converged or divergent. Conidia were round or ellipsoid in shape and intercalary or terminal chlamydospores were also present.

Dull green to bluish green sporulation with a pale yellow or colourless reverse side was observed in *T. koningii* fungal culture. Conidiophores were broad to narrow and appeared in whorls with frequent branching. Phialides took a lageniform or ampulliform shape with the terminal phialide more elongated. Conidia were sub- cylindrical to ellipsoidal in appearance. Intercalary or terminal chlamydospores were present in some cases. *T. reesei* culture showed minute tufts in the colony with pale yellowish colour. The reverse side of the plates showed pale yellow colour. Conidiophores were verticillate with rare branching habit. Cylindrical or moderately inflated and divergent phialides were observed under microscope. Chlamydospores were formed frequently in terminal or intercalary position. *T. harzianum* showed dark green areas intermingled with white mycelium. Dull yellowish colour was observed on the reverse side of the culture. Conidiophores appeared in whorls with numerous branching. Phialides were lageniform and convergent. Conidia appeared subglobose to obovoid shape. The colony of *T. aureoviride* showed complete dull green colour with discoloured reverse side. Conidiophores were ovoid and were arranged in Intercalary or terminal chlamydospores were formed frequently.

2.3. SCREENING FOR ABIOTIC STRESS TOLERANCE OF *Trichoderma*spp.

The biocontrol efficacy of *Trichoderma* species are known to be altered when they are exposed to extreme environmental conditions. However, there are a few recent reports which demonstrate that these fungi can alleviate abiotic stresses and may confer resistance to drought stress, chilling injury, high

temperature, acidity, salinity and agrochemicals.

2.3.1. High temperaturetolerance

Widden and Hsu (1987) elaborated the ability of *Trichoderma* species to colonize maple and pine litter under different temperature levels where they noticed that penetration time of the isolates decreased with increase in temperature. Eastburn and Butler (1991) evaluated the effect of soil temperature on the growth and development of *Trichoderma* species. Optimum temperature required for the growth of *T. harzianum* isolate 1059 was recorded between 27^0C and 30^0C. Moreover, they noticed that there was no substantial growth beyond 37^0C and the host root colonization by the antagonist was found maximum at a temperature range between 30 and 33^0C.

Amalraj *et al.* (2010) assessed the thermotolerance of *Trichoderma viride* under *in vitro* conditions and observed a significant reduction in the population of the fungus when temperature was raised from 30^0C to 45^0C (12×10^{-7} cfu g^{-1} and 3.8×10^{-7} cfu g^{-1}) and was further declined to 1×10^{-7} cfu g^{-1} when the temperature was increased to 50^0C. Similar observations were also noticed by Reetha *et al.* (2014) where they screened *T. harzianum* for high temperature tolerance and found that radial growth was recorded highest when the cultures were grown at 25- 30^0C (0.9065 ± 0.05) and minimum at 35^0C (0.330 ± 0.043), whereas at 40^0C, no growth was recorded.

Poosapati *et al.* (2014) observed that most of the *Trichoderma* isolates except *T. asperellum* TaDOR673, *T. asperellum* TaDOR7316 and *T. harzianum* TaDOR671 did not show any growth at 35^0C and when the temperature was increased to 37^0C, germination rates of all isolates with an exception to *T. asperellum* TaDOR673 was reduced greatly. In addition, they also noticed that when the cultures were exposed to extreme temperature such as 52^0C for 4 h, only *T. asperellum* TaDOR673 was able to tolerate this extreme temperature.

Singh *et al.* (2014) compared production of mycelial biomass at various temperature levels. The highest mycelia biomass was produced by *T. harzianum* (1.42 g) when exposed to 25^0C, compared to 20 and 35^0C where only of 0.97 and 0.82 g biomass was produced respectively. The second highest biomass production was seen with *T. viride* (1.35 g), followed by *T. asperellum* (1.27 g) and *T. longibrachiatum* (1.24g). Meanwhile, *T. atroviride* and *T. koningii* produced a biomass of 1.23 and 1.21 g respectively at 25^0C and *T. virens* recorded the lowest biomass production of 1.18 g at the same temperature.

El Badawy *et al.* (2016) performed thermotolerance studies on different *Trichoderma* species isolated from various parts of Qatar. The cultures were initially exposed to extreme temperature levels of 70^0C and 90^0C for 24 h followed by incubation at 25^0C. All the isolates *viz., T. viride, T. harzianum* and *T. longibrachiatum* exposed to 70^0C grew normally. However, *T. longibrachiatum* showed growth at 25^0C even after exposure to 90^0C for 24 h.

Different isolates of *Trichoderma* spp. were screened for thermotolerance at 28, 37, 41 and 50^0C by Vithya (2017). She observed that the isolate CKT from Chelakkara of Thrissur, showed highest mycelia weight of 12.6 g at 37^0C and the isolate CHT and VKT from Chaukka and Vellanikkara showed lower mycelial weight (0.06 and 0.07 g) at 50^0C.

2.3.2. Drought tolerance

Drought stress causes a change in membrane permeability in the plant cell which results in leakage of solutes (Premchandra *et al.*, 1990; Deshmukh *et al.*, 1991). Chandra *et al.* (2004) observed that, root colonization by *T. harzianum* strain T 22 resulted in increased concentration of antioxidant enzymes such as peroxidases, chitinases etc. They act as scavengers of reactive oxygen species (ROS) which is responsible for membrane disorganization and

thus causing membrane stability under drought stress.

Jackson *et al.* (1991) subjected different isolates of *Trichoderma* spp. to various water potential levels so as to study the drought tolerance. In all cases, the growth of the isolates in PEG (polyethylene glycol) amended PDA media was lesser when compared to control plates. They observed that the hyphal extension rate (mm day^{-1}) in the Petri dishes decreased from 13.2 mm day^{-1} to 0 mm day^{-}1 when the water potential decreased from -0.7 to -14 M Pa. Spore germination was also decreased when the water potential was decreased and complete spore germination occurred at -0.7 M Pa for all isolates, whereas at -14.0 M Pa, there was no spore germination at all.

Bae *et al.* (2009) reported that *T. hamatum* increased tolerance of cocoa plants towards drought stress by increasing root growth thereby delaying the onset of water deficit in plants.Ability to survive different levels of drought by *Trichoderma* spp. has been evaluated by Amalraj *et al.* (2010) where he reported that the performance of *T. viride* was recorded maximum at 10 per cent polyethylene glycol (PEG) with only 1 per cent inhibition of growth over control. Meanwhile, at 20 per cent PEG, 94.3 per cent growth was observed, which decreased gradually to 79.5 and 68 per cent in 25 and 30 per cent PEG concentration respectively. Sporulation of the fungus was also reduced with increase in PEG concentration.

Shukla *et al.* (2012) identified five *T. harzianum* isolates which produced higher mycelial biomass at lower moisture regimes. They were also associated with better colonization of host root leading to higher water acquisition in the root system. There was also increased production of stress induced metabolites in better colonized plants. Gusain *et al.* (2014) observed enhanced drought tolerance in rice due to *T. harzianum* T35 colonisation and they evidenced that *T. harzianum* promoted activity of antioxidant enzymes.

According to Ahmad *et al.* (2015) *Trichoderma* spp. have the potential to induce host plant tolerance to several biotic and abiotic stresses such as salinity and drought, through its involvement in root growth promotion and

29

maintenance of nutritional uptake. Rawat *et al.* (2016) compared the growth response of six isolates of *Trichoderma* at six moisture levels *viz.*, 5, 10, 20, 40, 70 and 90 per cent. They noticed that there was a general increase in growth of isolates with increase in moisture levels. Most of the isolates showed maximum growth at higher moisture levels such as 40 and 70 per cent. However, the growth of the fungus decreased drastically with lesser moisture levels and only two isolates *viz.,* Rani Th- 14 and Rani Th- 25 could survive and grow under a moisture level of five per cent.

Vithya (2017) subjected ten isolates of *Trichoderma* species obtained from different parts of Thrissur to various osmotic stress levels *viz.*, -0.15 M Pa and -1.03 M Pa to test the drought tolerance. She found that at -0.15 M Pa, *Trichoderma* sp. isolated from Chelakkara (CKT) and the isolate obtained from Panenchery (PCT) showed the lowest mycelial weight of 3.30 g. At 30 per cent PEG concentration, a mycelial biomass of 11.65 g wasrecorded with the isolate CKT (Chelakkara).

2.3.3. Aciditytolerance

Upadhyay and Rai (1979) earlier reported that *Trichoderma* spp. can grow well in soils with acidic pH and high organic matter and hence it is desirable to screen and assess the growth and development of *Trichoderma* spp. under low pH levels Biomass production of isolates of *Trichoderma* species (*T. virens, T. pseudokoningii, T. viride*) at different levels of pH was carried out by Jackson *et al.* (1991). They reported that mycelial biomass yield of all the isolates was higher when the fungus was grown at pH levels 4.6 and 6.8. Meanwhile, lower biomass production was observed in the two extreme pH values *viz.*, 2.5 and 7.4.

Bailey and Tahtiharju (2003) reported that increase in pH reduces the availability of carbohydrate source in culture medium of *T. reesei*. Similar studies have also confirmed that the growth of *Trichoderma* spp. was better at acidic rather than in alkaline soils (Benitez *et al.,* 2004; Rousk *et al.,*2009).

Reetha *et al.* (2014) studied the nature of growth of *Trichoderma harzianum* at different pH levels such as 5.0, 6.0, 7.0 and 7. 5. The mycelial weight of the fungus at pH 7- 7. 5 was found to be at the maximum level (1.46 g) followed by growth at pH 6.0 (1.38 g). Lowest mycelial weight was recorded in *T. harzianum* grown at pH 5.0 (1.27g).Seven species of *Trichoderma* isolated from rhizosphere soils of chickpea were evaluated for acidity tolerance by Singh *et al.* (2014). Major difference in mycelia growth was observed by the fungal cultures when they were grown at various pH levels from 4.0 to 8.0. The ideal pH range for the growth and development of the fungal isolates was found between 5.5 and 7.5, which yielded a dry weight of 1.41 g and 1.35 g respectively.Likewise, Ali *et al.* (2015) tested the variation in growth of different isolates of *Trichoderma* spp. at different levels of pH *viz.,* 4, 6 and 8 and the results showed that pH 6.0 was the ideal condition for the growth and sporulation of the fungus. Meanwhile, pH 8.0 resulted in significant reduction in growth and sporulation compared to pH 6.0.

Daryaei *et al.* (2016) compared conidia production of *T. atroviride* at different pH levels *viz.,* 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5 and they recorded significant conidia production at pH 6.5 compared with lower pH levels like 3.5, 4.5 and 5.5. Conidia production decreased drastically when pH was increased to 8.5 and the number of conidia formed at pH 4.5 and 3.5 were comparable, whereas, the number of conidia produced at pH 5.5 was significantly less. Highest number of conidia was formed at pH 6.5 and with further increase of pH to 7.5 and 8.5, conidia formation was also reduced.

Vithya (2017) also subjected 10 native *Trichoderma* spp. isolated from Thrissur to various pH levels to study their acidity tolerance. It was found that the isolate vKT (Vellanikkara) showed maximum mycelia weight of 3.75 g at the high acidic condition of pH 3.5.

2.3.4. Salinitytolerance

Increase in salt concentration of agricultural soil is a major issue which

disturbs the crop health and soil microbial community. Hence, there is a need to explore such isolates of *Trichoderma* species, which can thrive under high saline conditions and still perform as better antagonists.

Ghildiyal and Pandey (2008) isolated *T. harzianum, T. koningii* and *T.viride* from the soil samples procured from higher altitudes of Indian Himalayan region. These isolates were further subjected to salinity screening at 5 per cent (w/v) salt concentration. They noticed that all the isolates were able to grow under this salt stressed environment and were considered as saline tolerant.

Amalraj *et al.* (2010) observed a general decline in the mycelial growth of the *Trichoderma* spp. when salt concentration was increased in PDA media. *Trichoderma viride* when inoculated on 0.1M NaCl amended medium showed 92.9 per cent mycelial growth, followed by 0.5 M (85.6%), 0.75M (69.4%), 1.25 M (35.2%) and 1.5M (21.1%) when compared to control plate. However, *T. viride* inoculated on 2 M NaCl amended medium showed no growth when compared tocontrol.Rawat *et al.* (2013) observed a significant difference in the growth of *Trichoderma* isolates to various salt concentrations such as 4, 6, 9 and 10 dS m^{-1}. All isolates showed full growth (9 cm) at 4 dS m^{-1} after an incubation period of four days and with increase in salt concentration growth of isolates was found to be reduced.

Similarly, the mycelial growth of *Trichoderma* spp. under different salt concentrations was evaluated by Poosapati *et al.* (2014) and they observed that two isolates of *T. asperellum* showed higher growth at 0.5 M NaCl amended medium with a mean colony diameter of 4.48 cm. However, at 0.75 M salt concentration 50 per cent of the isolates lost their viability whereas, the isolates *T. asperellum* and *T. harzianum* showed highest colony growth of 3.0 cm and 2.73 cm respectively owing to their osmotolerance. Likewise, certain isolates were able to grow under high osmotolerance levels and showed only 65 - 70 per cent reduction in growth.

Kumar *et al.* (2017) subjected 70 isolates of *Trichoderma* spp. to various levels of NaCl concentrations to study their salinity tolerance and theyobserved

that 92.8 per cent of the isolates were able to survive under 5 per cent NaCl concentration. With increase in NaCl concentration to 10 per cent, only 45.7 per cent isolates showed mycelial growth.

2.3.5. Tolerance towards copper fungicides

Attempts have been made by several researchers to study the interaction between pesticides and those fungi which are antagonistic to various plant pathogens. However, there have been few reports on the effect of copper fungicides on naturally occurring strains of *Trichoderma* spp.Karpagavalli (1997) assessed the sensitivity of *T. harzianum* and *T. viride* towards the fungicide copper oxychloride @ 500, 1250 and 2500 ppm and they observed that both the species showed lower inhibition under this condition.

Another study was conducted by Haritha (2003) where she evaluated the compatibility of copper oxychloride @ 50, 100, 250, 500 and 1000 ppm with *Trichoderma* spp. and noticed that at 1000 ppm the growth of fungus was inhibited by 44.44 per cent. Beevi *et al.* (2005) confirmed that copper oxychloride @ 0.20 per cent is compatible with *T. harzianum.*

The tolerance of *T. harzianum, T. pseudokoningii, T. longibrachiatum* and *T. viride* towards various concentrations of copper fungicides were studied by Khan and Shahzad (2007). They reported that *T. harzianum, T. viride, T. longibrachiatum* were able to grow at copper oxychloride concentrations of 1, 10 and 100 ppm. Meanwhile, the growth was diminished when the concentration was further increased to 1000 and 10,000 ppm. However, a reduction in growth of *T. pseudokoningii* was noticed with 1- 100 ppm concentration of copper oxychloride.

Nallathambi *et al.* (2009) reported that *T.citrinoviride* showed zero per cent inhibition when exposed to copper oxychloride @ 250 μg g^{-1} in PDA medium, whereas, *T. viride T.v*-CIAH149 and *T. viride* SBI48 showed tolerance upto 500 μg g^{-1}of the fungicide.Sarkar *et al.* (2010) also noticed that contact fungicides

such as copper oxychloride and copper hydroxide showed compatibility with *T. harzianum*. Nonetheless, with the increase in concentration of the fungicide per cent inhibition of the bioagent also increased.Bhai and Thomas (2010) evaluated the effect of fungicides on the growth of *T. harzianum* and they observed that Bordeaux mixture (1 %) showed higher cent per cent inhibition in the growth of fungus when compared with copper oxychloride (0.25%).

Similarly, the effect of different fungicides on the growth of *T. harzianum* was studied by Pandya (2010). A per cent inhibition of 92.16 was recorded at 3000 ppm of copper oxychloride, which reflected the lower levels of tolerance of *T. harzianum* to copper fungicide. In an experiment conducted by Islam *et al.* (2011), *T. harzianum* and *T. viride* showed a general decline in the mycelial growth when the concentration of copper oxychloride 50 WP was increased from 750 ppm to 1000, 1250, 1500 and 1750 ppm. *T. harzianum* attained complete growth when the medium was poisoned with 750 and 1000 ppm copper oxychloride which was gradually reduced to 8.6, 3.1 and 2.0 cm when the concentration of the fungicide was increased to 1250, 1500 and 1750 ppm respectively. In the case of *T. viride,* the growth was retained at 9 cm even upto 1250 ppm of copper oxychloride and it was then reduced to 2.3 and 2.1 cm when the fungicide concentration was increased to 1500 and 1700 ppmrespectively.

Tapwal *et al.* (2012) compared the compatibility per cent of *T. viride* with copper oxychloride at concentrations such as 50, 100, 200 and 300 ppm. It was observed that compatibility per cent reduced from 97.9 to 34.9 per cent with increase in fungicide concentration. Sonavane and Venkataravanappa (2017) observed that copper oxychloride at 500, 1000, 1500 and 2000 ppm were found compatible with *T. harziaum* and showed no inhibition in growth. Waskale (2017) also reviewed the effect of copper oxycloride @ 250 ppm and 500 ppm to *Trichoderma* spp. where he observed a decrease in colony area when the fungicide concentration was increased from 250 ppm to 500ppm. Karaoglu *et al.* (2018) observed that *T. harzianum* PBT 23 was found

34

compatible with copper hydroxide @ 100 µg ml^{-1}.

2.4. BIOCHEMICAL CHARACTERIZATION OF *Trichoderma* spp.

Many workers have conducted several studies on the enzymes and biomolecules synthesized by *Trichoderma* spp. involved in antagonism and stress tolerance. Enzymes such as cellulase, *β*- 1, 3- glucanse and protease are some of the enzymes involved in the antagonism of *Trichoderma* spp.

2.4.1. Assay of extracellularenzymes

2.4.1.1. Cellulase activity of Trichoderma spp.

Montenecourt and Eveleigh (1977) observed a stable extracellular cellulase enzyme produced by *T. viride* which is capable of degrading the crystalline cellulose. Similarly, they evaluated the cellulase enzyme production of *T. reesei* by filter paper activity and recorded an activity of 4.65 U ml^{-1}.Ryu and Mandels (1980) also studied the production of cellulase enzyme by *T. reesei* OM6a strain, where they noticed 5 U ml^{-1} of enzyme activity when filter paper was used as the substrate. Likewise, Bailey and Nevalainen (1981) documented the cellulase enzyme production of *T. reesi* isolate by filter paper degrading activity, where they recorded 3.4 U ml^{-1} and 4.4 U ml^{-1} enzyme activities after 75 h and 96 h of incubation.

Domingues *et al.* (2001) noticed that *Trichoderma reesei* recorded a higher production of cellulase enzyme (2.8 FPU ml^{-1}) when glucose (30 g l^{-1}) and lactose (15 g l^{-1}) were used as substrates. Wen *et al.* (2005) reported that *T. reesei* recorded a cellulase activity of 1.74 U ml^{-1} when it was grown on agricultural waste.

Ahamed and Vermette (2008) studied the cellulose enzyme production

of *T. reesei* RUT-C30 and recorded an activity of 2.3± 0.6 U ml^{-1} when it was grown in cellulose–yeast nitrogen base culturemedia. Gajera and Vakharia (2012) conducted cellulase enzyme production from *T. harzianum, T. viride* and *T. virens* by following DNS (Dinitrosalicylic acid) method and they recorded specific activity of cellulase in different isolates of *T. harzianum* ranging from 2.26 to 2.71 U ml^{-1} at six days after inoculation.

2.4.1.2. *β- 1,3- glucanase activity of Trichodermaspp.*

De La Cruz *et al.* (1995) studied the production of extracellular enzyme *β*- 1, 3- glucanase in *T. harzianum* pre- grown in 10 per cent glucose following an incubation period of 48 h with different carbon sources. When yeast was used as the substrate, an enzyme activity of 1 U mg^{-1} was recorded and also the concentration of the enzyme was found to increase with increase in the incubation time.

El-Katatny *et al.* (2001) conducted enzymatic studies on *T. harzianum rifai* T24, where the specific activity of *β*-1, 3 glucanase was found to be 0.096 U ml^{-1}. Likewise, Bara *et al.* (2003) reported *β*-1, 3 glucanase production in *T. asperellum* by employing different carbon sources in the culture media. When 0.5 per cent cellulose was used in the media, the fungus recorded an enzyme activity of 0.14 ± 0.01 U ml^{-1}. Similarly in *T. koningii* Monteiro and Ulhoa (2006) studied the production of *β*-1, 3 glucanase enzyme by using different carbon sources and they observed that when cellulose was used as the carbon source, the fungus showed an activity of 0.5 U ml^{-1}.

The ability of different strains of *T. harzianum* for the production of *β*- 1, 3 glucanase enzyme was evaluated by dos Reis Almeida *et al.* (2007). They noticed that *T. harzianum* ALL44 recorded highest enzyme activity and the lowest was observed in *T. harzianum* ALL16 strain.According to Martin *et al.* (2007) *β*- 1,3- glucanase is involved in the mycoparaistism and saprophytic mode of nutrition of *Trichoderma* spp., as they are considered as the

biochemical weapon of the antagonist against pathogens.

Ahamed and Vermette (2008) conducted studies on *Trichoderma reesei* RUT- C30 and recorded 3.8 ± 0.9 U ml^{-1} and 2.0 ± 0.3 activity of β- 1,3-glucanase when the fungus was grown in cellulose-yeast extract-peptone media and cellulose-yeast nitrogen base mediarespectively.Marcello *et al.* (2010) evaluated the difference in β-1,3 glucanase enzyme production when the substrate used was changed. Maximum level of enzyme activity was recorded when starch (0.020 U mg^{-1}) was used as the substrate and minimum when cellulose (0.005 U mg^{-1}) was used.

2.4.1.3 Protease activity of Trichoderma spp.

The role of protease in the biocontrol ability of *Trichoderma* spp. has been described by Rodriguez-Kabana (1969), where he reported that increased protease production of *T. viride* resulted in reduction of enzyme activity of the pathogen, *Sclerotiumrolfsii*. Similarly, Elad *et al.* (1982) also implied the significance of enzyme protease in the destruction of cell wall thereby limiting the growth of pathogen. In mycoparasitism of plant pathogens by *Trichoderma* spp. the enzyme protease plays a key role in cell wall lysis, as the fungal cell wall contains chitin and/ or β- glucans fibrils which are embedded in a protein matrix (Wessels, 1986).

Geremia *et al.* (1993) reported an alkaline protease enzyme to be involved in the mycoparasitism exhibited by *T.harzianum.*De Marco *et al.* (2003) studied the production of hydrolytic enzymes by *Trichoderma* spp. where, protease activity was assayed using casein as the substrate for degradation and they observed that there was significant difference in the enzyme activity. Maximum protease activity was recorded in *T. harzianum* isolate 1051 (2.0 U ml^{-1}) followed by *Trichoderma* sp. isolate TVC and *T. harzianum* 39 (1.70 U ml^{-1}, 0.98 U ml^{-1} respectively) after 72 h of incubation. They also reported a general increase in the enzyme activity when the

incubation period was extended from 0 to 72h.

Shakeri and Foster (2007) studied the production of protease enzyme by *T. harzianum* by employing different growth supplements followed by three days of incubation at 25^0C. When 1 per cent (w/v) casein was used as the supplement an activity of 8.4 and 11.3 U ml^{-1} was recorded by *T. harzianum* 101645 *T. harzianum* 206040respectively.Gajera and Vakharia (2012) studied the antagonism exhibited by *Trichoderma* spp. on the plant pathogen by the synthesis of extracellular enzyme, protease. They reported that there was maximum enzyme production by *T. harzianum* 2J (5.63 Uml^{-1}) followed by *T. viride* 60 (5.4 U ml^{-1}). The lowest secretion of protease enzyme was seen in the isolate *T. viride* 62 with an activity of 1.20 Uml^{-1}.

Smitha *et al.* (2014) conducted experiment on the ability of *T. viride* to produce hydrolytic enzymes when encountered with plant pathogens. They recorded variation in the protease activity with increase in days of incubation and maximum activity was noticed on the seventh day (0.003 U ml^{-1}) followed by ninth and fifth day (0.002 and 0.001 U ml^{-1}). Parmar *et al.* (2015) observed general increase in the protease activity in all isolates of *Trichoderma* spp. with increase in the incubation period. Maximum protease activity was detected in *T.viride* NABII Tv23 (8.34 U ml^{-1}) followed by *T. koningii* MTC 796 (7.54 U ml^{-1}) and the minimum enzyme activity of 2.36 U ml^{-1} was shown by *T.virens* NABII TvS 12.

Cherkuppally *et al.* (2017) noticed a major difference in the secretion of extracellular enzymes among the various isolates of *Trichoderma* spp., where they concluded that very high protease activity was shown by *T.harzianum* followed by *T. koningii* and *T. atroviride* showing high enzyme activity. Whereas, the isolates *T. viride*, *T. reesei*, *T. virens* and *T. pseudokoningii* showed low protease activity.

2.4.2. Biomolecules imparting drought tolerance

2.4.2.1.Screening for ACC Deaminase in Trichoderma spp.

1- aminocyclopropane 1- carboxylate (ACC) act as the precursor for the synthesis of plant volatile hormone ethylene, which is engaged in plant physiological activities such as tissue differentiation, leaf and flower abscission, development of root hair, ripening of fruits and production of volatiles responsible for aroma in fruits. However, in response to biotic and abiotic stresses, the plant often significantly increases the endogenous ethylene production which has adverse effect on plant growth and is thought to be responsible for senescence in plants (Abeles *et al.*, 1992; Nayani *et al.*, 1998; Ali *et al.*, 2012). Interestingly, there are several reports that the plant growth promoting microbes can cleave the ethylene precursor ACC to ammonia and α-ketobutyrate consequently, lowering ethylene levels under stressed environments (Glick *et al.*, 1998). This process may occur under any unfavourable condition such as salt stress (Cheng *et al.*, 2007), flood stress (Grichko and Glick, 2001), drought stress (Mayak *et al.*, 2004) and pathogen invasion (Wang *et al.*, 2000).

According to Harman *et al.* (2004) under normal conditions plants will produce only required levels of ethylene, nevertheless under stressed conditions, its production may go beyond the required amount which is detrimental to the plant. *Trichoderma* spp. is one among the beneficial microbe which can reduce the unnecessary build up of ethylene by producing the enzyme ACC deaminase which is capable of cleaving ACC thereby blocking the synthesis of ethylene.

Gravel *et al.* (2007) reported that the growth promotion imparted by *T. atroviride* on tomato seedlings is attributed to the production of enzyme ACC deaminase by the fungus. The enzyme helps in lowering of ethylene levels in the plant system as it cleaves ACC, the precursor of ethylene thereby reducing its concentration. He also reported that ACC deaminase enzyme produced by beneficial soil micro organisms such as *Trichoderma* spp. is indirectly

promoting the growth and development of plants.

Likewise, Zhang *et al.* (2017) conducted a study on *Trichoderma harzianum* T- soybean and observed that the isolate has plant growth promotion ability because of the production of ACC deaminase enzyme.Ali *et al.* (2014) conducted studies on drought tolerant and ACC deaminase producing *Pseudomonas* spp. on DF (Dworkin and Foster) salt minimal media where they observed that all the nine drought tolerant isolates were able to grow in the treatment plates where ACC was used as the nitrogen source. Among the total nine isolates, one showed excellent growth on DF salt minimal media when compared to negative control plate without any nitrogen source.

2.4.2.2. Production of cytokinin

Cytokinins are plant growth regulators chemically classified as N^6 substituted aminopurines which enhances celldivision, root elongation, seed germination, chlorophyll accumulation and delay of senescence (Ross and Salisbury, 1992). In a study by Harman (2000) revealed that *T. harzianum* can induce root growth and plant development. Such growth promotion effect may be attributed to the production of growth promoting hormones such as cytokinin (Windham *et al.*, 1986).

Benitez *et al.* (2004) recorded an increase in root length and branching when tobacco seeds were inoculated with *T. harzianum* CECT2413 when compared with uninoculated control. Inoculation was done with the pathogen, *Rhizoctonia solani* along with *T. harzianum* where there was significant root production when compared with control.

Ortiz-Castro *et al.* (2009) observed that at the time of root colonization by beneficial microflora, secrete certain plant growth regulators such as auxins or cytokinins. A significant formation of root system in *Arabidopsis thaliana* seeds grown in Murashige- Skoog medium inoculated with *T. atroviride* and *Bacillus megatherium* was observed when compared to the uninoculated control plate.

Resende *et al.* (2014) made similar observation on inoculation with different isolates of *Trichoderma* spp. on *Calophyllum brasiliense*. The rhizosphere isolate *Trichoderma* RC14M and the endophytic isolate *Trichoderma* EC09M recorded maximum cotyledon biomass of 0.050 g and the lowest biomass was recorded in the rhizosphere isolate *Trichoderma* RC15M (0.015g) which may be attributed to the production of cytokinin by these isolates.

2.5. ANTAGONISM EXHIBITED BY *Trichoderma* spp. TOWARDS PLANT PATHOGENS

Elad *et al.* (1980) reported the efficacy of various *Trichoderma* isolates against pathogens *viz., Fusarium oxysporum, Rhizoctonia solani* and *Sclerotium rolfsii* isolated from naturally infected vanilla plants. Two isolates of *T. harzianum* and *T. virens* recorded 80 to 90 per cent inhibition in the growth of pathogens.

Bell *et al.* (1982) studied the antagonistic potential of 77 isolates of *Trichoderma* spp. against six fungal pathogens *viz., Sclerotium rolfsii, Ceratobasidium cornigerum, Phytophthora parasitica, Pythium aphanidermatum, P. myriotylum* and *R. solani* wherethey noticed that each pathogen was anatgonised by one or other isolate of *Trichoderma* spp.

Antagonistic ability of *T. longibrachiatum* and *T.harzianum* was evaluated against *Sclerotium rolfsii* by Yaquab and Shahzad (2005). They noticed that hyphae of *Trichoderma* spp. coiled around the pathogen causing lysis and destruction of the pathogen. Other antagonists such as *T. polysporum, T. pseudokoningii* also reduced the growth of fungal pathogen.

Biocontrol efficiency of five isolates of *Trichoderma* spp. collected from rhizosphere region of groundnut plants were studied against the pathogen, *Macrophomina phaseolina* under *in vitro* conditions by Sreedevi *et al.* (2011).

They observed maximum inhibition percentage in the growth of the pathogen (64.4 and 61.1%) with *T. harzianum* and *T. viride*respectively.Gajera *et al.* (2012) recorded maximum inhibition in growth of the test pathogen *M. phaseolina* by antagonist *T. koningii* MTC796 (74.3%) followed by *T. harzianum* NBAII Th1(61.4%).

The antagonistic potential of six isolates of *Trichoderma* spp. *viz.*, TCVSI 1, TCVSI 3, TCVSI 5, TCVSI 8 and TCVSI 10 were evaluated against the pathogen *Fusarium monoliforme* under *in vitro* conditions by Gawade *et al.* (2012) using liquid culture filtrate of the antagonist. According to them, volatile and non volatile compounds produced by *Trichoderma* spp. played a major role in the antagonism of plant pathogens. Bhale *et al.* (2013) elucidated the biocontrol effect of different *Trichoderma* spp. towards fruit rot pathogen *Aspergillus niger* of *Manilkhara zapota* and they noticed maximum per cent inhibition with *T. koningii* (57.70%) followed by *T. harzianum* (54.40%). Similar results were observed with *T. koningii* (67.07%) when grown along with the pathogen *R. solani*.

Sundaramoorthy and Balabaskar (2013) studied the antagonism of *T. harzianum* against the soil borne pathogen *Fusarium oxysporum* f. sp. *lycopersici*. They reported that per cent inhibition was higher for *T. harzianum* ANR 1 isolate (53%) when evaluated against the pathogen and under pot culture conditions also, this isolate produced the least disease incidence of 15.33 per cent.Biocontrol of collar rot of chick pea caused by *S. rolfsii* by using isolates of *Trichoderma* spp. was studied by Sab *et al.* (2014). Among the antagonists used, *T. harzianum* 55IIHR showed maximum per cent inhibition (70%) followed by *T. harzianum* NBAII (63%).

2.6. *In vivo* EVALUATION OF *Trichoderma* spp. FOR GROWTH PROMOTION AND BIOCONTROLEFFICACY

Antagonistic potential of *Trichoderma* spp. vary under *in vivo* conditions when compared to controlled laboratory conditions. The disease incidence caused by the pathogen may be reduced when *Trichoderma* is used for pot culture experiments.

2.6.1. Mass multiplication

A perusal through the literature revealed that there are several reports on the mass production of *Trichoderma* spp. using different inorganic inert material and organic substrates which are used as carrier or delivery media during formulation. Some of the commonly used locally available substrates are agro - industrial cellulosic wastes like wheat straw, paddy straw, saw dust, paper waste, vegetable and fruit waste, coffee waste, tea waste, soybean bagasse and organic manure like farm yard manure, neem cake, vermicultite, and spent mushroom substrate and cereal bran such as wheat bran, rice bran, grain of sorghum (Lewis *et al.*, 1991; Prakash *et al.*, 1999; Saju *et al.*, 2002) apart from inorganic inert materials like talc and lignite (Mandhare and Suryawanshi, 2005).

Thangavelu *et al.* (2004) conducted studies to find out the best suited substrate which can be employed for the mass multiplication of *T. harzianum* used against *Fusarium* wilt of banana. Five different organic substrates were used *viz.*, rice bran, rice chaffy grain, farmyard manure, banana pseudostem and dried banana leaves. Among them, dried banana leaves were found best substrate for the growth of *T. harzianum*. Colonization of the leaves was completed within few days and better buildup of microbial population (4.6 x 10^{32} cfu g^{-1} of leaf) was recorded.

Similarly, Bhagat and Pan (2006) also compared different substrates which can be used for the mass multiplication of *Trichoderma* spp. Rice bran,

wheat bran and pulse bran showed better sporulation and growth of fungi when compared to other substrates. Substrate evaluation was done for the mass multiplication of *Trichoderma* spp. by Rini and Sulochana (2007). Various substrates such as cow dung, neem cake, coir pith, sorghum grains, sawdust and rice bran either alone or in combination with additives such as jaggery and wheat flour were exploited for the study. They found that pre- boiled sorghum grains, coirpith + neem cake taken in the ratio1:1, cowdung + neemcake (1:1) + wheat flour (10%) maintained high populations of *T. harzianum* and *T. viride* within 10 days of inoculation.

Singh *et al.* (2014) evaluated locally available substrates for the mass multiplication of *Trichoderma* spp. against *R. solani* causing collar rot of cowpea. Seven days after inoculation, sorghum seeds had retained maximum population of *T. harzianum* (22.5×10^7 cfu g^{-1}) followed by broken pieces of maize and wheat. Meanwhile, minimum population of 12.7×10^{-7} cfu g^{-1} was recorded when vermicompost was used as the substrate.

2.6.2. Evaluation of *Trichoderma* spp. for growth promotion and biocontrol efficacy

Biological control of soil borne pathogens by adopting *Trichoderma* is an ecofriendly and sustainable method of diseases management. In addition, the fungus is also responsible for the synthesis of plant growth regulators such as indole acetic acid (IAA) and cytokinin which are responsible for growth promotion.

Chung and Hoitink (1990) studied the interaction between *T. hamatum* and *Rhizoctonia solani* under pot culture experiment. Enumeration of microbial population revealed that there was higher population of *Trichoderma* spp. in the pot than the pathogen. It may be due to the better colonization character of the antagonist. Bankole and Adebanjo (1996) reported the antagonistic effect of *T. viride* on *Colletotrichum truncatum*. They observed that two isolates of *T. viride* treatments showed a significant increase in seed germination when the seeds

were dipped in spore suspension of the antagonist followed by drenching with the same. Moreover, there was reduction in the per cent seedlings infected in the treatment plants (27.6 % and 45.2%) when compared to control plants (85.5%).

Aly *et al.* (2007) examined the antagonism of *Trichoderma* spp. against *M. phaseolina* of cotton plants under *in vivo* conditions. Results revealed that, the isolate *Trichoderma* 1 could drastically reduce the incidence of pre-emergence damping off. Variation in the height of cotton plants and the dry matter production was noticed in plants treated with *Trichoderma* spp. compared to that of pathogen inoculatedcontrol. Berber *et al.* (2009) reported that *T. harzianum* controlled the growth of *Bipolaris* sp., *F. oxysporum* and *R. solani* at different rates during pot culture studies.

Mokhtar and Dehimat (2015) evaluated *in vivo* biocontrol efficacy of *T. harzianum* against *Phoma* sp. and *Gliocladium* sp. where the results confirmed that *T. harzianum* arrested the growth and sporulation of the pathogens. Raut *et al.* (2017) evaluated biocontrol efficacy of *Trichoderma* spp. against *Phytophthora parasitica* in black pepper. The results implied that treatment with *T. asperellum* T36 showed the lowest per cent (54%) of *Phytophthora* infected pepper seedlings.

Padder and Sharma (2011) conducted studies on the antagonism of *Trichoderma* spp. against bean anthracnose pathogen, *Colletotrichum lindemuthianum*. They observed that seed treatment with *Trichoderma* isolates showed better results than soil drenching. In pot culture experiment, *T. viride* showed maximum increase in seed germination and prevention of seed borne infection followed by *T. harzianum*. Similar study on application of *Trichoderma* spp. for the management of *F. solani* causing black root rot was carried out by Belete *et al.* (2015). According to them, disease suppression caused by the antagonist under *in vivo* condition ranged from 69 to 74 per cent. However, the maximum control of disease was seen when the plants were treated with isolate TS025. Meanwhile, the lowest disease suppression was recorded when isolate TS058 was applied in the pots.

Materials & Methods

3. MATERIALS AND METHODS

The present study on "Exploitation of abiotic stress tolerant strains of *Trichoderma* spp. for the management of soil borne fungal pathogens" was carried out in the Department of Plant Pathology, College of Horticulture, Vellanikkara during the period 2016- 2018. The description of the materials used and methods followed during the course of the experiment are given below.

3.1 SURVEY AND COLLECTION OF SOIL SAMPLES FROM DIFFERENT STRESSED ECOSYSTEMS ACROSS KERALA

Intensive sampling surveys were conducted across different abiotic stressed ecosystems of Kerala. Six locations *viz.,* Wayanad, Palakkad, Thrissur, Vytilla, Alappuzha and Kumarakom were selected for the survey and the details regarding the places surveyed are depicted in Table 3.1 (Plate 3.1). Soil samples, approximately weighing 500 g, were collected from different locations representing areas of salinity, drought, acidity, fungicide tolerance and high temperature. Dry litter was removed from the top and random soil samples were drawn, pooled and sieved to remove coarse stones and root fragments. It was air dried for 72 h at room temperature and then used for estimation of pH and electrical conductivity (EC) and enumeration of native *Trichoderma* spp.

3.1.1. Estimation of pH and electrical conductivity (EC) of the soil samples

pH value is a measure of hydrogen ion activity of the soil water system and it represents the alkalinity and acidity of the soil sample. It determines the nutrient availability, microbial activity and physical properties of soil. pH of the soil samples were estimated as per the protocol of Jackson (1958) where soil and distilled water

Table 3.1 Locations of survey for soil sample collection

Sl. No.	Districts	Locations	Cropping systems
1.	Palakkad	1.Aalathoor-1	Coconut
		2. Aalathoor- 2	Coconut
		3. Aalathoor- 3	Coconut
		4. Aalathoor- 4	Coconut
		5.Pazhambalakode- 1	Rubber
		6. Pazhambalakode- 2	Rubber
		7.Pazhambalakode- 3	Rubber
		8. Pazhambalakode-4	Rice
		9. Athipotta- 1	Rice
		10. Athipotta- 2	Rice
		11. Tarur-1	Rice
		12. Tarur- 2	Rice
		13. Tarur- 3	Rice
		14. Tarur- 4	Rice
2.	Alappuzha	1.Cherthala- 1	Rice
		2. Cherthala-2	Rice
		3. Kanjikuzhi-1	Rice
		4. Muhamma-1	Rice
		5. Muhamma- 2	Rice
3.	Ernakulam	1.Vytilla- 1	Rice
		2. Vytilla- 2	Rice
		3. Vytilla- 3	Rice
		4. Vytilla- 4	Rice
		5. Vytilla- 5	Rice
4.	Kottayam	1.Kumarakom-1	Rice
		2. Kumarakom- 2	Rice
		3. Kumarakom- 3	Rice
		4. Kumarakom- 4	Rice
		5. Kumarakom- 5	Rice
5.	Wayanad	1. Bathery- 1	Forest ecosystem
		2. Bathery- 2	Forest ecosystem
		3. Bathery- 3	Forest ecosystem
		4.Muthumala- 1	Forest ecosystem
		5.Ambukuthi- 1	Forest ecosystem
		6. Ambukuthi- 2	Forest ecosystem
		7.Tholpetty- 1	Forest ecosystem
		8. Tholpetty- 2	Forest ecosystem
		9. Tholpetty- 3	Forest ecosystem
		10. Tholpetty-4	Forest ecosystem
		11. Kurichiyad- 1	Forest ecosystem

35

|		12. Kalloor- 1	Forest ecosystem
		1. Puzhakkal- 1	Rice
		2. Puzhakkal- 2	Rice
		3. Puzhakkal- 3	Rice
		5. Kanjani- 1	Rice
6.	Thrissur	6. Kanjani- 2	Rice
		7. Kanjani- 3	Rice
		8. Adat- 1	Rice
		9. Adat- 2	Rice
		10. Adat- 3	Rice
		11. Alappat- 1	Rice

are taken in the ratio 1:2.5. Air dried soil sample of 10 g each was taken in a 50 ml beaker and 25 ml of distilled water was added to it. The suspension was stirred intermittently for 30 min by using Orbitek magnetic stirrer at 260 rpm and was left for settling. A digital pH meter was employed to estimate the pH of the samples.

Electrical conductivity (EC) represents soluble salts present in the soil. The electrical conductivity of soil samples were determined by immersing digital electrical conductivity bridge of Jenway conductivity meter in the supernatant of the soil suspension which was prepared in the same manner as that for pH.

3.2 ISOLATION AND ENUMERATION OF *Trichoderma spp.* FROM SOIL

Isolation of *Trichoderma* spp. was carried out using serial dilution and pour plate method on a suitable selective media (Parkinson *et al.*, 1971). Ten gram of soil was weighed out and was transferred aseptically to a 90 ml sterile water blank taken in a 250 ml conical flask. Further, the soil suspension in the conical flasks were agitated vigorously for 15 min using Orbitek shaker at 260 rpm. The well blended soil suspension was then diluted upto 10^{-4} dilution. From 10^{-3} and 10^{-4} dilutions 1 ml suspension was pipetted out into sterile Petri plates and the respective media were poured into the plates. Different media *viz.*, *Trichoderma* selective medium (TSM), Malt extract agar (MEA), Oat meal agar, Potato dextrose agar (PDA) and Special nutrient agar (SNA) (Appendix- I) were

Plate 3.1 Collection of soil samples from different locations across Kerala
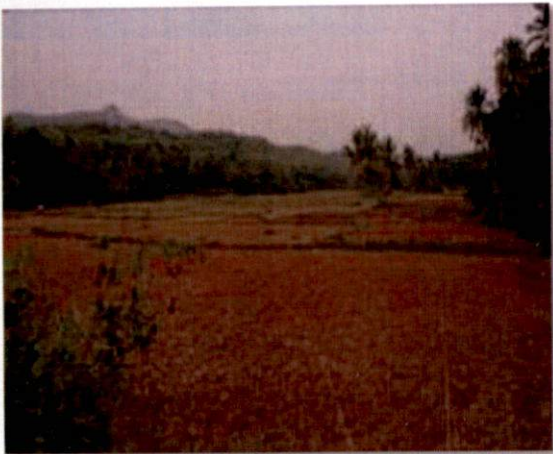
a) Palakkad

b) Alappuzha

c) Ernakulam

d) Kottayam

e) Wayanad

f) Thrissur

evaluated to study the efficiency of culture media in the isolation of *Trichoderma* spp. The plates were swirled to ensure an even distribution of the suspension in the media and were incubated at 26 ± 2^0C for six days. The colonies developed were studied for their cultural and morphological characters.

3.2.1 Purification and preservation of *Trichoderma* spp.

After the incubation period morphologically distinct colonies appearing on the plates resembling *Trichoderma* spp. were purified by hyphal tip method (Grove *et al.*, 1970). Pure cultures of the fungi were maintained on PDA slants by periodic subculturing and were preserved at 4^0C under refrigerated conditions for further studies.

Cultures of various isolates of *Trichoderma* spp. were also preserved in glycerol for long term storage. For this, vials with glycerol (15%) were autoclaved for 20 min and the moisture from glycerol solution was removed by keeping them in hot air oven at 65^0C for 2 h. A five mm mycelial disc of the fungal culture was inoculated into these vials aseptically and was kept in refrigerator at 4^0C (Paul *et al.*, 2015).

3.2.2. Characterization of *Trichoderma* isolates

Various isolates of *Trichoderma* spp. were studied for their cultural and morphological characteristics for the purpose of tentative identification. Two techniques, visual identification on Petri dishes and micromorphological studies in slide culture were adopted for this.

3.2.2. a. *Cultural characters*

The cultural characters exhibited by different *Trichoderma* species were studied by visual observation. The isolates were grown aseptically in sterile Petri plates on PDA medium and were kept for incubation at 26 ± 2^0Cand the distinguishing characters were recorded upto six days at an interval of 24 h. Variations in colony colour, texture, odour, days to sporulation and pigmentation

were recorded.

3.2.2. b. *Morphological characters*

For micromorphological studies, slide culture technique was used by following the protocol by Riddell (1950). Microscopic observations on shape, size, arrangement and development of conidiophores and phialides, hyphal width, and size, shape and colour of conidia were recorded. The characters of isolates were compared to a taxonomic key suggested by Sekhar *et al.* (2017) for the genus *Trichoderma* for identification. Photomicrographs of the isolates were also taken using Carl Zeiss AxioLab A1 Image analyzer of College of Agriculture, Padanakkad.

3.3. *In vitro* SCREENING OF *Trichoderma spp.* FOR ABIOTIC STRESS TOLERANCE

The effect of temperature, drought, pH, fungicides and salinity on the growth of *Trichoderma* isolates were studied under *in vitro* conditions.

3.3.1. *In vitro* screening of *Trichoderma spp.* for temperature tolerance

Isolates of *Trichoderma* species were subjected to different temperature conditions to study the best suited temperature level for the growth of the fungus and also to screen for thermo tolerance as per the protocol of Reetha *et al.* (2014). A five mm mycelial disc of the fungus was inoculated aseptically to sterile potato dextrose broth (PDB) prepared in 100 ml conical flask. The inoculated flasks were incubated at various temperature levels of 25, 30, 35 and 40^0C for five days in Rotek water bath. Mycelial growth formed after incubation period was taken out and filtered through Whatman No. 1 filter paper to remove the excess broth. Thereafter, fresh weight of these mycelial mats was recorded.

3.3.2. *In vitro* screening of *Trichoderma spp.* for drought tolerance

Drought tolerance of the isolates of *Trichoderma* spp. was assessed as per

the protocol of Amalraj *et al.* (2010). A five mm disc of the fungal isolate was inoculated into sterile PDB amended with polyethylene glycol (PEG 6000 Da) at different concentrations *viz.,* 10 per cent(100 g PEG added in 1 l media that contributes an osmotic pressure of -0.15 M Pa), 20 per cent (200 g PEG added in 1 l medium that contributes an osmotic pressure of -0.49 M Pa) and 30 per cent (300 g PEG added in 1 l medium that contributes an osmotic pressure of -1.03 M Pa). The inoculated flasks were kept for incubation at 26 ± 2^0C for five days. The fresh weight of the mycelial mats was recorded after filtering off the excess broth as per 3.3.1.

3.3.3. *In vitro* screening of *Trichoderma spp.* for acidity tolerance

The effect of acidity on the growth of different isolates of *Trichoderma* species were studied following the protocol of Reetha *et* al. (2014). The reaction of the medium was adjusted to the desired pH by using 0.1 N HCl and/ or 0.1 N NaOH according to the requirement. The flasks were autoclaved for 20 min before inoculation with the isolates. A five mm mycelial disc of the fungal isolate was aseptically transferred to PDB at different pH levels of 3.5, 4.5 and 5.5. The inoculated flasks were incubated at 26 ± 2^0C for five days and after the completion of growth, the mycelial mat was removed, filtered and the fresh weight was recorded.

3.3.4 *In vitro* screening for salinity tolerance

Tolerance of different isolates of *Trichoderma* species to salinity evaluated as per the protocol of Amalraj*et* al. (2010). Mycelial disc of five mm diameter was placed in the centre of NaCl amended potato dextrose agar (PDA) and the growth was compared with the PDA plate without NaCl which served as control. Four concentrations *viz.,* 0.5M, 1M, 1.5M and 2M were employed for the experiment. The plates were incubated at 26 ± 2^0C for six days. Percentage reduction of growth in salt amended media was calculated using the formula (C-T) x100 /C, where C is the radial growth of the isolate in control plate (cm) and T

is radial growth in salt amended plate (cm) (Vincent, 1927).

3.3.5. *In vitro* screening for fungicide tolerance

The tolerance of various isolates of *Trichoderma* species to different fungicides were tested by employing the method of poisoned food technique (Grover and Moore, 1962). For this purpose, different concentrations of copper fungicides *viz.*, copper oxy chloride and copper hydroxide at 0.2%, 0.25% and 0.3% were used. An appropriate quantity of the fungicide was weighed out and was added to 100 ml of the sterile molten PDA so as to get the desired concentration. The medium was thoroughly mixed and about 20 ml of the medium was poured on to before plating. After solidification, a five mm mycelial disc of the fungal isolate was kept in the centre of the plate and the plates were kept for incubation at 26 ± 2^0C. A fungicide free inoculated plate was kept as control for comparison. Observations on radial growth were recorded daily until the control plates showed full growth. The per cent reduction of mycelial growth was calculated using the formula given in 3.3.4.

3.4. BIOCHEMICAL CHARACTERIZATION OF STRESS TOLERANT ISOLATES OF *Trichoderma*

The isolates of *Trichoderma* spp. obtained through *in vitro* evaluation for stress tolerance were further subjected to biochemical analysis for determination of cellulase, β- 1,3- glucanase and protease for determining their antagonistic potential. Biomolecules involved in imparting drought tolerance like ACC-deaminase (1-aminocyclopropane-1-carboxylate deaminase) and cytokinin were also assessed.

3.4.1. Cellulase assay

Production of cellulase enzyme by *Trichoderma* spp. was evaluated by the method suggested by Sadasivam and Manickam (2008). The selected fungal

isolates were grown in carboxy methyl cellulose (Guevara and Zambrano, 2006) (CMC) (Appendix II) (1%) medium for five days at $26\pm2^{0}C$. After the incubation period, 0.5 ml of the enzyme extract was pipetted out into 32 mg of dry Whatman No.1 filter paper discs (substrate) taken in boiling tubes. The mixture was incubated for 1 h at $50^{0}C$. The enzyme- substrate complex was removed from the water bath and 0.5 ml dinitrosalicylic acid (DNS) reagent was added immediately. This mixture was heated till boiling in a water bath for 5 min. Thereafter, one ml of Rochelle salt solution (K-Na- tartarate) (40%) was added to this while the tubes were still warm. The mixture was cooled to room temperature and the volume was adjusted to 5 ml with distilled water. The absorbance was measured at 540 nm assisted by spectrophotometer. A standard graph was plotted with glucose in the range 50 to 1000 µg ml^{-1}.

3.4.1. a. Calculation of cellulase activity

Glucose stock solution (1000 ppm) was prepared by taking 100 mg glucose and the volume was made upto 100 ml with distilled water. From this stock solution, 100, 300, 500, 600 and 750 ppm solutions were made.

Glucose solution (0.5 ml) and same amount of DNS reagent were added to boiling tubes and were kept in boiling water bath for 5 min. Procedure was continued in the same way as in 3.4.1. Absorbance was plotted against concentration of glucose and thus a standard graph and a regression equation was established.

$$\text{Cellulase activity (U/ml)} = \frac{\Delta E \times Vf}{\Delta t \times \Sigma \times Vs \times d}$$

ΔE = Absorbance at 540 nm

Vf = Final volume including DNS

Vs = Volume (mL) enzyme extract used

Δt = Time of hydrolysis

Σ = Extinction coefficient

d = Diameter of cuvette (1 cm for standard cuvette)

Extinction coefficient (Y) = 0.001X+ 0.069   (X= 0.18 mg= 1μM glucose)

3.4.2. *β- 1,3- glucanase assay*

β- 1, 3- glucanase activity of *Trichoderma* spp. was assayed as per the protocol of Sadasivam and Manickam (2008). For this the isolates were grown in carboxy methyl cellulose (1%) medium for five days at 26 ± 2^0C. One per cent CMC solution (substrate) (0.45 ml) and 0.05ml of enzyme extract were pipetted into the tubes and it was incubated at 55^0C for 15 min. Immediately after removing the enzyme- substrate complex, 0.5 ml of dinitrosalicylic acid (DNS) reagent was added. The tubes were kept in boiling water bath for 5 min. One ml of 40 per cent Rochelle salt (K-Na tartarate) solution was added to this, while the tubes were still warm. Further the tubes were cooled to room temperature and the volume was made upto 5 ml using distilled water. The absorbance was measured at 540 nm wavelength assisted by spectrophotometer. A standard graph was plotted using glucose in the concentration range of 50 to 1000μg ml^{-1}. Calculations of the *β-* 1, 3- glucanase activity was done following the method followed in 3.4.1.a.

3.4.3. Protease assay

The proteolytic ability of the fungal isolates was assessed by employing modified procedure of Tsuchida *et al.* (1986). The activity was determined with casein as substrate (Appendix II). Casein solution (2%) was prepared by using 0.2 M carbonate buffer (pH 10). Enzyme solution (0.5 ml) and an equal volume of casein solution were pipetted into the tubes and kept at 55^0C for 10 min. The reaction was ceased by adding 1 ml of 10 per cent trichloroacetic acid. Thereafter, this mixture was transferred to eppendorf tubes and centrifuged at 10,000 rpm for 10 min. The supernatant was collected and to this, five ml of 0.44 M Na_2CO_3 and one ml of two-fold diluted Folin- Ciocalteau reagent were added. Colour developed was read at 660 nm against the blank prepared. Standard graph was prepared using tyrosine in the concentration range of 50 to 1000μg ml^{-1}. One unit

of enzyme activity was defined as the amount of enzyme that released one μg of tyrosine per ml per min.

3.4.3. a. Calculation of protease activity

Stock solution of tyrosine (100 ppm) was prepared by taking 10 mg glucose and making it upto 100 ml with distilled water. From this stock solution, 20, 40, 60 and 80 ppm solutions were made. A standard graph was plotted with absorbance value on Y axis and concentration of tyrosine on X axis. Protease enzyme activity (units/ ml) was calculated using the formula,

$$\text{Protease activity (U/ ml)} = \frac{\text{Tyrosine equivalents released x Vf}}{\text{Ve x T x Vc}}$$

Vf = Final volume used in assay

Ve = Volume of enzyme extract used in assay

T = Time of assay as per the unit definition

Vc = Volume used in colorimetric determination

3.4.4. Screening for ACC Deaminase production in *Trichoderma* spp.

The ACC (1-Amino Cyclopropane-1-Carboxylate) deaminase activity of drought tolerant *Trichoderma* isolates was screened based on the ability of the isolates to use ACC as a sole nitrogen source (Ali *et al.*, 2014). All the isolates were grown in 100 ml potato dextrose broth for five days at $26 \pm 2^{0}C$. After completing full growth, the cells were collected by centrifugation at 10,000 rpm for 10 min and washed twice with sterile 0.1 M Tris- HCl. After washing, the cells were resuspended in 1ml of 0.1 M HCl and were spot inoculated on modified DF (Dworfkin and Foster) salts minimal medium (Appendix iii) (Dworkin and Foster, 1958). The plate with DF media amended with $(NH_4)_2SO_4$ (0.2% w/v) as the nitrogen source served as positive control. The plate with DF media, devoid of any nitrogen source served as negative control. Growth of isolates on ACC

supplemented plates was compared to positive and negative control plates and was selected based on growth by utilizing ACC as the nitrogen source.

3.4.5. Screening for cytokinin production in *Trichoderma* spp.

Production of cytokinin by selected isolates of *Trichoderma spp.* was evaluated by following the protocol of Cattelan *et al.* (1999). Fungal isolates were grown in sterile potato dextrose broth (PDB) for eight days at 26 ± 2^0C, followed by centrifugation at 10,000 rpm for 10 min.

Cowpea (var. Bhagyalakshmi) seeds were germinated on moistened filter paper kept in sterile Petri plates. After germination, hypocotyl and cotyledon were carefully separated from the seeds and were transferred to Petri plates containing filter paper moistened with the supernatant from each isolate after centrifugation. Specifically five seeds with intact cotyledons and hypocotyls were kept in each Petri plate. The plates were incubated for four days at 26 ± 2^0C to compare the biomass of the cotyledons, rate of secondary rootlet formation and length of the root or hypocotyl produced. The treatments were compared with control plates which consisted of filter paper moistened with sterile medium alone.

3.5.   *In vitro* EVALUATION OF STRESS TOLERANT ISOLATES OF *Trichoderma* spp. FOR THEIR ANTAGONISTIC POTENTIAL

The selected *Trichoderma spp.* with abiotic stress tolerance were tested for antagonistic property against five major soil borne pathogens *viz., Phytophthora capsici, Pythium aphanidermatum, Scelrotium rolfsii, Fusarium solani* and *Rhizoctonia solani* under *in vitro* conditions by employing the dual culture technique (Dennis and Webster, 1971). A five mm mycelial disc was taken from a five day old *Trichoderma* culture and was inoculated aseptically two cm away from the periphery of the sterile Petri plate containing PDA medium. Similarly, a five mm mycelial disc of the pathogen culture was inoculated in same PDA plate 3.5 cm away from the *Trichoderma* mycelial disc. The plates were incubated at 26 ± 2^0C. The fungal pathogen and each isolate of *Trichoderma* spp. were inoculated as dual culture after giving due consideration on the rate of growth of

pathogen and the antagonist. PDA mediated Petri dishes inoculated with 5 mm disc of pathogen alone served as control.Three replications were maintained and the growth of the pathogen was compared with the control plate. Measurements on radial growth were taken at regular intervals after 24 h of inoculation of the antagonist till the control plates attained full growth.Per cent inhibition of the growth of pathogen over control was calculated using the formula given in 3.3.4.

The type of interaction between *Trichoderma spp.* and the test pathogen was studied by the method proposed by Purkayastha and Bhattacharya (1982).

Types of reactions:

Homogenous (H)   : Free intermingling of hyphae
Overgrowth (O)   : Pathogen grown over by antagonist
Cessation of growth (C) : Cessation of growth at the line of contact
Aversion (A)   : Development of clear zone of inhibition

3.6 MOLECULAR CHARACTERIZATION OF STRESS TOLERANT *Trichoderma* spp.

Based on the previous studies, four *Trichoderma spp.* showing high abiotic stress tolerance *viz.*, salinity, acidity, drought, fungicide and thermotolerance and better enzyme activity as well as antagonistic property toward major pathogens were selected for further studies. The molecular characterization of these isolates was carried out at Rajiv Ganghi Centre for Biotechnology (RGCB), Thiruvananthapuram by ITS sequencing to identify the isolates upto species level. Sequence analysis and nucleotide homology of each isolate were analyzed through the BLASTn programme of National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov).

3.6.1 DNA isolation using NucleoSpin® Plant II Kit (Macherey-Nagel)

About 100 mg of the tissue/mycelium is homogenized using liquid nitrogen and the powdered tissue is transferred to a microcentrifuge tube. Four

hundred microlitres of buffer PL1 is added and vortexed for 1 minute. Ten microlitres of RNase A solution is added and inverted to mix. The homogenate is incubated at 65°C for 10 minutes. The lysate is transferred to a Nucleospin filter and centrifuged at 11000 x g for 2 minutes. The flow through liquid is collected and the filter is discarded. Four hundred and fifty microlitres of buffer PC is added and mixed well. The solution is transferred to a Nucleospin Plant II column, centrifuged for 1 minute and the flow through liquid is discarded. Four hundred microlitre buffer PW1 is added to the column, centrifuged at 11000 x g for 1 minute and flow though liquid is discarded. Then 700 µl PW2 is added, centrifuged at 11000 x g and flow through liquid is discarded. Finally 200 µl of PW2 is added and centrifuged at 11000 x g for 2 minutes to dry the silica membrane. The column is transferred to a new 1.7 ml tube and 50 µl of buffer PE is added and incubated at 65°C for 5 minutes. The column is then centrifuged at 11000 x g for 1 minute to elute the DNA. The eluted DNA was stored at 4°C.

3.6.2 Agarose Gel Electrophoresis for DNA Quality check

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.6.3 PCR Analysis

PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X Phire PCR buffer (contains 1.5 mM $MgCl_2$), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, 0.2 µl Phire Hotstart II DNA

polymerase enzyme, 0.1 mg/ml BSA and 3% DMSO, 0.5M Betaine, 5pM of forward and reverse primers.

Primers used

Target	Primer Name	Direction	Sequence (5' → 3')
ITS	ITS-1F	Forward	TCCGTAGGTGAACCTGCGG
	ITS-4R	Reverse	TCCTCCGCTTATTGATATGC

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

PCR amplification profile

ITS

98 °C - 30 sec

98 °C - 5 sec
60 °C - 10 sec } 40 cycles
72 °C - 15 sec

72 °C - 60 sec

4 °C - ∞

3.6.4 Agarose Gel electrophoresis of PCR products

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 5 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.6.5 ExoSAP-IT Treatment

ExoSAP-IT (GE Healthcare) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications.

Five micro litres of PCR product is mixed with 2 μl of ExoSAP-IT and incubated at 37°C for 15 minutes followed by enzyme inactivation at 80°C for 15 minutes.

3.6.6 Sequencing using BigDye Terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems , USA) following manufactures protocol.

The PCR mix consisted of the following components:

PCR Product (ExoSAP treated)	-	10-20 ng
Primer Reverse)	-	3.2 pM (either Forward or
Sequencing Mix	-	0.28 μl
5x Reaction buffer	-	1.86 μl
Sterile distilled water	-	make up to 10μl

The sequencing PCR temperature profile consisted of a 1ˢᵗ cycle at 96°C for 2 minutes followed by 30 cycles at 96°C for 30 sec, 50°C for 40 sec and 60°C for 4 minutes for all the primers.

3.6.7 Post Sequencing PCR Clean up

1. Make master mix I of 10μl milli Q and 2 μl 125mM EDTA per reaction

2. Add 12μl of master mix I to each reaction containing 10μl of reaction

contents and are properly mixed.

3. Make master mix II of 2 µl of 3M sodium acetate pH 4.6 and 50 µl of ethanol per reaction.

4. Add 52 µl of master mix II to each reaction.

5. Contents are mixed by inverting.

6. Incubate at room temperature for 30 minutes

7. Spin at 14,000 rpm for 30 minutes

8. Decant the supernatant and add 100 µl of 70% ethanol

9. Spin at 14,000 rpm for 20 minutes.

10. Decant the supernatant and repeat 70% ethanol wash

11. Decant the supernatant and air dry the pellet.

The cleaned up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems).

3.6.8 Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems).

3.7. *In vivo* EVALUATION OF PROMISING STRESS TOLERANT ISOLATES OF *Trichoderma* spp. FOR BIOCONTROL EFFICACY AND GROWTH PROMOTION

The most promising isolates of *Trichoderma* spp. obtained through abiotic stress screening, biochemical characterization and dual culture assay were subjected to a pot culture experiment so as to test the antagonism potential and growth promotion capability of the selected isolates. A pot culture experiment with cowpea as the test crop and *Rhizoctonia solani* as the test pathogen was laid

out. The isolates were compared with a positive control (*Trichoderma viride*) and a negative control (without *Trichoderma*). The experiment was carried out during March- June 2018 at College of Horticulture, Vellanikkara. The details of the experiment are as follows.

Design	: CRD
Treatments	: 6
Replications	: 3
Number of plants per replication	: 3
Crop	: Cowpea
Variety	: Bhagyalakshmi

3.7.1 Preparation of potting mixture

Grow bags of size $60 \times 30 \times 30$ cm the bags were filled with potting mixture prepared with soil, sand and cow dung in the ratio 1:1:1. The potting mixture was subjected to chemical sterilization with five per cent formalin solution (40%). Approximately 1l of formalin solution was used for drenching the soil in one polybag and they were covered with a polythene sheet for 10 days. Thereafter, the bags were kept open and given a waiting period of seven days so as to let the fumes of formalin escape from the grow bags. Afterwards the seeds of cowpea var. Bhagyalakshmi were sown in the grow bags and raised the crop.

3.7.2 Mass multiplication and application of *Trichoderma* spp.

Promising isolates of *Trichoderma spp.* and reference culture of KAU (*Trichoderma viride*) were grown in potato dextrose broth separately for 10 days at 26 ± 2^0C. Rice grains (var. Uma), locally available organic substrate were used for the mass multiplication of each isolate of *Trichoderma* spp. The grains were washed thoroughly and half boiled prior to sterilization. Thereafter, 500 g of half boiled rice grains were filled in 100 ml conical flasks and plugged with non absorbent cotton and then sterilized at 121^0C, 15 psi pressure for 60 min. After cooling, the mycelial discs of *Trichoderma* spp. were inoculated into the rice grains aseptically. These bags were incubated for 14 days at 26 ± 2^0C. After

incubation a green fungal mat formed inside the flask on the grains were used to inoculate the pots under study (Khandelwal *et al.*, 2012). The plants were inoculated 30 days after sowing with the mass multiplied *Trichoderma* spp. in rice grains @ 20 g per polybag with a spore concentration of 2×10^5 cfu g^{-1} (Singh *et al.*, 2014).

3.7.3. Mass multiplication of pathogen and it's challenge inoculation

The performance of the isolates of *Trichoderma* spp. was evaluated against cowpea collar rot disease which is caused by the pathogen *Rhizoctonia solani*. A five mm mycelial disc of the pathogen, *Rhizoctonia solani* was inoculated on fresh PDA medium under laminar air flow and incubated for 48 h at 26 ± 2^0C. The substrate selected for mass multiplication of *R. solani* was rice grains (var. Uma) and the same method of sterilization was followed as depicted in 3.7.2. Inoculation with fungal disc of the pathogen was done in 100 ml conical flask which was allowed to grow for 14 days at 26 ± 2^0C. As white fungal mat grew over the substrate after the completion of incubation period, the plants were challenge inoculated with the mass multiplied *R. solani* (spore concentration 2 $\times 10^5$ cfu g^{-1}) @ 20 g, 10 days after inoculation of *Trichoderma* (Bhagat and Pan, 2006).

3.7.4. Germination percentage

To study germination percentage, cowpea seeds were treated with spore suspensions of selected *Trichoderma* isolates and were sown in pathogen inoculated potting mixture. The pathogen was mass multiplied in the same manner as explained in 3.7.3. The mass multiplied rice grains were inoculated @ 5 g per pot prior to sowing. Cowpea seeds (var. Bhagyalakshmi) coated with the selected isolates of *Trichoderma spp.* were sown @ 3 seeds per pot. Observations such as number of seeds germinated, earliness in germination, pre- germination rot and per cent germination were recorded and compared with a positive control (*Trichoderma viride*, KAU isolate) and a negative control (without *Trichoderma*

spp.).

Design	: CRD
Treatments	: 6
Replications	: 5
Number of plants per replication	: 5
Crop	: Cowpea
Variety	: Bhagyalakshmi

3.7.5. Biometric observations

Observations on plant height, number of leaves, number of flowers, days to flowering, number of pods and yield were recorded and compared among the treatments.

3.7.5.2.a. Height of the plant

The height of each plant was taken before and after the treatment application at 30 days after sowing (DAS), 45 DAS and 60 DAS.

3.7.5.2.b. Number of leaves

Number of leaves was counted before and after the treatment application at 30, 45 and 60 DAS.

3.7.5.2.c. Days to first flowering

Days to flowering were noted and were recorded for each treatment and compared among the various treatments.

3.7.5.2.d. Yield obtained

Weight and number of pods obtained from each treatment were measured and recorded.

3.7.6. Assessment of disease incidence

After one week of challenge inoculation with the pathogen *R. solani,*

collar region of the plants was checked to see symptom appearance and accordingly per cent disease incidence was calculated by following the formula given by Wheeler (1969).

$$\text{Per cent disease incidence (PDI)} = \frac{\text{Number of plants infected}}{\text{Total number of plants observed}} \times 100$$

3.8. Statistical analysis

Data was subjected to Analysis of Variance (ANOVA), which was carried out by employing Web Agri Stat Package (WASP 2.0). Levels of significance, means and standard error were obtained for various data sets. Appropriate transformation was done according to the method suggested by Gomez and Gomez (1984). Multiple comparison between the treatment means where the F test was significant was done with Duncan's Multiple Range Test (DMRT).

Results

4. RESULTS

The research on "Exploitation of abiotic stress tolerant strains of *Trichoderma* spp. for the management of soil borne fungal pathogens" was conducted to screen abiotic stress tolerant isolates of *Trichoderma* spp. under *in vitro* and also to evaluate the growth promotion and biocontrol ability of the promising isolates against soil borne pathogen under *in vivo* conditions. The results of the experiment carried out during 2016-2018 are given below.

4.1 SURVEY, COLLECTION AND ESTIMATION OF ELECTROCHEMICAL PROPERTIES OF SOIL SAMPLES FROM DIFFERENT STRESS PRONE AREAS OF KERALA

Intensive sampling surveys were conducted across different abiotic stressed ecosystems of Kerala *viz.,* Wayanad, Palakkad, Thrissur, Vytilla, Alappuzha and Kumarakom. pH and electrical conductivity of the samples were estimated under *in vitro* conditions.

4.1.1. Collection of soil samples

A total of 52 representative soil samples were collected from different locations of six districts across Kerala. A total of 12, 14 and 11 samples were collected from Wayanad, Palakkad and Thrissur respectively and five samples each from Alappuzha, Ernakulam and Kottayam were also procured. The properties of soil such as soil reaction (pH), electrical conductivity (EC) and soil texture are depicted in Table 4.1.

Table 4.1 Details of soil samples collected from different locations across Kerala

Sl. No.	Districts	Locations	Soil sample code	Soil texture	pH	EC (dS m^{-1})
1.	Palakkad	1.Alathur- 1	P1	Sandy clay loam	6.20	0.01
		2. Alathur- 2	P2	Sandy clay loam	5.80	0.01
		3. Alathur- 3	P3	Sandy clay loam	6.00	0.01
		4. Alathur- 4	P4	Sandy clay loam	5.50	0.02
		5.Pazhambalakode- 1	P5	Sandy clay loam	5.40	0.02
		6. Pazhambalakode- 2	P6	Sandy clay loam	5.20	0.02
		7.Pazhambalakode- 3	P7	Sandy clay loam	5.60	0.02
		8. Pazhambalakode-4	P8	Sandy clay loam	5.80	0.01
		9. Athipotta- 1	P9	Sandy clay loam	5.20	0.02
		10. Athipotta- 2	P10	Sandy clay loam	5.30	0.02
		11. Tarur-1	P11	Sandy clay loam	5.70	0.02
		12. Tarur- 2	P12	Sandy clay loam	6.00	0.02
		13. Tarur- 3	P13	Sandy clay loam	5.90	0.02
		14. Tarur- 4	P14	Sandy clay loam	5.20	0.02
2.	Alappuzha	1.Cherthala- 1	A1	Sandy	3.00	7.61
		2. Cherthala-2	A2	Sandy	2.90	5.26
		3. Kanjikuzhi-1	A3	Sandy	3.30	5.50
		4. Muhamma-1	A4	Sandy	2.80	5.50
		5. Muhamma- 2	A5	Sandy	3.50	3.00
3.	Ernakulam	1.Vytilla- 1	E1	Clay	4.10	3.73
		2. Vytilla- 2	E2	Clay	4.10	2.21
		3. Vytilla- 3	E3	Clay	4.30	3.25
		4. Vytilla- 4	E4	Clay	4.30	2.09
		5. Vytilla- 5	E5	Clay	4.20	2.05
4.	Kottayam	1.Kumarakom-1	K1	Clay loam	4.20	1.37
		2. Kumarakom- 2	K2	Clay loam	4.70	1.76
		3. Kumarakom- 3	K3	Clay loam	3.90	1.29
		4. Kumarakom- 4	K4	Clay loam	4.40	2.35
		5. Kumarakom- 5	K5	Clay loam	4.20	1.34
5.	Wayanad	1. Bathery- 1	W1	Sandy loam	5.80	0.19
		2. Bathery- 2	W2	Sandy loam	7.10	0.11
		3. Bathery- 3	W3	Sandy loam	6.20	0.18
		4. Muthumala- 1	W4	Sandy loam	6.30	0.05
		5. Ambukuthi- 1	W5	Sandy clay	6.50	0.04
		6. Ambukuthi- 2	W6	Sandy clay	6.10	0.22
		7. Tholpetty- 1	W7	Sandy loam	5.40	0.14

70

Sl. No.	Districts	Locations	Soil sample code	Soil texture	pH	EC (dS m^{-1})
		8. Tholpetty- 2	W8	Sandy loam	6.10	0.08
		9. Tholpetty- 3	W9	Sandy loam	7.00	0.14
		10. Tholpetty-4	W10	Sandy loam	6.10	0.16
		11. Kurichiyad- 1	W11	Sandy clay loam	6.00	0.11
		12. Kalloor- 1	W12	Sandy clay loam	5.90	0.10
6.	Thrissur	1. Puzhakkal- 1	T1	Clay	5.30	0.10
		2. Puzhakkal- 2	T2	Clay	5.40	0.09
		3. Puzhakkal- 3	T3	Clay	5.20	0.86
		4. Kanjani- 1	T4	Clay	5.00	0.09
		5. Kanjani- 2	T5	Clay	5.10	0.09
		6. Kanjani- 3	T6	Clay	4.40	0.26
		7. Adat- 1	T7	Clay	4.60	0.25
		8. Adat- 2	T8	Clay	4.50	0.48
		9. Adat- 3	T9	Clay	4.30	0.55
		10. Alappat- 1	T10	Clay	4.90	0.56
		11. Alappat-2	T11	Clay	4.40	0.56

4.1.2 Estimation of soil reaction (pH)

Rhizosphere soil samples were collected and pH was estimated under laboratory conditions. The soil samples collected were serially numbered with respect to location and designated with a sample code representing the district. Code P1 to P14 represent samples from Palakkad district, A1 to A5 represent Alappuzha district, E1 to E5 were assigned for samples collected from Ernakulam, K1 to K5 represent Kottayam district, W1 to W12 represent Wayanad district and T1 to T11 represent Thrissur district (Table 4.1). Among the 52 soil samples collected, the lowest pH of 2.8 was recorded from the sample Muhamma-1 collected from Alappuzha district and the highest of 7.10 from Bathery- 2 of Wayanad district. The data recorded is depicted in Table 4.1.

pH of the 14 soil samples collected from different cropping system of Palakkad district were estimated. In general, it was observed that the soils were having sandy to clay loam texture with pH value ranging from 5.20 to 6.20 making them moderately to slightly acidic. The highest pH value of 6.20 was noticed from Alathur-1 and lowest value of 5.20 from Pazhambalakode- 2 and Athipotta- 1.

All the five soil samples collected from rice fields of Alappuzha was sandy in texture and showed a pH value in the range of 2.80- 3.50 making them very strongly acidic. Lowest pH value of 2.80 was recorded from Muhamma-1 and highest of 3.50 from Muhamma-2. Soil samples from rice fields of Vytilla Rice Research Station of Ernakulam district revealed a clayey texture with pH values ranging between 4.10 and 4.30 rendering them strongly acidic. Among the five samples, Vytilla- 3 and Vytilla- 4 recorded highest pH of 4.30 and Vytilla-1 and Vytilla-2 recorded the lowest of 4.10.

In addition, five representative soil samples collected from rice fields of Kumarakom of Kottayam district depicted a clayey loam texture, where the pH of the soil samples were found to be strongly acidic with a pH value ranging from 3.90 to 4.70. The soil sample, Kumarakom- 2 recorded the highest pH of 4.70 and

the lowest value of 3.90 was reported from Kumarakom-3. Twelve soil samples were collected from different locations of Wayanad and pH was recorded. Soil texture of the samples varied among different locations and it was noticed that except for Ambukuthi-1, Ambukuthi-2, Kalloor-1 and Kurichiyad- 1 all other samples were sandy loam in texture. However, Ambukuthi- 1 and 2 were sandy to clayey in texture, whereas Kalloor-1 and Kurichiyad-1 had sandy clay loam texture. pH of the samples revealed that the soils were moderately acidic to neutral with values ranging from 5.40- 7.10. The lowest pH of 5.40 was recorded in soils collected from Tholpetty and the highest of 7.10 was recorded from Bathery- 2.

Similarly, 11 representative soil samples were collected from *Kole* lands of Thrissur district and pH were estimated under laboratory conditions. The data revealed clayey texture and showed strongly to moderately acidic reaction with values ranging from 4.30 to 5.40. Soil sample collected from Adat-3 recorded lowest pH value of 4.30 and highest value of 5.40 was recorded in Puzhakkal-2.

4.1.3. Estimation of electrical conductivity (EC)

Electrical conductivity (EC) of soil sample collected from different locations was estimated and it was expressed in dS m^{-1}. The details are also presented in Table 4.1. In general, the highest EC of 7.61 dS m^{-1} was observed from Cherthala-1 sample collected from Alappuzha district and the lowest of 0.01 dS m^{-1} from Alathur- 1, Alathur- 2, Alathur- 3 and Pazhambalakode-4 of Palakkad district.

Electrical conductivity of soil samples collected from Palakkad district showed an EC ranging from 0.009 dSm^{-1} (Alathur- 2) to 0.24 dSm^{-1} (Tarur- 2 and Tarur-4) which were also considered as non- saline. However, it was observed that soil samples collected from Alappuzha district recorded high EC value ranging between 3.00 to 7.61 dSm^{-1} rendering them slightly saline. Moreover, samples

from Vytilla and Kumarakom also depicted an EC ranging from 2.05- 3.73 dS m^{-1} and 1.34- 2.35 dS m^{1} respectively which shows that the soil samples were found to be slightly saline.

Similarly, soil samples collected from Wayanad district ranged from 0.04 to 0.22 dSm^{-1} with the highest EC value from Ambukuthi- 2 and the lowest value from Ambukuthi -1 making them non saline. Likewise, 11 soil samples collected from *Kole* lands of Thrissur revealed that EC ranged from 0.09 to 0.562 dS m^{-}^{1}which also makes them non saline. The highest EC value of 0.562 dS m^{-1} and the lowest value of 0.09 dS m^{-1} were recorded from Puzhakkal- 2 and Kanjani- 1 and Alappat-2 of Thrissur district.

4.2. ISOLATION, ENUMERATION AND CHARACTERIZATION OF *Trichoderma* spp.

Native *Trichoderma* species were isolated from soil samples using different selective media. Five different media, *viz.*, *Trichoderma* selective medium (TSM), Malt extract agar (MEA), Oatmeal extract agar (OEA), Special nutrient agar (SNA) and Potato dextrose agar (PDA) were used for the isolation. After screening of these five media, TSM and MEA were selected for further isolation of *Trichoderma* spp. as these media yielded significantly numerous colonies of *Trichoderma* spp. Three dilutions of 10^{-2}, 10^{-3} and 10^{-4} were used initially and it was narrowed down to the latter two due to better formation of single colonies of *Trichoderma* spp. in those dilutions. Population of *Trichoderma* spp. from different soil samples obtained after serial dilution in MEA and TSM are presented in Table 4.2.

A total of 24 isolates of *Trichoderma* spp. were obtained from different locations selected under the study. Among the six locations, soil samples collected from Wayanad recorded maximum number of eight isolates followed by Palakkad, Thrissur, Alappuzha, Vytilla and Kumarakom with 6, 4, 3, 2 and 1 isolate respectively. Here again, based on the number of *Trichoderma* spp.

74

obtained from each district, they were serially numbered and abbreviated based on the name of the location. Accordingly, PAT 1 to PAT 6 represent number of isolates of *Trichoderma* spp. from Palakkad district, ALT 1 to ALT 3 from Alappuzha, VYT 1 and VYT 2 from Ernakulam district, KUT 1 from Kottayam district, WAT 1 to WAT 8 from Wayanad and THT 1 to THT 4 from Thrissur district.

From the Table 4.2, it is clear that there was a significant difference in the population of *Trichoderma* spp. among the locations. Highest population of the fungus using both selective media of Wayanad district was obtained from W7 with a cfu of 32.52×10^3 and 22.22×10^4 in TSM and 31.22×10^3 and 17.61×10^4 in MEA medium. Lowest population of 4.18×10^3 and 2.17×10^4 cfu g^{-1} was obtained from P 10 of Palakkad district in TSM and MEA respectively.

In Palakkad district, population of *Trichoderma* spp. ranged from 5.87 to 12.75×10^3 cfu g^{-1} and 1.77 to 6.09×10^4 cfu g^{-1} in TSM media. While, a population of 4.18 to 10.18×10^3 cfu ml^{-1} and 2.17 to 5.77×10^4 cfu g^{-1} was noticed when MEA medium was used. Highest population of *Trichoderma* spp. was isolated from P 5 and P 14 and lowest from P 6 and P 10 samples at 10^{-3} in TSM media. From 10^{-4} dilution, maximum number of *Trichoderma* colonies was obtained from P 5 and minimum from P 10 using the same medium. When MEA media was employed for the enumeration of *Trichoderma* spp., maximum number of colonies was retrieved from P 5 in both dilutions, nonetheless, minimum number of colonies was obtained from P 6 and P 10 from 10^{-3} and 10^{-4} dilution respectively. Some of the samples collected from Palakkad district *viz.,* P1 to P4, P8, P9, P12 and P 13 did not show any colonies of *Trichoderma* spp. in both media. Isolates of *Trichoderma* spp. obtained from the soil samples P5, P6, P7, P10, P11 and P14 were thereby abbreviated as PAT1, PAT2, PAT3, PAT4, PAT5 and PAT6 respectively and were selected for further studies.

75

Table 4.2 Enumeration of *Trichoderma* spp. from soil samples collected from different locations

Sl. No.	Districts	Soil samples	Isolates of *Trichoderma* spp.	*Population of *Trichoderma* spp. (CFU g^{-1})			
				TSM		MEA	
				x 10^3	x 10^4	x 10^3	x 10^4
1.	Palakkad	P1	Nil	0.00(0.00)^d	0.00(0.00)^g	0.00(0.00)^e	0.00(0.00)^e
		P2	Nil	0.00(0.00)^d	0.00(0.00)^g	0.00(0.00)^e	0.00(0.00)^e
		P3	Nil	0.00(0.00)^d	0.00(0.00)^g	0.00(0.00)^e	0.00(0.00)^e
		P4	Nil	0.00(0.00)^d	0.00(0.00)^g	0.00(0.00)^e	0.00(0.00)^e
		P5	PAT1	12.70(1.14)^a	6.09(0.78)^a	10.07(1.00)^a	5.77(0.76)^a
		P6	PAT2	5.87(0.84)^c	2.57(0.41)^e	4.27(0.63)^d	2.39(0.38)^d
		P7	PAT3	6.11(0.85)^c	2.97(0.47)^d	5.90(0.77)^c	3.077(0.49)^c
		P8	Nil	0.00(0.00)^d	0.00(0.00)^g	0.00(0.00)^e	0.00(0.00)^e
		P9	Nil	0.00(0.00)^d	0.00(0.00)^g	0.00(0.00)^e	0.00(0.00)^e
		P10	PAT4	5.87(0.84)^c	1.77(0.24)^f	4.18(0.62)^d	2.17(0.33)^d
		P11	PAT5	10.86(1.07)^b	4.39(0.64)^c	9.54(0.98)^b	4.38(0.64)^b
		P12	Nil	0.00(0.00)^d	0.00(0.00)^g	0.00(0.00)^e	0.00(0.00)^e
		P13	Nil	0.00(0.00)^d	0.00(0.00)^g	0.00(0.00)^e	0.00(0.00)^e
		P14	PAT6	12.75(1.14)^a	5.11(0.71)^b	10.18(1.01)^a	4.24(0.63)^b
	CD (0.05)			0.012	0.030	0.014	0.034
2.	Alappuzha	A1	ALT1, ALT2	16.11(1.21)^b	10.00(1.00)^b	18.08(1.26)^b	14.93(1.17)^b
		A2	Nil	0.00(0.00)^c	0.00(0.00)^c	0.00(0.00)^c	0.00(0.00)^c
		A3	Nil	0.00(0.00)^c	0.00(0.00)^c	0.00(0.00)^c	0.00(0.00)^c
		A4	ALT3	27.73(1.46)^a	14.20(1.15)^a	23.16(1.37)^a	15.45(1.19)^a
		A5	Nil	0.00(0.00)^c	0.00(0.00)^c	0.00(0.00)^c	0.00(0.00)^c
	CD (0.05)			0.009	0.045	0.014	0.011
3.	Ernakulam	V1	Nil	0.00(0.00)^c	0.00(0.00)^c	0.00(0.00)^c	0.00(0.00)^b
		V2	VYT1, VYT2	17.00(1.26)^a	10.56(1.02)^a	18.07(1.26)^a	10.42(1.02)^a
		V3	Nil	0.00(0.00)^c	0.00(0.00)^c	0.00(0.00)^c	0.00(0.00)^c
		V4	Nil	0.00(0.00)^c	0.00(0.00)^c	0.00(0.00)^c	0.00(0.00)^c
		V5	Nil	0.00(0.00)^c	0.00(0.00)^c	0.00(0.00)^c	0.00(0.00)^c
	CD (0.05)			0.033	0.017	0.02	0.043
4.	Kottayam	K1	Nil	0.00(0.00)^c	0.00(0.00)^b	0.00(0.00)^c	0.00(0.00)^b
		K2	KUT1	13.91(1.17)^a	8.72(0.94)^a	12.50(1.10)^a	9.00(0.95)^a
		K3	Nil	0.00(0.00)^c	0.00(0.00)^b	0.00(0.00)^c	0.00(0.00)^b
		K4	Nil	0.00(0.00)^c	0.00(0.00)^b	0.00(0.00)^c	0.00(0.00)^b

Sl. No.	Districts	Soil samples	Isolates of *Trichoderma* spp.	*Population of *Trichoderma* spp. (CFU g^{-1})			
				TSM		MEA	
				x 10^3	x 10^4	x 10^3	x 10^4
		K5	Nil	0.00(0.00)^c	0.00(0.00)^b	0.00(0.00)^c	0.00(0.00)^b
CD (0.05)				0.011	0.002	0.010	0.034
5.	Wayanad	W1	WAT 1	4.47(0.65)^f	2.72(0.43)^f	3.43(0.54)^f	2.46(0.38)^f
		W2	Nil	0.00(0.00)^c	0.00(0.00)^b	0.00(0.00)^c	0.00(0.00)^b
		W3	Nil	0.00(0.00)^c	0.00(0.00)^b	0.00(0.00)^c	0.00(0.00)^b
		W4	Nil	0.00(0.00)^c	0.00(0.00)^b	0.00(0.00)^c	0.00(0.00)^b
		W5	WAT2,WAT3, WAT4	25.27(1.40)^c	15.00(1.18)^c	23.161.37)^c	9.67(0.99)^c
		W6	WAT5	26.33(1.42)^b	18.00(1.26)^b	26.07(1.42)^b	12.73(1.11)^b
		W7	WAT6	32.52(1.51)^a	22.22(1.35)^a	31.22(1.50)^a	17.61(1.25)^a
		W8	Nil	0.00(0.00)^c	0.00(0.00)^b	0.00(0.00)^c	0.00(0.00)^b
		W9	WAT7	14.82(1.17)^d	8.33(0.92)^d	13.47(1.13)^d	7.49(0.87)^d
		W10	Nil	0.00(0.00)^c	0.00(0.00)^b	0.00(0.00)^c	0.00(0.00)^b
		W11	Nil	0.00(0.00)^c	0.00(0.00)^b	0.00(0.00)^c	0.00(0.00)^b
		W12	WAT8	9.17(0.96)^e	5.08(0.71)^e	9.97(1.00)^e	4.08(0.61)^e
CD (0.05)				0.021	0.046	0.010	0.034
6.	Thrissur	T1	Nil	0.00(0.00)^c	0.00(0.00)^b	0.00(0.00)^c	0.00(0.00)^b
		T2	Nil	0.00(0.00)^c	0.00(0.00)^b	0.00(0.00)^c	0.00(0.00)^b
		T3	THT1	11.98(1.11)^b	6.62(0.82)^b	12.50(1.10)^b	8.40(0.92)^b
		T4	Nil	0.00(0.00)^c	0.00(0.00)^b	0.00(0.00)^c	0.00(0.00)^b
		T5	Nil	0.00(0.00)^c	0.00(0.00)^b	0.00(0.00)^c	0.00(0.00)^b
		T6	THT2	16.11(1.21)^a	8.66(0.94)^a	13.40(1.13)^a	10.60(1.03)^a
		T7	Nil	0.00(0.00)^c	0.00(0.00)^b	0.00(0.00)^c	0.00(0.00)^b
		T8	THT3	13.52(1.13)^b	6.78(0.83)^b	9.67(0.98)^b	9.11(0.96)^b
		T9	THT4	16.90(1.23)^a	9.72(0.99)^a	13.77(1.14)^a	10.89(1.04)^a
		T10	Nil	0.00(0.00)^c	0.00(0.00)^b	0.00(0.00)^c	0.00(0.00)^b
		T11	Nil	0.00(0.00)^c	0.00(0.00)^b	0.00(0.00)^c	0.00(0.00)^b
CD (0.05)				0.014	0.033	0.039	0.022

*Mean of three replications

In each column figure followed by same letter do not differ significantly according to DMRT

Log transformed values are given in parentheses.

Similarly, population of *Trichoderma* spp. from Alappuzha soils in TSM medium ranged from 16.11 to 27.33 x 10^3 cfu g^{-1} and 10 to 14.20 x 10^4 cfu g^{-1} at 10^{-3} and 10^{-4} dilutions respectively. Meanwhile, when MEA medium was used, the number of colonies ranged from 18.08 to 23.16 x 10^3 cfu g^{-1} and 14.93 to 15.45 x 10^4 cfu g^{-1} and it was observed that in both the medium, maximum number of colonies was isolated from A 4 and minimum from A 1 from both dilutions. Soil samples *viz.,* A 2, A 3 and A 5 showed no colony of *Trichoderma* spp. in both media. Two isolates were acquired from the soil sample A1 and they were named as ALT1 and ALT2 and the isolate obtained from the soil sample A4 was abbreviated as ALT3 and these were used for further studies.

Soil sample E 2 from Vytilla of Ernakulam district was only noticed with a population of 17.00 x 10^3 and 10.56 x 10^4 cfu g^{-1} in TSM medium. In MEA medium, a population of 18.07 x 10^3 and 10.42 x 10^4 cfu g^{-1} were recorded from the same sample E 2 and none of the samples other than E 2 showed a population of *Trichoderma* spp. The isolates of *Trichoderma* spp. acquired from E 2 soil sample were named as VYT1 and VYT2. In addition, population of *Trichoderma* spp. isolated from Kumarakom of Kottayam district recorded values of 13.91 x 10^3 and 8.72 x 10^4 cfu g^{-1} in TSM media. Whereas, the population in MEA medium was found to be 12.50 x 10^3 and 9 x 10^4 cfu g^{-1} from the soil sample K 2. The isolate of *Trichoderma* spp. obtained from K2 soil sample was named as KUT1. It was also noticed that all the other soil samples were devoid of *Trichoderma* colonies.

From Wayanad, *Trichoderma* population ranging from 4.47 to 32.52 x 10^3 cfu g^{-1} and 2.72 to 22.22 x 10^4 cfu g^{-1} at 10^{-3} and 10^{-4} dilutions while using TSM as the selective media. Whereas, when MEA media was used for the isolation, 3.43 - 31.22 x 10^3 cfu g^{-1} and 2.46 - 17.61x 10^4 cfu g^{-1} was acquired from 10^{-3} and 10^{-4} dilutions respectively. In both the cases, highest population of *Trichoderma* spp. was isolated from soil sample W7 followed by W 6 and the lowest population from W1. From W2, W 3, W 4, W 8, W 10 and W 11, no *Trichoderma* colonies were obtained. Isolates of *Trichoderma* spp. obtained from W1, W6, W7, W9 and

W10 were abbreviated as WAT1, WAT5, WAT6, WAT7 and WAT8 respectively. Three morphologically different *Trichoderma* isolates were obtained from W5 soil sample and they were named as WAT2, WAT3 and WAT4 and all these isolates were used for further studies.

Likewise, population of *Trichoderma* spp. from *Kole* lands of Thrissur district ranged from 11.98 - 16.90 x 10^3 cfu g^{-1} and 6.62 - 9.72 x 10^4 cfu g^{-1} when TSM was employed. Maximum number of colonies was noticed in T 9 and T 6 at both dilutions and minimum in T 3 at 10^{-3} dilution in the same medium. Meanwhile, in MEA medium, population ranged from 12.50 - 13.77 x 10^3 cfu g^{-1} and 8.40 - 10.89 x 10^4 cfu g^{-1}. Number of colonies isolated from T 9 and T 6 were on par and they were found to be the maximum, whereas, minimum number of colonies were noticed in T 8 and T 3 in both the dilutions when MEA was used. No colony of *Trichoderma* spp. were noticed from soil samples collected from T 1and T 2, T 4 and T 5, T 7, T 10 and T 11. *Trichoderma* isolates obtained from T3, T6, T8 and T9 were designated as THT1, THT2, THT3 and THT4 respectively and were used for later studies.

4.2.1. Characterization of *Trichoderma* spp.

Cultural and morphological characterization of *Trichoderma* spp. isolated from different locations was carried out under *in vitro* conditions. A detailed description of each isolate is described below and the observations are also presented in Table 4.3.

4.2.1.1. *Trichoderma* spp. isolated from Palakkad

a) PAT 1

The isolate PAT1 from P 5 soil sample (Pazhambalakode-1) of Palakkad district, was characterized by a white fluffy mycelium in the initial stage which later turned green after three days due to sporulation. On the fifth day after inoculation, the plate was completely covered with a green spore mass [Plate

4.1(i) 1]. More conidial formation was obtained towards the margin of the fungal colony rather than in the centre. Reverse side of the plate appeared cream or buff coloured. Hyphae hyaline with septations, conidia round, 2.95 μm size and appeared at the tip of the subglobose to ovoidal penicillate phialides measuring a size of 10.42 μm [Plate 4.2 (i) 1].

b) PAT 2

PAT 2 isolated from P 6 sample (Pazhambalakode-2) from Palakkad district was noticed with a white feeble mycelial growth which after third day of inoculation which turned green colour due to sporulation. Five days after inoculation, the colour of the conidia became dark green and a concentric pattern of spores were visible in the media [Plate 4.1(i) 2]. The fungus appeared buff coloured on the reverse side of the Petri plate. Hyphae hyaline, septate, conidia round, dark green colour, 2.77μm and arranged at the tip of penicillate phialides of 15.73μm [Plate 4.2(i) 2].

c) PAT 3

This isolate was obtained from soil sample P 7 location (Pazhambalakode-3), where the isolate produced fluffy white mycelium initially which after four days of incubation turned green due to the formation of conidia [Plate 4.1 (i) 3]. Growth was completed after five days of incubation at room temperature. The reverse side of the plate had buff or cream colour. Hyphae septate, hyaline, round conidia of 2.64 μm attached to the tip of solitary elongated phialides of 11.61 μm seen in clusters [Plate 4.2 (i) 3].

80

Table 4.3 Cultural and morphological characters of various isolates of *Trichoderma* spp. from different locations

Sl. No.	*Trichoderma* isolate	Cultural characters	Reverse side of the Petri plate	Morphological characters						
				Hyphae	Shape of conidia	Colour of conidia	Diameter of conidia (μm)	Arrangement of conidia	Shape of phialides	Size of phialides (μm)
1	PAT1	White fluffy mycelium green sporulation	Cream or buff colour	Hyaline, septate	Round	Green	2.95	Clusters at tip of phialides	Subglobose to ovoidal	10.42
2	PAT2	White feeble mycelial growth with dark green sporulation	Buff colour	Hyaline, septate	Round	Dark green	2.77	In whorls as clusters	Subglobose to ovoidal	15.73.
3	PAT3	Fluffy white mycelium with green sporulation, concentric ring growth pattern	Cream or buff colour	Hyaline, septate	Round	Green	2.64	Attached to tip of solitary phialides seen in clusters	Elongate	11.61
4	PAT4	Sparse white mycelial growth with light green conidia	Amber colour	Hyaline, septate	Round	Light green	2.25	Arranged in cluster at tip of penicillate phialides	Subglobose to ovoidal	7.67
5	PAT5	Cream coloured sparse mycelial growth with dark green sporulation	Cream or buff colour	Hyaline, septate	al to globose	Dark green	2.77	Arranged in cluster at tip of penicillate phialides	Ellipsoidal	6.20
6	PAT6	White fluffy mycelial growth, which later transformed into green colour	Cream or buff colour	Hyaline, septate	Globose to round	Green	2.82	In clusters at tip of phialides.	Ellipsoidal	11.936

Sl. No.	Trichoderma isolate	Cultural characters	Reverse side of the Petri plate	Morphological characters						
				Hyphae	Shape of conidia	Colour of conidia	Diameter of conidia (µm)	Arrangement of conida	Shape of phialides	Size of phialides (µm)
7	ALT1	Cream coloured sparse mycelium with green sporulation	Cream or buff colour	Hyaline, septate	Smooth, round to globose,	Green	2.86	In clusters to tip of penicillate phialides	Elongate	8.26
8	ALT2	White fluffy mycelium which later turned into pale green growth due to sporulation.	Cream or buff colour	Hyaline, septate	Globose to ovoid,	Light green	3.30	In clusters to tip of phialides	Elongate and cylindrical phialides	13.23
9	ALT3	Sparse white mycelium which later turned to dark green sporulation	Cream or buff colour	Hyaline, septate	Smooth, round	Green	2.47	In whorls at tip of the penicillate phialides	Subglobose to ovoidal	9.89
10	VYT1	White to cream sparse mycelium with dark green sporulation	Cream or buff colour	Hyaline, septate	Globose to round	Dark green	2.13	Attached to tip of phialides in whorls as cluster	Ellipsoidal phialides	6.221
11	VYT2	White sparse mycelium, later dark green spores covered the plate &few spores in centre	Cream or buff colour	Hyaline, septate	Smooth, ovoidal	Green	3.86	Attached to spindle shaped conidiophores seen in pairs at the tip	Spindle shaped	6.88

68

Sl. No.	Trichoderma isolate	Cultural characters	Reverse side of the Petri plate	Morphological characters						
				Hyphae	Shape of conidia	Colour of conidia	Diameter of conidia (µm)	Arrangement of conida	Shape of phialides	Size of phialides (µm)
12	KUT1	White fluffy mycelium initially with green concentric rings after sporulation	Cream or buff colour	Hyaline, septate	ovoid to globose	Green	0.98	In clusters attached to tip of phialides arranged in whorls	Ellipsoidal to pin shaped	9.54
13	WAT1	Cream mycelium with dark green conidia	Cream or buff colour	Hyaline, septate	Round to oval	Dark green	2.54	Attached to tip of penicillate phialides in clusters	Ellipsoid, plump	14.23
14	WAT2	White fluffy growth with light green sparse sporulation	Cream or buff colour	Hyaline, septate	Round to ovoid	Light green	2.95	Attached in clusters to the tip of penicillate phialides	Short, plump	10.31
15	WAT3	Light cream coloured mycelia with dull green sporulation	Cream or buff colour	Hyaline, septate	Round to globose	Light green	2.33	Attached to tip of penicillate phialides	Ellipsoid	11.94
16	WAT4	Light cream coloured sparse mycelium with green spore mass	Cream or buff colour	Hyaline, septate	Smooth, round to globose,	Green	2.36	Attached to tip of penicillate phialides	Short, plump	7.67
17	WAT5	White mycelial growth with green and concentric growth pattern	Cream or buff colour	Hyaline, septate	Globose to round	Green	2.30	Attached to phialides in clusters	Short ellipsoidal	4.11

| | | | | Morphological characters | | | | | | |
Sl. No.	Trichoderma isolate	Cultural characters	Reverse side of the Petri plate	Hyphae	Shape of conidia	Colour of conidia	Diameter of conidia (µm)	Arrangement of conida	Shape of phialides	Size of phialides (µm)
18	WAT6	White mycelium turned into green and white colony with concentric pattern	Cream colour	Hyaline, septate	Smooth walled and round	Green	2.36	Attached to phialides in clusters	Small short ellipsoidal	5.46
19	WAT7	White mycelium dark green spores	Cream colour	Hyaline, septate	Round to globose	Dark green	2.56	Attached to phialides in clusters	Ellipsoidal	9.14
20	WAT8	Light cream mycelial growth, green sporulation and yellow patches	Cream colour	Hyaline, septate	Round to ovoid	green	2.34	Attached to phialides in clusters	Ellipsoidal	7.74
21	THT1	Cream sparse mycelia, light green conidia.	Cream colour	Hyaline, septate	Smooth, round to oval	Light green	2.33	Attached to penicillate phialides	Ellipsoidal	8.08
22	THT2	White mycelia green coloured conidia	Cream colour	Hyaline, septate	Round	Green	2.41	Attached to penicillate phialides	Ellipsoidal	6.79
23	THT3	White fluffy mycelia, light green conidia	Cream colour	Hyaline, septate	Round to globose	Light green	2.03	Attached in clusters to phialides	Solitary elongated	9.07
24	THT4	Sparse white mycelia with green conidia	Cream colour	Hyaline, septate	Round to globose	Green	2.13	Attached to tip of phialides	Ellipsoidal to ovoidal	8.41

84

Fig. 4.1 (i) Cultural characters of *Trichoderma* spp. isolated from different locations

1) PAT 1

2) PAT 2

3) PAT 3
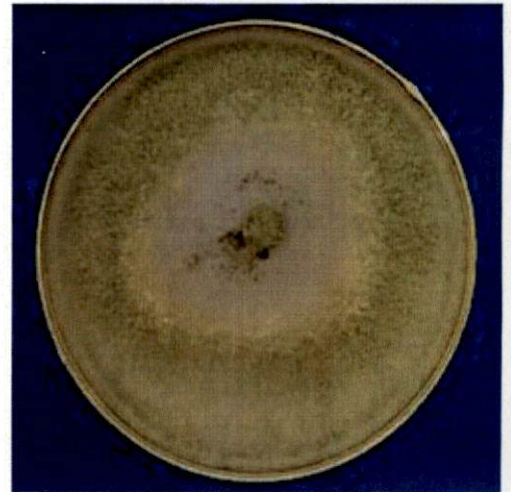
4) PAT 4
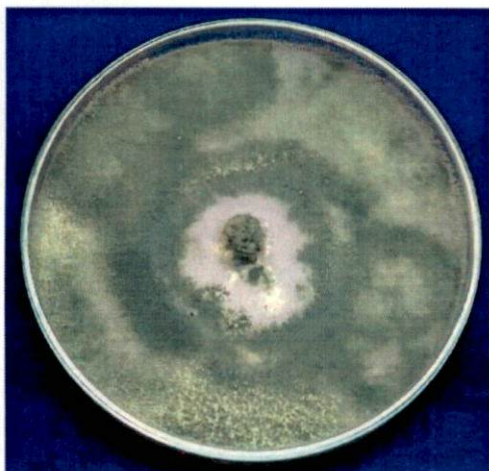
4) PAT 5

5) PAT 6

Fig. 4.1 (ii) Cultural characters of *Trichoderma* spp. isolated from different locations
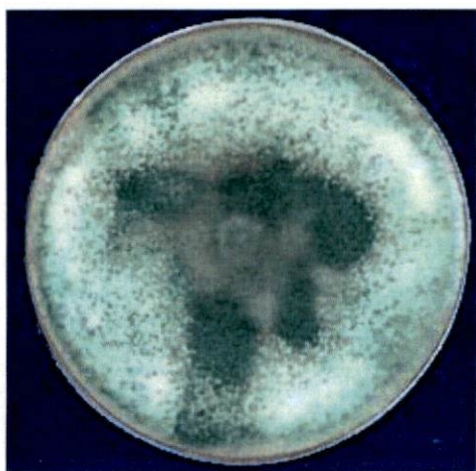
7) ALT 1

8) ALT 2

9) ALT 3

10) VYT 1
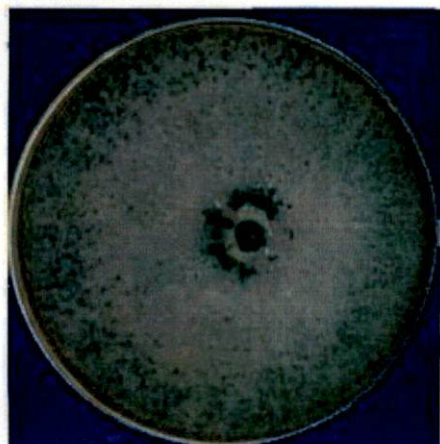
11) VYT 2

12) KUT 1

Plate 4.1 (iii) Cultural characters of *Trichoderma* spp. isolated from different locations
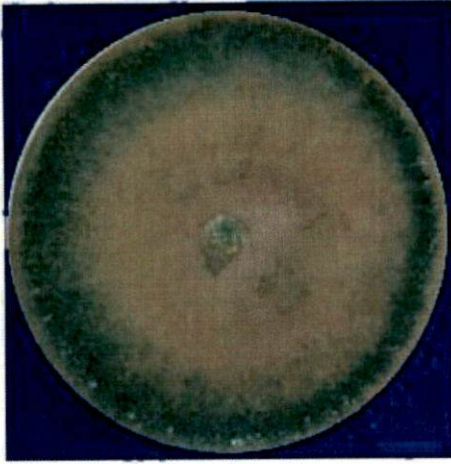
13) WAT 1

14) WAT 2

15) WAT 3
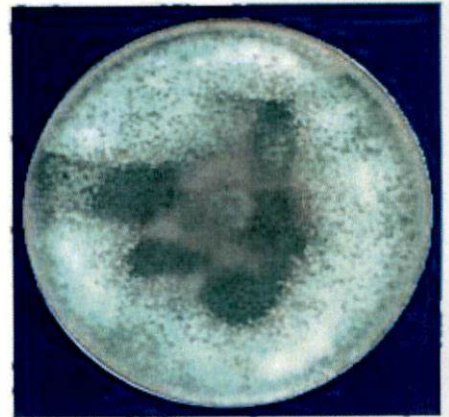
16) WAT 4

17) WAT 5

18) WAT 6

Plate 4.1 (iv) Cultural characters of *Trichoderma* spp. isolated from different locations

19) WAT 7

20) WAT 8

21) THT 1

22) THT 2

23) THT 3

24) THT 4

88
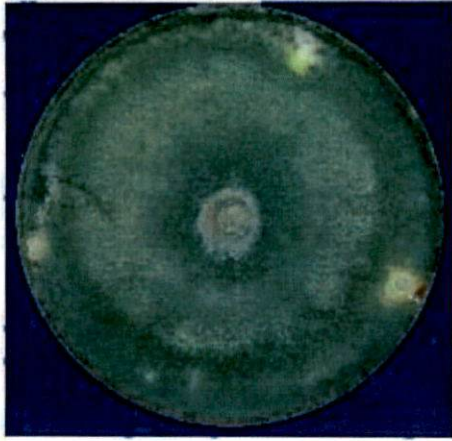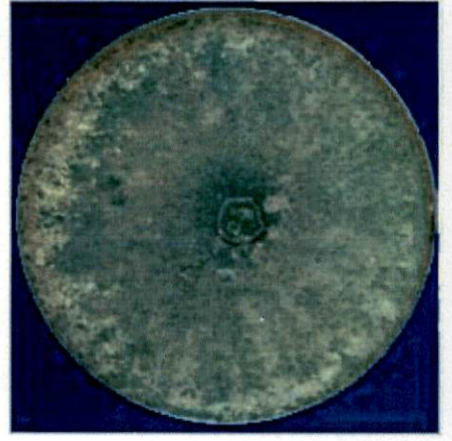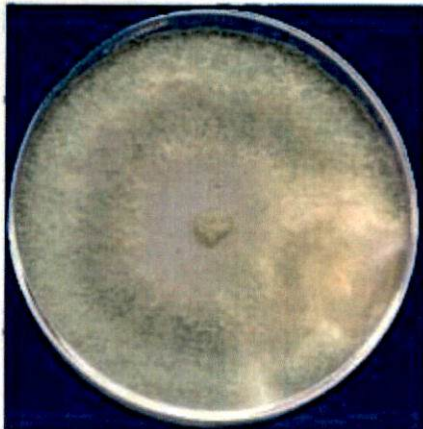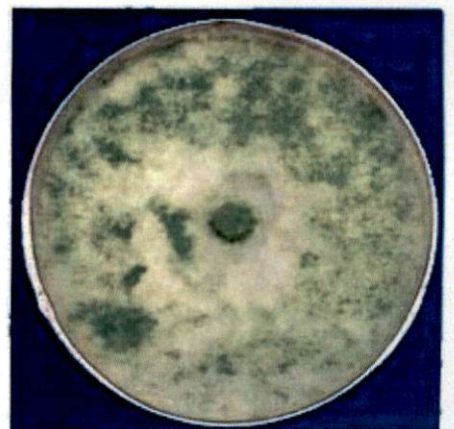
Plate 4.2 (i) Photomicrographs of isolates of *Trichoderma* spp.
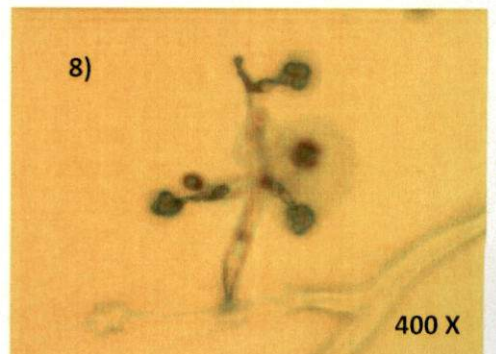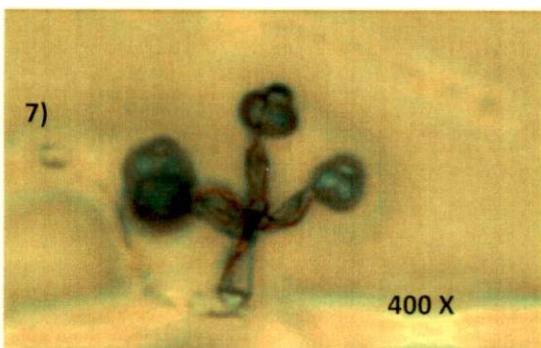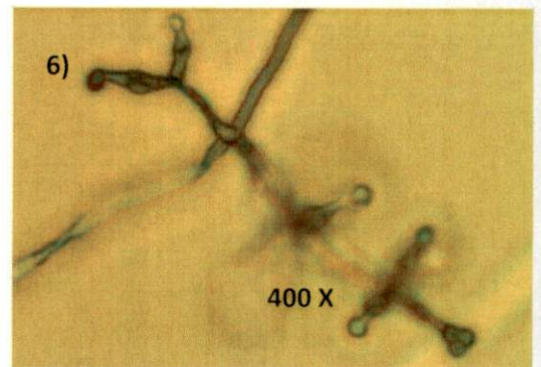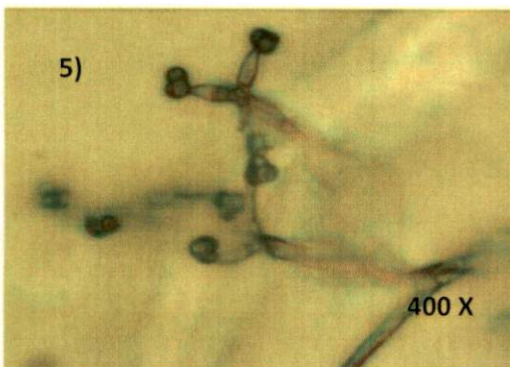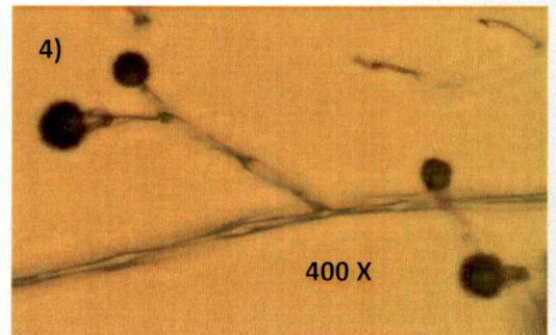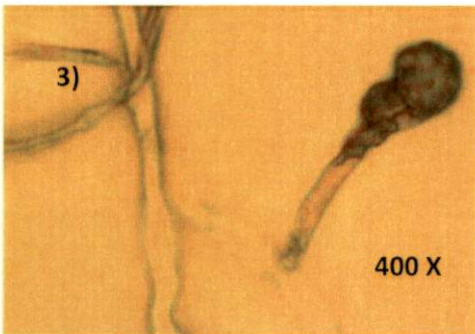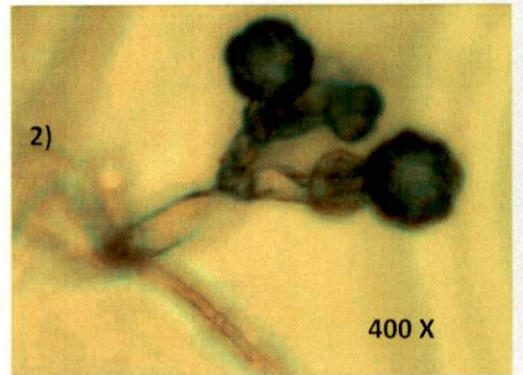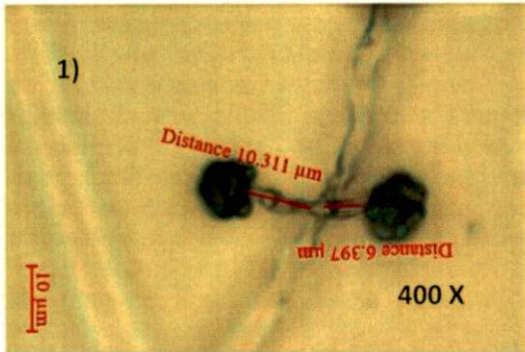
1) PAT 1; 2) PAT 2; 3) PAT 3; 4) PAT 4; 5) PAT 5; 6) PAT 6; 7) ALT 1; 8) ALT 2

Plate 4.2 (ii) Photomicrographs of isolates of *Trichoderma* spp.

9) ALT 3; 10) VYT1; 11) VYT 2; 12) KUT 1; 13) WAT 1; 14) WAT 2; 15) WAT 3; 16) WAT 4
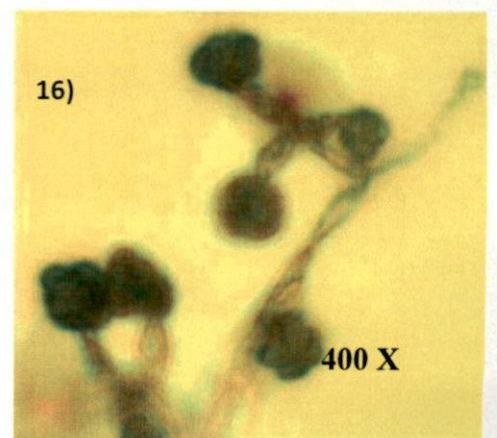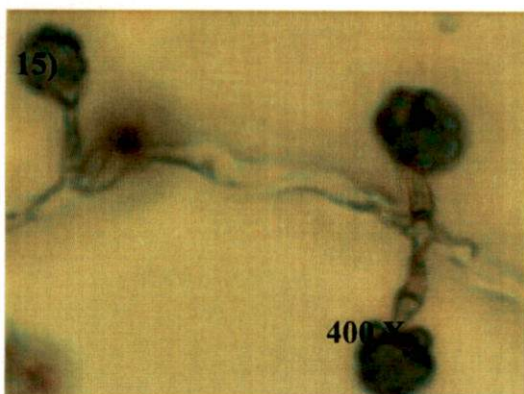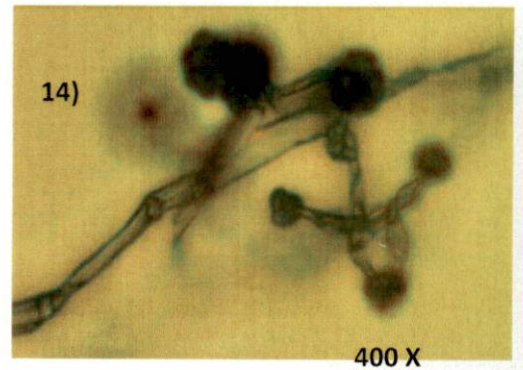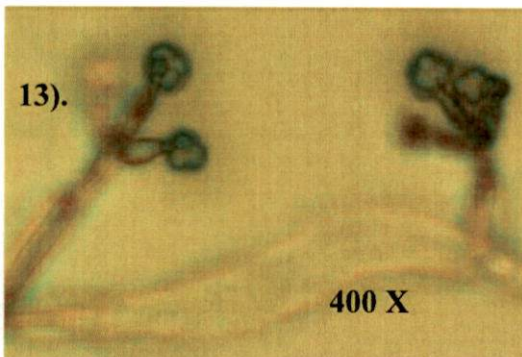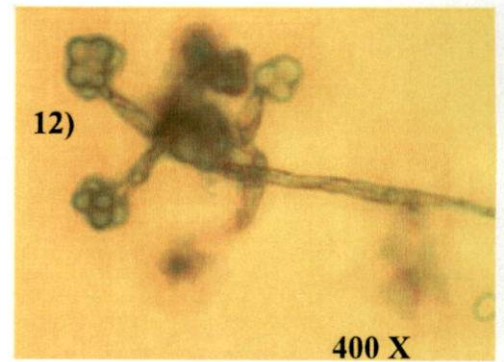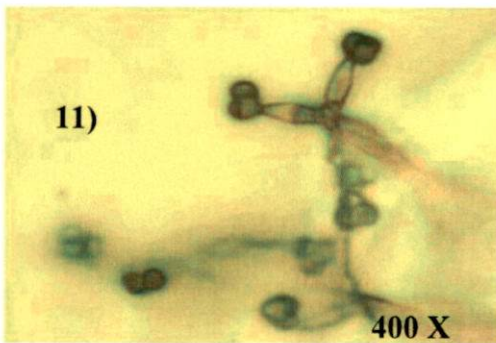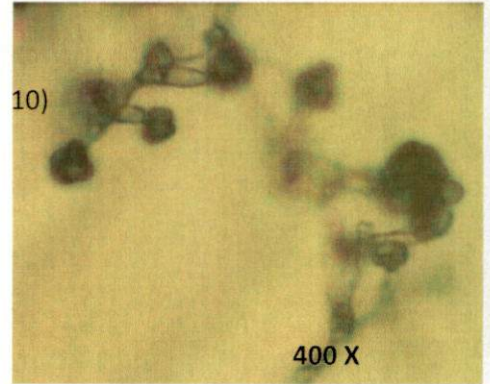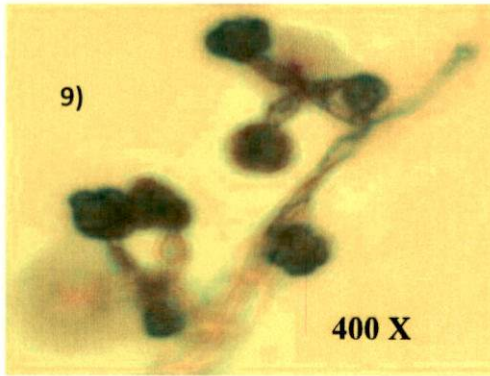
Plate 4.2 (iii) Photomicrographs of isolates of *Trichoderma* spp.

18) WAT 6; 19) WAT 7; 20) WAT 8; 21) THT1; 22) THT 2; 23) THT3; 24) THT4

91
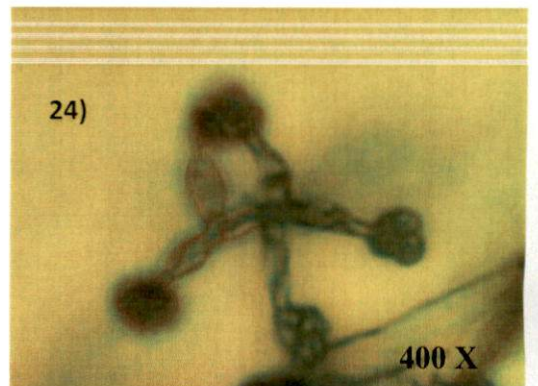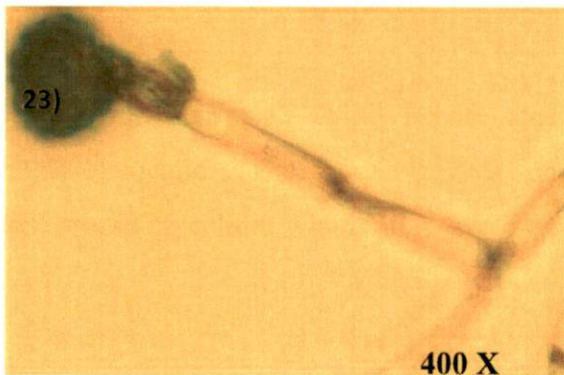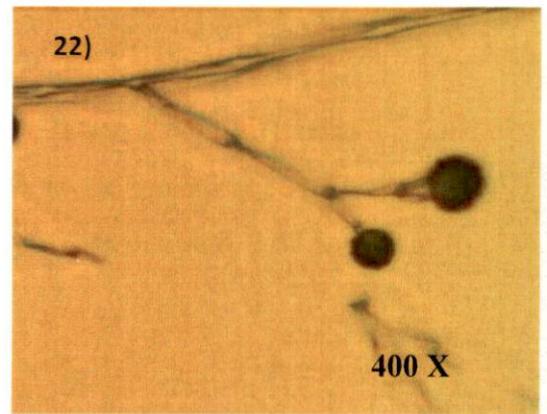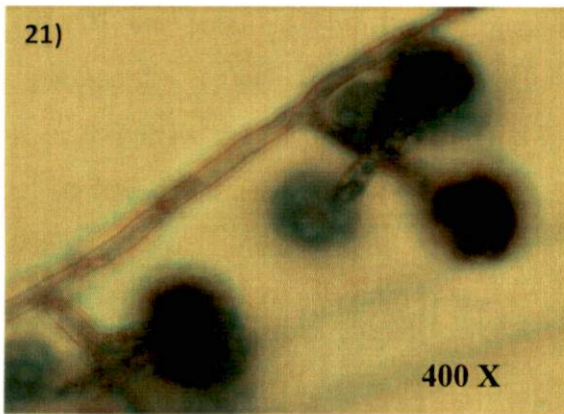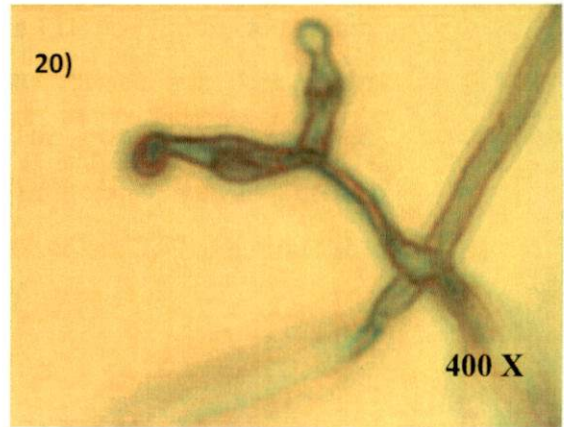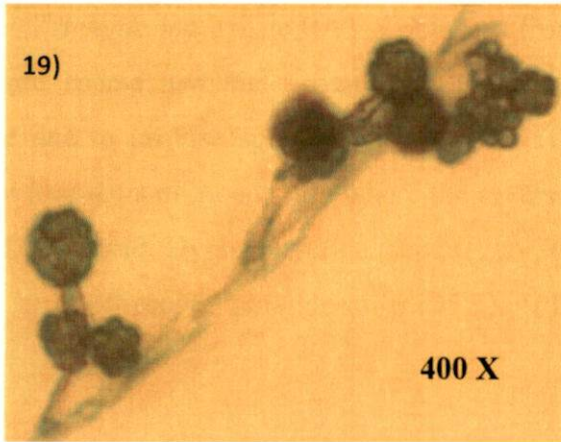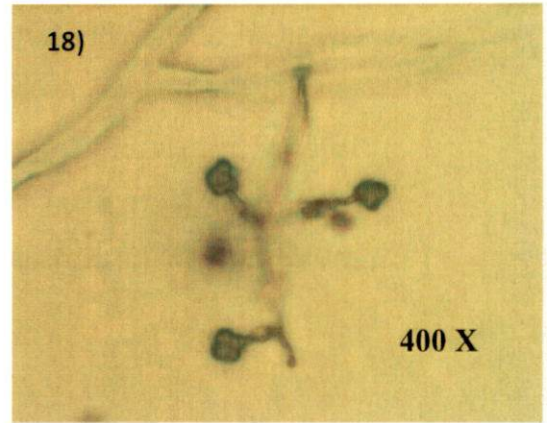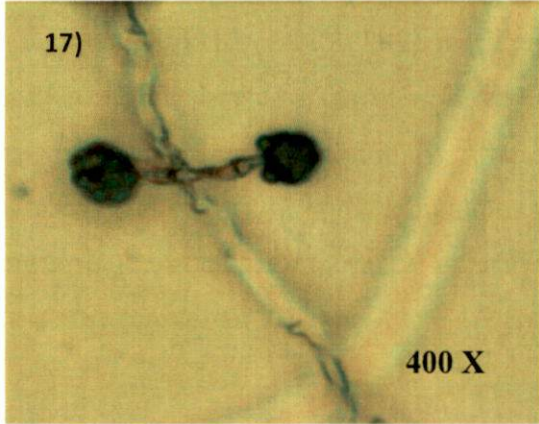
d) PAT 4

Soil sample P 10 (Athipotta-2) when subjected to serial dilution acquired PAT4 isolate which showed a sparse white mycelial growth on the media initially and later produced light green conidia after fifth day of inoculation at room temperature [Plate 4.1 (i) 4]. Hyphae hyaline, septate, conidia round, light green, 2.25 µm arranged in clusters at the tip of penicillate phialides of 7.67 µm which are subglobose to ovoidal in shape [Plate 4.2 (i) 4].

e) PAT 5

Trichoderma isolate PAT 5 obtained from P 11 (Tarur-1) produced cream coloured sparse mycelial growth and the colony later turned into dark green colour due to profuse sporulation [Plate 4.1 (i) 5]. The growth was completed within four days of inoculation where the reverse side of the plate appeared cream or buff coloured. Hyphae hyaline, septate, conidia dark green, 2.77 µm, arranged at the tip of ellipsoidal phialides of 6.195 µm [Plate 4.2 (i) 5].

f) PAT 6

PAT6 was obtained from the soil sample P 14 of location, Tarur-4. The isolate produced a typical white fluffy mycelial growth, which later transformed into green colour with a coconut odour in the old culture [Plate 4.1(i) 6]. Hyphae hyaline, septate, phialides narrower at the base, broader in the middle and again narrowed at the tip with a length of 11.94 µm. Conidia globose to round, 2.82 µm seen in clusters at the tip of phialides [Plate 4.2 (i) 6].

4.2.1.2. *Trichoderma spp. isolated from Alappuzha*

a) ALT1

Trichoderma isolate ALT 1 was acquired from the soil sample A 1 (Cherthala-1) location. The growth of this fungus primarily started as a cream coloured sparse mycelium which after five days of incubation formed undulating

mycelia with green spore mass, where reverse side of the colony appeared buff or cream coloured [Plate 4.1 (ii) 7]. Hyphae hyaline, septate, conidia smooth, round to globose, 2.86 μm attached in clusters to the tip of penicillate phialides, where the length of phialide was measured as 8.26 μm [Plate 4.2 (i) 7].

b) ALT2

This isolate was obtained from soil sample A 1 (Cherthala-1) where the growth of the isolate was noticed as white fluffy mycelium which later turned into pale green growth due to sporulation [Plate 4.1 (ii) 8]. Conidia were distributed as a compact mass in the Petri plate and the reverse side of the plate appeared buff coloured, hyphae hyaline, septate, conidia globose to ovoid, 3.30 μm and attached to the tip of elongated cylindrical phialides of 13.23 μm [Plate 4.2 (i) 8].

c) ALT 3

The isolate ALT 3 was obtained from A 4 (Muhamma-2) of Alappuzha district. The fungal colony appeared as sparse white mycelium which later turned to dark green due to conidia formation [Plate 4.1 (ii) 9]. Reverse side of the colony appeared buff coloured. Conidia smooth, round, 2.47 μm arranged in whorls at the tip of the penicillate phialides of 9.89 μm [Plate 4.2 (ii) 9].

4.2.1.3. *Trichoderma spp. isolated from Ernakulam*

a) VYT 1

The isolate VYT 1 from E 2 soil sample (Vytilla-2) produced white to cream sparse mycelium which later turned into dark green colour after five days of incubation [Plate 4.1 (ii) 10]. Reverse side of the fungal culture appeared cream coloured. Conidia globose to round 2.13 μm attached to ellipsoidal phialides of 6.221 μm arranged in whorls [Plate 4.2 (ii) 10].

b) VYT 2

Trichoderma isolate from the soil sample E 2 acquired from Vytilla- 2 location initially appeared as white sparse mycelium and later dark green spores covered the plate with few spores in the centre[Plate 4.1 (ii) 11]. Conidia smooth, ovoidal to subglobose, 3.86 μm attached to spindle shaped conidiophores seen in pairs in apical portion [Plate 4.2 (ii) 11].

4.2.1.4. Trichoderma sp. isolated from Kottayam

a) KUT 1

The isolate obtained from K 1 (Kumarakom-1) produced white fluffy mycelium initially which later appeared as white and green concentric rings after five days of incubation [Plate 4.1 (ii) 12]. Conidia ovoid to globose in cluster, 0.98 μm, attached to ellipsoidal to pin shaped conidiophores arranged in whorls, length of phialides 9.54 μm [Plate 4.2 (ii) 12].

4.2.1.5. Trichoderma spp. isolated from Wayanad

a) WAT 1

The isolate WAT 1 from W 1 sample (Bathery-1) produced a cream mycelium after five days of incubation which was transformed into dark green conidia [Plate 4.1 (iii) 13]. Reverse side of the colony appeared cream or buff coloured. Numerous spores were formed towards the margin rather than at the centre. Conidia dark green, round to oval, 2.54 μm attached to the tip of ellipsoid, plump phialides with a length of 14.23 μm [Plate 4.2 (ii) 13].

b) WAT 2

This isolate was procured from W 5 (Ambukuthi-1) showed white fluffy growth initially which after five days of incubation produced light green coloured spores [Plate 4.1 (iii) 14]. Reverse side of the culture plate appeared cream colour. Conidia round to ovoid, 2.95 μm attached to short, plump phialides of size 10.31 μm arranged in whorls [Plate 4.2 (ii) 14].

c) WAT 3

The isolate WAT 3 was acquired from soil sample W 5 also collected from Ambukuthi-1. The fungus produced light cream coloured mycelia which turned into dull green colour after six days of incubation [Plate 4.1 (iii) 15]. Reverse side of the fungal colony appeared cream or buff coloured. Conidia round to globose, 2.33 μm, attached to ellipsoidal phialides of 11.936 μm seen in pairs [Plate 4.2 (ii) 15].

d) WAT 4

WAT4 was obtained also from W 5 (Ambukuthi-1) initially appeared as a light cream coloured sparse mycelium and after five days of incubation formed undulating mycelia with green spore mass [Plate 4.1 (iii) 15]. Reverse side of the colony appeared buff or cream coloured. Hyphae hyaline, septate, conidia smooth, round to globose, 2.36 μm attached in clusters to the tip of short, plump phialides of 7.674 μm [Plate 4.2 (ii) 16].

e) WAT 5

The isolate WAT5 acquired from W 6 (Ambukuthi-2) appeared as white mycelial growth primarily, which after five days of inoculation produced green colour due to sporulation. Concentric pattern of spores were formed after the incubation period [Plate 4.1 (iii) 17]. Conidia globose to round, 2.30 μm, attached to short ellipsoidal phialides of 4.11 μm in clusters [Plate 4.2 (iii) 17].

f) WAT 6

The isolate WAT6 acquired from W 7 (Tholpetty-1), primarily produced a white mycelium which later turned into green and white colony with conidia formation. Concentric pattern of spores were observed on completion of growth [Plate 4.1 (iii) 18]. Reverse side of the culture appeared cream or buff coloured. Conidia smooth walled, round, 2.36 μm, attached to the tip of small, short ellipsoidal phialides of 5.46 μm [Plate 4.2 (iii) 18].

g) WAT 7

Another fungal colony WAT7 isolated from W 9 (Tholpetty-3), produced a light cream mycelial growth which turned uniform green sporulation with yellow patches after an incubation of four days [Plate 4.1 (iv) 19]. Hyphae hyaline and septate, conidia round to ovoid, attached to ellipsoid phialides of 7.74 μm [Plate 4.2 (iii) 19].

h) WAT 8

Trichoderma sp. WAT8 isolated from W 12 (Kalloor-1) initiated as a white mycelium and turned into dark green with the production of conidia after five days of incubation. Uniform sporulation was seen throughout the plate after completion of growth [Plate 4.1 (iv) 20]. Hyphae hyaline, sepatate, conidia round to globose, 2.56 μm attached to ellipsoid phialides of 9.14 μm [Plate 4.2 (iii) 20].

4.2.1.6. Trichoderma spp. isolated from Thrissur

a) THT 1

Trichoderma sp., THT 1 isolated from soil sample T 3 (Puzhakkal-3) started its growth by producing light cream sparse mycelia where the growth was completed within four days of inoculation with scattered sporulation by forming light green conidia [Plate 4.1 (iv) 21]. Reverse side of the culture plate appeared buff or cream colour. Hyphae hyaline, septate, conidia smooth, round to oval, 2.33 μm size, attached to penicillate ellipsoidal phialides of length 8.08 μm [Plate 4.1 (iii) 21].

b) THT2

The isolate THT 2 was acquired from soil sample T 6 (Kanjani-3) which appeared as a white fluffy mycelia initially and sporulated only after five days of incubation at room temperature. Green coloured conidia appeared towards the margin of the fungal culture than in the centre [Plate 4.1 (iv) 22]. Reverse side of the culture appeared cream or uncoloured. Hyphae hyaline and septate, dark

green, round conidia, attached to the tip of penicillate phialides of 6.749 µm [Plate 4.2 (iii) 22].

c) THT 3

The isolate THT 3 from soil sample of T 8 (Adat-2) appeared as white fluffy mycelial growth on the plate which turned into light green conidia after five days of incubation period [Plate 4.1 (iv) 23]. Hyphae hyaline, septate, pattern of conidia formation was developed in concentric circles on the plate. Conidia round to globose, 2.03 µm attached to solitary elongated phialides of 9.07µm [Plate 4.2 (iii) 23].

d) THT 4

This isolate was obtained from the soil sample T 9 (Adat-3) where the growth of the fungus initiated as sparse white mycelia which later transformed into scattered green conidia after the incubation of five days [Plate 4.1 (iv) 24]. Reverse side of the culture appeared cream or uncoloured. Hyphae hyaline and septate, conidia round to globose, 2.13 µm attached to ellipsoidal to ovoidal penicillate phialides of 8.41 µm [Plate 4.1 (iii) 24].

4.3 *In vitro* SCREENING OF *Trichoderma* spp. FOR ABIOTIC STRESS TOLERANCE

For studying the abiotic stress tolerance of *Trichoderma* spp., all the 24 selected isolates from different locations were subjected to varying levels of temperature, drought, salinity, pH and fungicides.

4.3.1 *In vitro* screening of *Trichoderma* spp. for high temperature tolerance

Twenty four isolates of *Trichoderma* spp. were subjected to various levels of temperature *viz.*, 25, 30, 35 and 40^0C to study the best suited temperature level for the growth of the fungus and also to screen for thermo tolerance. It was

observed that in general, that there was a significant difference among the isolates when grown at a temperature varying from 25- 40^0C and mycelial weight of the fungus increased when the temperature was increased from 25 to 30^0C and thereafter it decreased (Table 4.4).

At 25^0C , mycelia produced by the isolates ranged from 0.003 to 0.44 g and it was noticed that the maximum mycelial weight of 0.44g was noticed with the isolate PAT6 followed by ALT1 (0.34 g), whereas minimum of 0.003 g was recorded with PAT3. When the temperature was increased to 30^0C, all the isolates showed an increase in mycelial biomass production which ranged from 0.02 to 0.78 g. Maximum biomass was produced by the isolate VYT1 (0.78 g) followed by PAT6 (0.53 g), while minimum was recorded with PAT4 (0.02 g) and PAT 5 (0.03 g).

Further at 35^0C, mycelial weight produced by the isolates showed a general reduction and it ranged from 0.002 to 0.33 g. Maximum mycelial biomass was produced by the isolate PAT 6 (0.33 g) followed by ALT 1 (0.25 g), while the minimum of 0.002 g was noticed with the isolates PAT 2 and PAT 3. In general, the mycelial biomass produced by the isolates at 40^0C ranged between 0.001 to 0.03 g. At this temperature, most of the isolates failed to grow except for PAT6, WAT2 and WAT7 which produced a mycelial biomass of 0.03, 0.03 and 0.01 g respectively. All other isolates except for the former three produced no mycelia when exposed to 40^0C.

The extent of sporulation at different temperature levels was also assessed and it is presented in Table 4.5. All the isolates except PAT3, PAT4, VYT2 and WAT2 at 25^0C showed high sporulation while these four showed medium sporulation. It was also noticed that when the temperature was elevated to 30^0C, all isolates except WAT3 and VYT2 showed high sporulation. With the further increase in temperature to 35^0C, PAT6, ALT3 and WAT2 showed moderate sporulation and rest of the isolates did not sporulate at all. At the highest

temperature level of 40^0C, PAT 6 alone showed moderate sporulation and WAT 2 was noticed with sparse sporulation (Plate 4.3).

It can be inferred that the isolates PAT6 and WAT2 produced the maximum mycelial biomass of 0.03 g and were able to sporulate at the highest temperature of 40^0C. Hence, the two isolates were considered as thermotolerant and were selected for further studies.

4.3.2. *In vitro* screening of *Trichoderma* spp. for drought tolerance

All the twenty four isolates were exposed to different osmotic potential such as -0.15, -0.49 and -1.03 M Pa by amending the media with 10, 20 and 30 per cent polyethylene glycol (PEG). It was observed that there was a significant difference among the isolates at different concentrations of PEG and in general, the mycelial weight was decreasing gradually with increase in PEG concentration (Plate 4.4). The results of the study are presented in Table 4.6.

At 10 per cent PEG concentration, the mycelial biomass formed by the isolates ranged from 0.43 to 2.61 g and the highest mycelial weight was recorded with WAT2 (2.61 g) followed by ALT3 (1.61 g), while the minimum biomass was recorded in the isolate PAT 3 (0.43 g). On increasing the PEG concentration to 20 per cent, the biomass showed a range from 0.33 to 1.94 g with the highest biomass produced by WAT2 followed by VYT2 (1.40 g) and the minimum of 0.33 g was produced by PAT3. Further when the concentration of PEG was increased to 30 percent, the mycelial biomass produced by the isolates showed a general reduction and it ranged from 0.14 to 2.43 g. The maximum biomass was produced by VYT 2 (2.43 g) followed by ALT 1 (0.90 g) and the minimum with WAT 8 (0.14 g) and PAT 3 (0.15 g).

Table 4.4 Effect of temperature on the growth of *Trichoderma* spp.

Sl No.	*Trichoderma* isolates	*Mycelial weight (g/ 50 ml)			
		25^0C	30^0C	35^0C	40^0C
1.	PAT1	0.01^{jkl}	0.19^g	0.01^i	0.001^d
2.	PAT2	0.02^j	0.123^m	0.002^i	0.003^c
3.	PAT3	0.003^l	0.04^n	0.002^i	0.001^d
4.	PAT4	0.004^{kl}	0.02^{opq}	0.003^i	0.003^c
5.	PAT5	0.004^{kl}	0.03^{nop}	0.003^i	0.002^{cd}
6.	PAT6	0.44^a	0.53^b	0.33^a	0.03^a
7.	ALT1	0.34^b	0.43^d	0.25^b	0.003^c
8.	ALT2	0.08^{fg}	0.12^m	0.01^i	0.003^c
9.	ALT3	0.13^e	0.19^g	0.07^e	0.002^{cd}
10.	VYT1	0.05^h	0.78^a	0.01^i	0.002^{cd}
11.	VYT2	0.09^f	0.15^{ij}	0.06^{fg}	0.001^d
12.	KUT1	0.13^e	0.17^h	0.10^d	0.003^c
13.	WAT1	0.08^{fg}	0.15^{ijk}	0.06^{efg}	0.003^c
14.	WAT2	0.13^e	0.20^g	0.13^d	0.03^a
15.	WAT3	0.01^{jkl}	0.13^{lm}	0.004^i	0.003^c
16.	WAT4	0.08^g	0.14^{jkl}	0.07^{ef}	0.003^c
17.	WAT5	0.04^i	0.52^b	0.03^h	0.002^{cd}
18.	WAT6	0.28^d	0.36^e	0.21^c	0.002^{cd}
19.	WAT7	0.33^c	0.47^c	0.21^c	0.01^b
20.	WAT8	0.04^i	0.14^{klm}	0.003^i	0.002^{cd}
21.	THT1	0.01^{jk}	0.15^{ij}	0.01^i	0.003^c
22.	THT3	0.06^h	0.16^{hi}	0.01^i	0.002^{cd}
23.	THT2	0.05^h	0.12^m	0.01^i	0.003^c
24.	THT4	0.13^e	0.21^f	0.05^g	0.002^{cd}
	CD (0.05)	0.010	0.014	0.012	0.001

* Mean of three replications

In each column figures followed by same letter do not differ significantly according to DMRT

100

Table 4.5 Effect of temperature on sporulation of *Trichoderma* spp.

Sl No.	*Trichoderma* isolates	Sporulation of isolates at different temperature			
		25^{0}C	30^{0}C	35^{0}C	40^{0}C
1.	PAT1	+++	+++	-	-
2.	PAT2	+++	+++	-	-
3.	PAT3	++	+++	-	-
4.	PAT4	++	+++	-	-
5.	PAT5	+++	+++	-	-
6.	PAT6	+++	+++	++	++
7.	ALT1	+++	+++	-	-
8.	ALT2	+++	+++	-	-
9.	ALT3	+++	+++	++	-
10.	VYT1	+++	+++	-	-
11.	VYT2	++	++	-	-
12.	KUT1	+++	+++	-	-
13.	WAT1	+++	+++	-	-
14.	WAT2	++	+++	++	+
15.	WAT3	+++	++	-	-
16.	WAT4	+++	+++	-	-
17.	WAT5	+++	+++	-	-
18.	WAT6	+++	+++	-	-
19.	WAT7	+++	+++	-	-
20.	WAT8	+++	+++	-	-
21.	THT1	+++	+++	-	-
22.	THT2	+++	+++	-	-
23.	THT3	+++	+++	-	-
24.	THT4	+++	+++	-	-

- : no sporulation, +: sparse sporulation, ++: medium population, +++: high sporulation

Plate 4.3 Growth and sporulation of isolates of *Trichoderma* spp. at 40^{0}C

a) WAT 2 ; b) PAT6; c) PAT 3

Variation in sporulation of *Trichoderma* spp. at different concentrations of PEG is depicted in Table 4.7. A general decrease in sporulation was observed when the concentration of PEG was increased from 10 to 30 per cent. At 10 per cent PEG concentration, except for the isolates PAT3, PAT4, VYT2 and THT1, all others produced high sporulation, while the isolate WAT 2 was noticed with sparse sporulation. With the increase of PEG concentration to 20 per cent, all the isolates showed medium sporulation except for PAT3, PAT4, VYT1, VYT2, WAT2, THT1 and THT 3 which were recorded with sparse sporulation. At the highest concentration of PEG, (30 %) the isolates PAT1, PAT3, PAT4, ALT2, VYT1, VYT2, WAT1, WAT2, WAT5, THT1, THT2 and THT3 exhibited only sparse sporulation while all others were noticed with medium sporulation.

From the study, it can be concluded that the isolates VYT2 (2.43 g) and ALT 1 (0.090 g) produced higher mycelial biomass and showed sparse to moderate sporulation at higher concentration of PEG (30 %) and thus the two isolates were considered as drought tolerant and were selected for further studies.

Table 4.6 Effect of drought on the growth of *Trichoderma* spp.

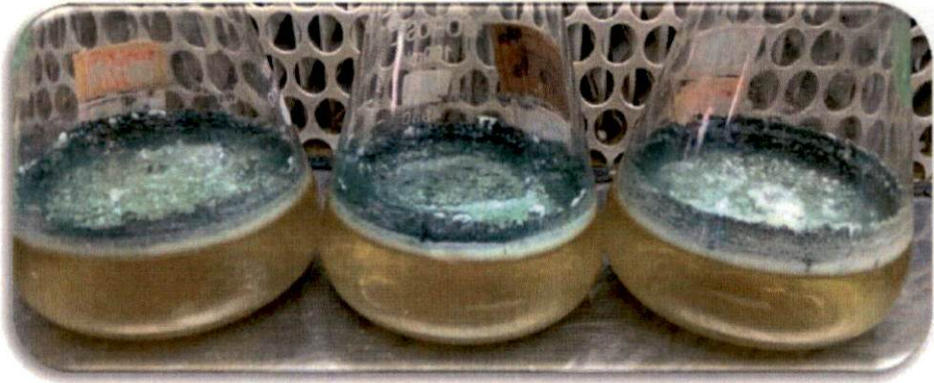| Sl No.	*Trichoderma* isolates	*Mycelial weight (g/ 50 ml)		
		10 %PEG	20% PEG	30 % PEG
1.	PAT1	0.72^{hi}	0.54^{lm}	0.21^{fgh}
2.	PAT2	0.47^{kl}	0.37^{opq}	0.27^{fg}
3.	PAT3	0.43^{l}	0.33^{pq}	0.15^{h}
4.	PAT4	0.51^{jkl}	0.39^{nop}	0.27^{fg}
5.	PAT5	0.76^{h}	0.74^{hij}	0.47^{e}
6.	PAT6	0.59^{j}	0.47^{mn}	0.27^{fg}
7.	ALT1	1.03^{f}	0.77^{hi}	0.90^{b}
8.	ALT2	1.00^{fg}	0.83^{gh}	0.63^{cd}
9.	ALT3	1.61^{b}	0.35^{pq}	0.29^{f}
10.	VYT1	0.90^{g}	0.59^{kl}	0.45^{e}
11.	VYT2	0.53^{jkl}	1.40^{b}	2.43^{a}
12.	KUT1	1.20^{e}	1.02^{de}	0.26^{fg}
13.	WAT7	1.39^{c}	1.25^{c}	0.17^{gh}
14.	WAT2	2.61^{a}	1.94^{a}	0.70^{c}
15.	WAT1	1.05^{f}	0.87^{fg}	0.18^{gh}
16.	WAT6	1.34^{cd}	1.12^{d}	0.15^{h}
17.	WAT3	0.63^{ij}	0.35^{pq}	0.18^{gh}
18.	WAT8	1.25^{de}	0.68^{ijk}	0.14^{h}
19.	WAT4	1.03^{f}	0.95^{ef}	0.17^{gh}
20.	WAT5	1.29^{cde}	0.61^{kl}	0.21^{fgh}
21.	THT1	0.61^{ij}	0.37	0.29^{f}
22.	THT3	0.77^{h}	0.65^{jk}	0.43^{e}
23.	THT2	1.39^{c}	0.78^{ghi}	0.59^{d}
24.	THT4	0.56^{jk}	0.28^{q}	0.16^{h}
CD value(0.05)		0.120	0.101	0.098

*Mean of three replications

In each column figures followed by same letter do not differ significantly according to DMRT

Table 4.7 Effect of drought on the sporulation of *Trichoderma* spp.

Sl No.	*Trichoderma* isolates	Sporulation of *Trichoderma* spp. at different PEG concentrations		
		10%	20%	30%
1.	PAT1	+++	++	+
2.	PAT2	+++	++	++
3.	PAT3	+	+	+
4.	PAT4	+	+	+
5.	PAT5	+++	++	++
6.	PAT6	+++	++	++
7.	ALT1	+++	++	++
8.	ALT2	+++	++	+
9.	ALT3	+++	++	++
10.	VYT1	+++	+	+
11.	VYT2	++	+	+
12.	KUT1	+++	++	++
13.	WAT1	+++	++	+
14.	WAT2	+	+	+
15.	WAT3	+++	++	++
16.	WAT4	+++	++	++
17.	WAT5	+++	++	+
18.	WAT6	+++	++	++
19.	WAT7	+++	++	++
20.	WAT8	+++	++	++
21.	THT1	++	+	+
22.	THT2	+++	++	+
23.	THT3	+++	+	+
24.	THT4	+++	++	++

-: no sporulation, +: sparse sporulation, ++: medium sporulation, +++: high sporulation

Plate 4.4 Effect of drought on growth and sporulation of *Trichoderma* spp.

a) 10% PEG

b) 20 % PEG

c) 30 % PEG

4.3.3 *In vitro* screening of *Trichoderma* spp. for acidity tolerance

Trichoderma species isolated from different stressed ecosystems were screened to study their growth at different pH levels such as 3.5, 4.5 and 5.5 to study their acidity tolerance. A significant difference in the mycelial biomass was noticed in the isolates and it was observed that as the pH of the culture media increased, an increase in the mycelial biomass was noticed (Table 4.8) (Plate 4.5).

At pH 3.5, the biomass produced by the fungal isolates ranged from 0.001 to 0.90 g, where the maximum biomass of 0.90 g was produced by ALT3 followed by ALT1 (0.67g) and the minimum biomass of 0.001 g was produced by the isolates PAT2, PAT3 and PAT4. When the isolates were grown at pH 4.5, the mycelial biomass ranged from 0.04 to 2.45 g. The maximum was recorded with the isolate WAT 3 (2.45 g) followed by PAT5 (1.98 g) and the minimum was observed in PAT3 (0.04 g) and THT1 (0.09 g). Mycelial biomass of the *Trichoderma* isolates ranged from 0.07 to 2.82 g at pH 5.5 with the maximum biomass was produced by WAT3 followed by PAT5 (2.48 g) and the minimum was recorded with THT1 (0.07 g).

Sporulation ability of the isolates with respect to different pH conditions are tabulated in Table 4.9. At pH 3.5, it was observed that the isolates PAT5, PAT6, ALT1 and ALT3 showed high sporulation. Isolates such as PAT2, PAT3, PAT4, ALT2, VYT1, VYT2, WAT1, WAT2 and THT1 showed sparse sporulation and rest of the isolates showed medium sporulation at 3.5 pH. Isolates at pH 4.5 *viz.*, PAT5, PAT6, ALT1, ALT3 and KUT1 showed high sporulation whereas PAT3, PAT4, ALT2, VYT1 and VYT2 was noticed with sparse sporulation and all others produced medium sporulation. Similarly, the effect of pH 5.5 on the sporulation of the fungus was also studied and it was noticed the sporulation ability of the isolates at pH 5.5 was found similar to those at pH 4.5.

Table 4.8 Effect of pH on growth of *Trichoderma* spp.

Sl No.	*Trichoderma* isolate	*Mycelial weight (g/ 50 ml)		
		pH 3.5	**pH 4.5**	**pH 5.5**
1.	PAT1	0.15^h	0.56^f	0.83^h
2.	PAT2	0.001^m	0.39^{gh}	0.57^j
3.	PAT3	0.001^m	0.04^k	0.23^n
4.	PAT4	0.001^m	0.23^{ij}	0.47^k
5.	PAT5	0.45^d	1.98^b	2.48^b
6.	PAT6	0.43^d	0.46^{fg}	0.53^{jk}
7.	ALT1	0.67^b	1.50^c	2.03^d
8.	ALT2	0.51^c	1.43^c	1.80^e
9.	ALT3	0.90^a	1.24^d	2.20^c
10.	VYT1	0.25^f	0.29^{hi}	0.36^l
11.	VYT2	0.50^c	1.12^d	1.23^f
12.	KUT1	0.18^g	0.26^{ij}	0.37^l
13.	WAT1	0.08^{kl}	0.51^{fg}	0.12^{op}
14.	WAT2	0.11^j	0.31^{hi}	0.55^{jk}
15.	WAT3	0.33^e	2.45^a	2.82^a
16.	WAT4	0.12^{ij}	0.24^{ij}	0.35^l
17.	WAT5	0.45^d	0.59^f	0.71^i
18.	WAT6	0.49^c	0.58^f	0.76^{hi}
19.	WAT7	0.14^{hi}	0.95^e	1.10^g
20.	WAT8	0.12^{ij}	0.47^{fg}	0.56^j
21.	THT1	0.08^l	0.09^k	0.07^q
22.	THT3	0.11^{ij}	0.27^{hij}	0.35^l
23.	THT2	0.11^{jk}	0.16^{jk}	0.25^{mn}
24.	THT4	0.12^{ij}	0.30^{hi}	0.32^{lm}
	CD (0.05)	0.028	0.125	0.085

*Mean of three replications
In each column figures followed by same letter do not differ significantly according to DMRT

Table 4.9 Effect of pH on sporulation of *Trichoderma* spp.

Sl No.	*Trichoderma* isolate	Sporulation at different pH conditions		
		3.5	4.5	5.5
1.	PAT1	++	++	++
2.	PAT2	+	++	++
3.	PAT3	+	+	+
4.	PAT4	+	+	+
5.	PAT5	+++	+++	+++
6.	PAT6	+++	+++	+++
7.	ALT1	+++	+++	+++
8.	ALT2	+	++	++
9.	ALT3	+++	+++	+++
10.	VYT1	+	+	+
11.	VYT2	+	+	+
12.	KUT1	++	+++	+++
13.	WAT1	+	++	++
14.	WAT2	+	++	++
15.	WAT3	++	++	++
16.	WAT4	++	++	++
17.	WAT5	++	++	+
18.	WAT6	++	++	++
19.	WAT7	++	++	++
20.	WAT8	++	++	++
21.	THT1	+	+	+
22.	THT2	++	++	+
23.	THT3	++	+	+
24.	THT4	++	++	++

-: no sporulation, +: sparse sporulation, ++: medium sporulation, +++: high sporulation

Plate 4.5 Effect of pH on growth and sporulation of *Trichoderma* spp.

a) pH 5.5

b) 4.5

c) pH 3.5

From the study it was found that the isolates ALT 3 and ALT 1 from Alappuzha showed the highest mycelial weight of 0.90 g and 0.67 g and were observed with high sporulation when grown at a low pH of 3.5. Thus, these two *Trichoderma* spp. were selected as acid tolerant isolates and were used for further studies.

4.3.4. *In vitro* screening for salinity tolerance

Tolerance of all 24 isolates to salinity was studied by exposing them to different concentrations of NaCl such as 0.5, 1 , 1.5 and 2 M. It was noticed that in general, there was a decline in the radial growth of the mycelia of the fungi when the concentration of NaCl was increased from 0.5 to 2 M [Plate 4.6 (i), (ii), (iii) and (iv)]. Details of the result are tabulated in Table 4.10.

When the isolates were exposed to 0.5 M NaCl concentration, the per cent inhibition varied between 0 to 94.44 per cent. It was observed that the isolates PAT2, PAT3, PAT4, ALT2, THT2, THT3 and THT4 were on par with each other with per cent inhibition ranging from 89.26 to 94.44 while, the minimum per cent inhibition was noticed with the isolates such as PAT1, ALT1, ALT3, WAT3 and WAT8. At 1.0 M salt concentration, the per cent inhibition exhibited by the isolates also varied from 0 to 94.44 per cent and the maximum inhibition was observed in isolates PAT3, PAT4, ALT2, THT3 and PAT2 and they were all on par with each other depicting a per cent inhibition of 87.96 to 94.44 . Minimum per cent inhibition of zero was noticed with the isolate WAT3 and it was on par with PAT1 (1.85 %), WAT8 (1.85 %) and ALT3 (3.70 %).

Table 4.10 Effect of salinity on growth of *Trichoderma* spp.

Sl No.	*Trichoderma* isolate	*Per cent inhibition over control (%)			
		0.5 M	1 M	1.5 M	2 M
1.	PAT1	0.00 (0.707)^k	1.85 (1.292)^i	94.44 (9.718)^a	94.44 (9.718)^a
2.	PAT2	89.26 (9.474)^a	87.96 (9.404)^a	94.44 (9.718)^a	94.44 (9.718)^a
3.	PAT3	94.44 (9.744)^a	94.44 (9.744)^a	94.44 (9.718)^a	94.44 (9.718)^a
4.	PAT4	94.44 (9.744)^a	94.44 (9.744)^a	94.44 (9.718)^a	94.44 (9.718)^a
5.	PAT5	40.74 (6.421)^c	47.04 (6.893)^{bc}	94.44 (9.718)^a	94.44 (9.718)^a
6.	PAT6	25.93 (5.139)^{de}	25.93 (4.851)^{efg}	94.44 (9.718)^a	94.44 (9.718)^a
7.	ALT1	0.00 (4.766)^k	28.51 (5.386)^{def}	90.00 (9.487)^c	94.44 (9.718)^a
8.	ALT2	91.11 (9.571)^a	94.44 (9.744)^a	94.44 (9.718)^a	94.44 (9.718)^a
9.	ALT3	0.00 (0.707)^k	3.70 (1.607)^i	90.74 (9.526)^b	94.44 (9.718)^a
10.	VYT1	23.33 (4.881)^{def}	25.37 (5.083)^{defg}	94.44 (9.718)^a	94.44 (9.718)^a
11.	VYT2	1.85 (1.292)^j	18.52 (4.314)^{fgh}	94.44 (9.718)^a	94.44 (9.718)^a
12.	KUT1	7.41 (2.801)^h	13.70 (3.520)^h	94.44 (9.718)^a	94.44 (9.718)^a
13.	WAT1	1.85 (1.292)^j	12.41 (3.589)^h	94.44 (9.718)^a	94.44 (9.718)^a
14.	WAT2	4.07 (2.064)^i	15.74 (4.027)^{gh}	94.44 (9.718)^a	94.44 (9.718)^a
15.	WAT3	0.00 (0.707)^k	0.000 (0.707)^i	94.44 (9.718)^a	94.44 (9.718)^a
16.	WAT4	20.37 (4.566)^f	34.44 (5.886)^{cde}	94.44 (9.718)^a	94.44 (9.718)^a
17.	WAT5	28.15 (5.351)^d	38.89 (6.276)^{cd}	94.44 (9.718)^a	94.44 (9.718)^a
18.	WAT6	22.22 (4.766)^{ef}	28.70 (5.398)^{def}	94.44 (9.718)^a	94.44 (9.718)^a

112

Sl. No.	*Trichoderma* isolate	*Per cent inhibition over control (%)*			
		0.5 M	1 M	1.5 M	2 M
19.	WAT7	11.11 (3.405)^g	23.52 (4.881)^{efg}	94.44 (9.718)^a	94.44 (9.718)^a
20.	WAT8	0.37 (0.895)^{jk}	1.85 (1.292)^i	94.44 (9.718)^a	94.44 (9.718)^a
21.	THT1	75.93 (8.742)^b	29.07 (5.407)^{def}	94.44 (9.718)^a	94.44 (9.718)^a
22.	THT2	90.93 (9.562)^a	56.85 (7.573)^b	94.44 (9.718)^a	94.44 (9.718)^a
23.	THT3	89.63 (9.494)^a	94.44 (9.744)^a	94.44 (9.718)^a	94.44 (9.718)^a
24.	THT4	94.44 (9.744)^a	46.30 (6.838)^{bc}	94.44 (9.718)^a	94.44 (9.718)^a
	CD (0.05)	0.573	1.245	0.023	NS

*Mean of three replications

In each column figures followed by same letter do not differ significantly according to DMRT.

$\sqrt{x+0.5}$ transformed values are given in parentheses.

Table 4.11 Effect of salinity on sporulation of *Trichoderma* spp.

Sl. No.	*Trichoderma* isolate	Sporulation of *Trichoderma* spp. at different concentrations of NaCl			
		0.5 M	1.0 M	1.5 M	2M
1.	PAT1	-	-	-	-
2.	PAT2	-	-	-	-
3.	PAT3	-	-	-	-
4.	PAT4	-	-	-	-
5.	PAT5	-	-	-	-
6.	PAT6	-	-	-	-
7.	ALT1	-	-	-	-
8.	ALT2	-	-	-	-
9.	ALT3	++	++	-	-
10.	VYT1	-	-	-	-
11.	VYT2	-	-	-	-
12.	KUT1	-	-	-	-
13.	WAT1	-	-	-	-
14.	WAT2	-	-	-	-
15.	WAT3	-	-	-	-
16.	WAT4	-	-	-	-
17.	WAT5	-	-	-	-
18.	WAT6	-	-	-	-
19.	WAT7	-	-	-	-
20.	WAT8	-	-	-	-
21.	THT1	-	-	-	-
22.	THT2	-	-	-	-
23.	THT3	-	-	-	-
24.	THT4	+	+	+	+

-: no sporulation, +: sparse sporulation, ++: moderate sporulation, +++: high sporulation

Plate 4.6(i) Effect of salinity on the growth and sporulation of *Trichoderma* spp.

1) PAT 1

2) PAT 2

3) PAT 3

4) PAT 4

5) PAT5

6) PAT6

115
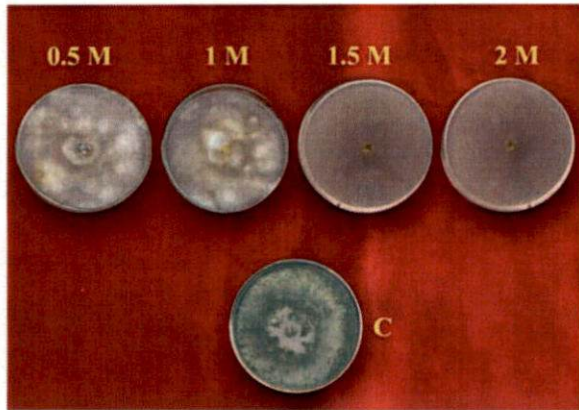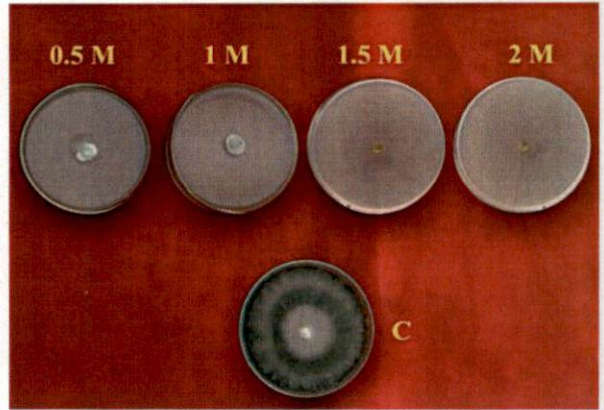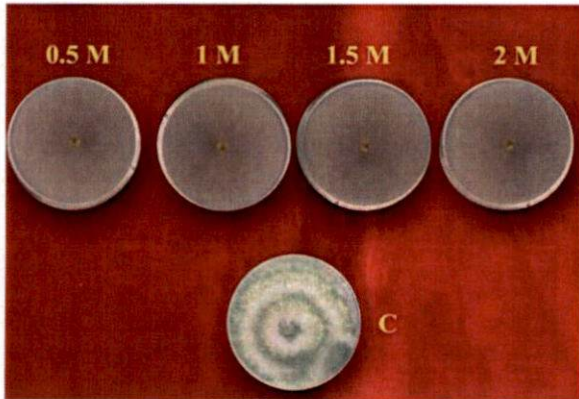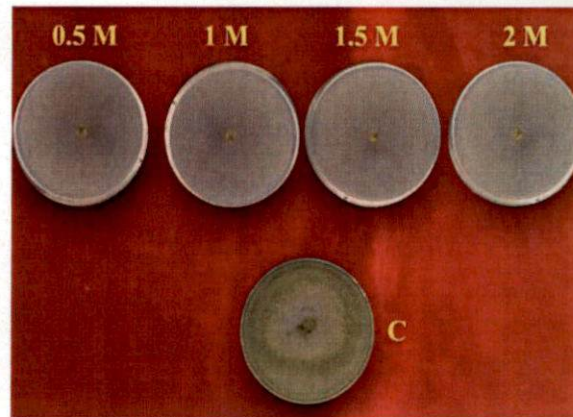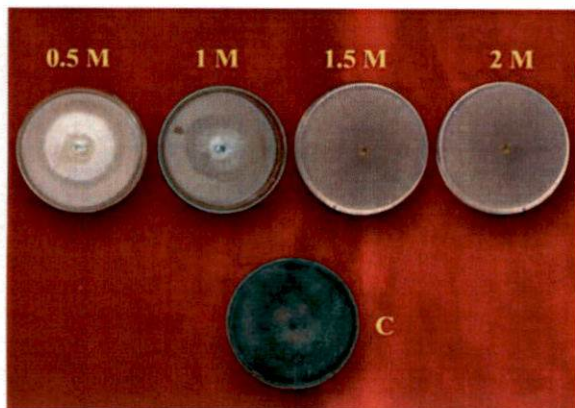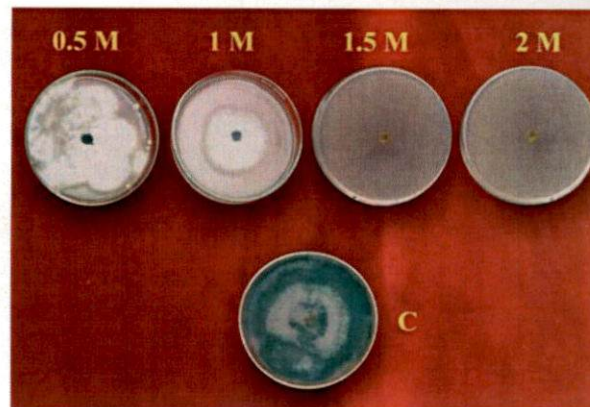
Plate 4.6(ii) Effect of salinity on the growth and sporulation of *Trichoderma* spp.
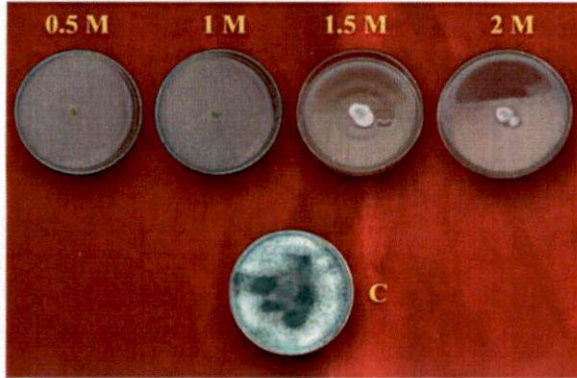
7) ALT1
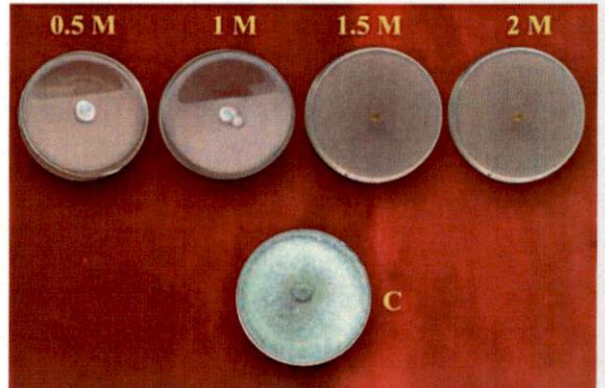
8) ALT2
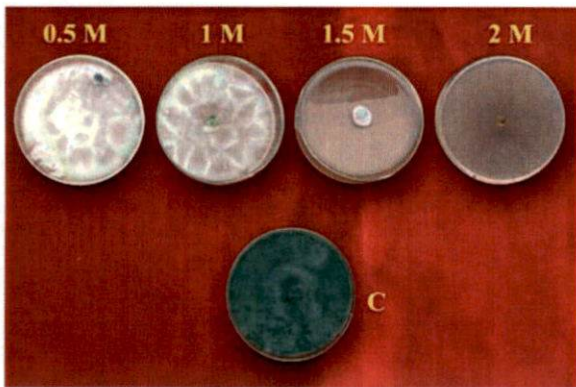
9) ALT3
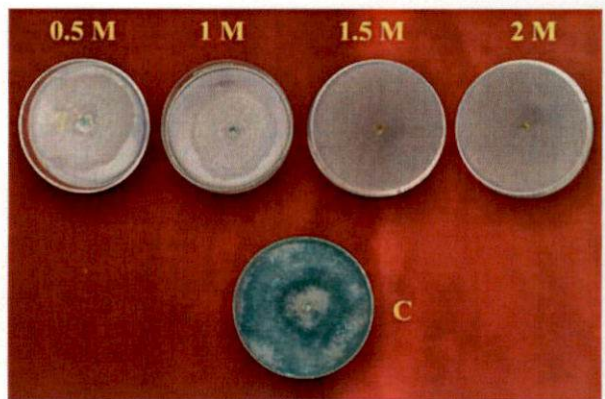
10) VYT1
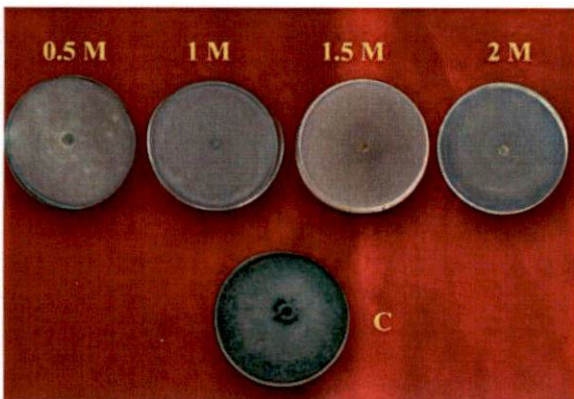
11) VYT2
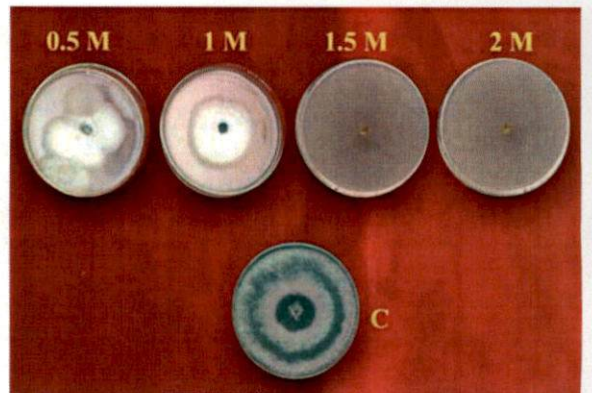
12) KUT1

Plate 4.6(iii) Effect of salinity on the growth and sporulation of *Trichoderma* spp.
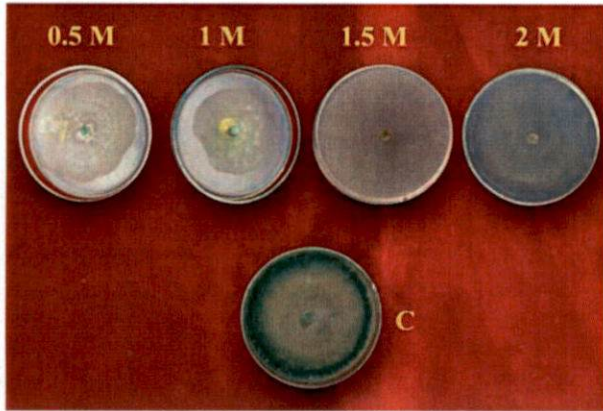
13) WAT 1
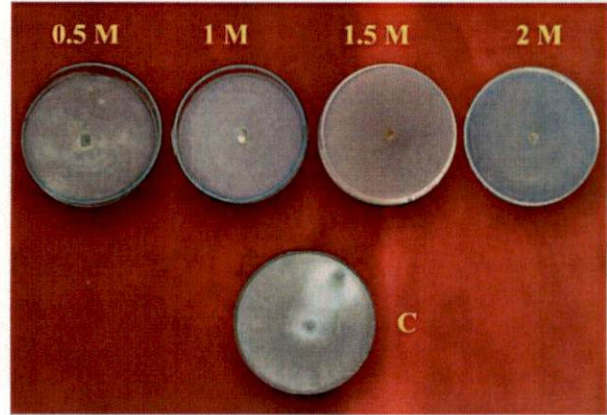
14) WAT 2
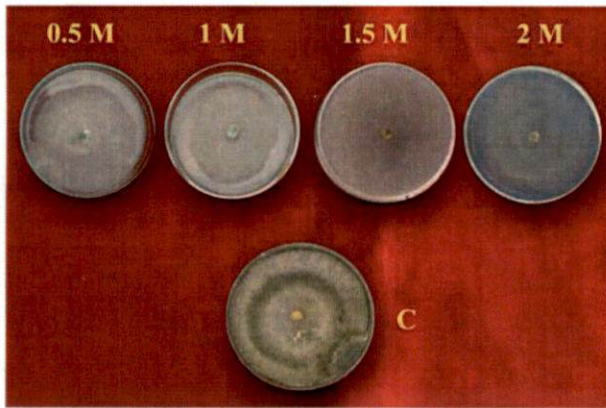
15) WAT 3
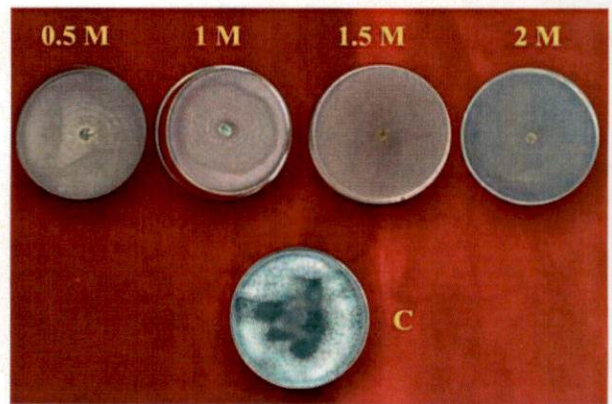
16) WAT 4
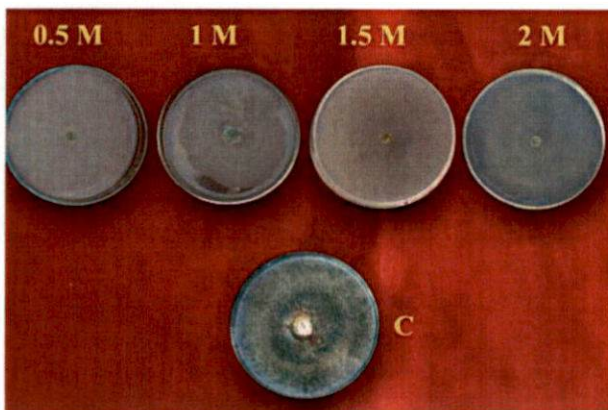
17) WAT 5
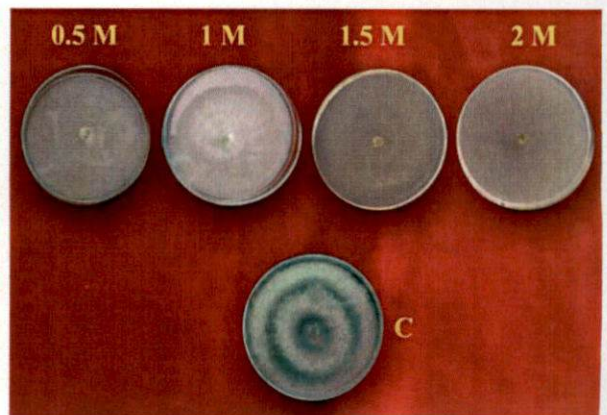
18) WAT 6

Plate 4.6(iv) Effect of salinity on the growth and sporulation of *Trichoderma* spp.
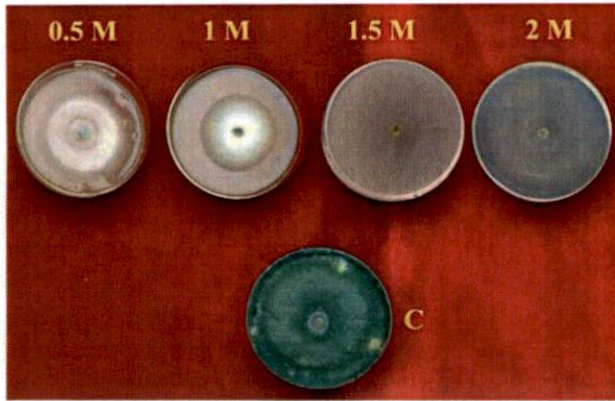
19) WAT 7
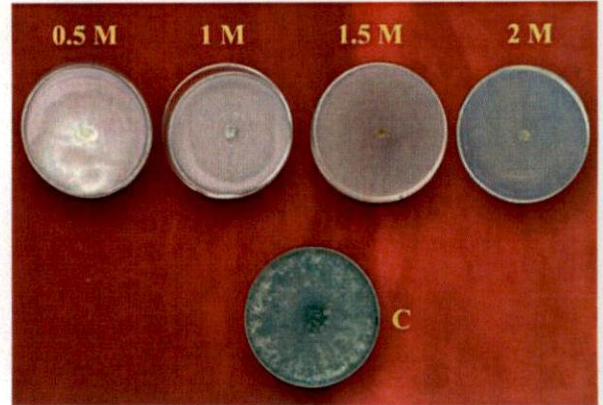
20) WAT 8
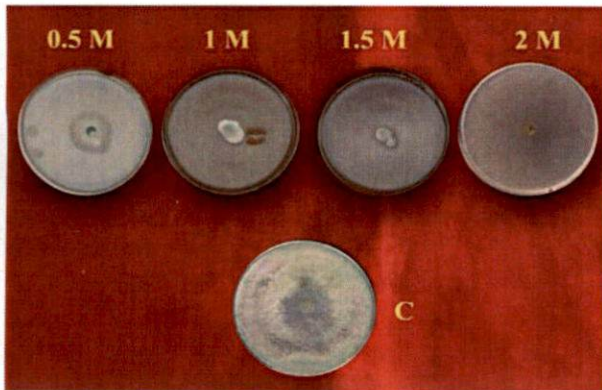
21)THT1
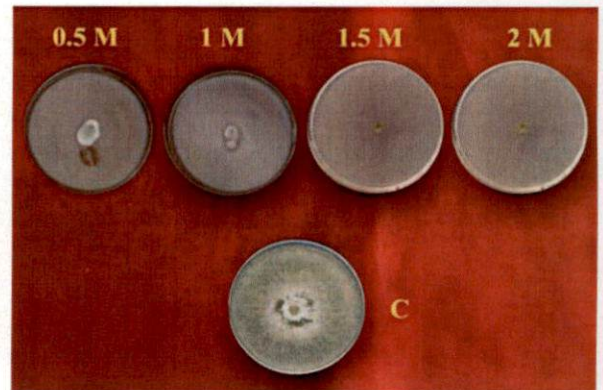
22) THT 2
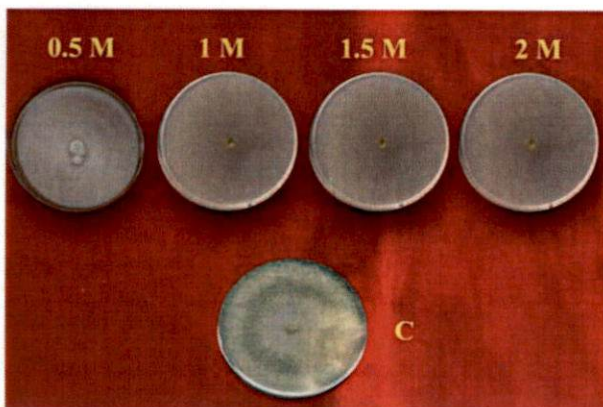
23) THT 3
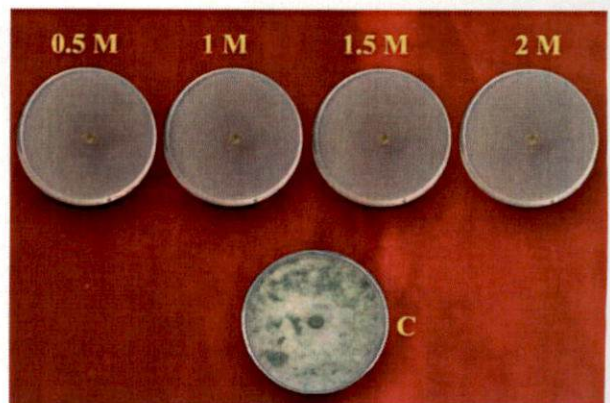
24) THT 4

When the concentration of NaCl was further increased to 1.5 M, the growth of almost all isolates were reduced drastically, and the percent inhibition ranged from 90.0 to 94.44 per cent, where the minimum value of 90 per cent was observed with the isolate ALT1 and followed by the isolate ALT3 with 90.74 per cent inhibition. All other isolates showed per cent inhibition of 94.44 when grown under the same salt concentration. None of the isolates showed any growth when they were exposed to 2.0 M concentration of NaCl as all showed a per cent inhibition of 94.44.

Sporulation of the fungus was also affected by the presence of NaCl in the media, and it was noticed that all isolates except ALT3 and THT 4 produced no spores at all concentrations of NaCl (Table 4.11). The isolate ALT3 was noticed with moderate sporulation at 0.5 and 1.0 M salt concentration and THT 4 expressed sparse sporulation at all concentration of NaCl. Hence after the study, it was concluded that since ALT1 and ALT3 showed good growth under 0.50 M, 1.0 M and 1.50 M with ALT3 producing spores at first two concentrations of NaCl, the two isolates were selected as salinity tolerant *Trichoderma* spp. and were used for further studies.

4.3.5 *In vitro* screening of *Trichoderma* isolates for fungicide tolerance

All 24 isolates of *Trichoderma* spp. were screened for fungicide tolerance against copper fungicides. Two fungicides, *viz.,* copper oxychloride 50 WP (Fytolan) and copper hydroxide 77 WP (Kocide) at three different concentrations (0.2 %, 0.25% and 0.3 %) were used for the study.

4.3.5.1. Screening for copper hydroxide tolerance

The 24 isolates of *Trichoderma* spp. were grown at different concentrations (0.2, 0.25 and 0.3%) of copper hydroxide to assess the tolerance of fungus against

Table 4.12 Effect of copper hydroxide 77 WP on growth of *Trichoderma* spp.

Sl. No.	*Trichoderma* isolates	*Per cent inhibition over control		
		0.2%	0.25%	0.3%
1.	PAT1	72.22 (8.489) de	90.00 (9.487)b	94.44 (9.718)a
2.	PAT2	94.44 (9.718)a	94.44 (9.718)a	94.44 (9.718)a
3.	PAT3	94.44 (9.718)a	94.44 (9.718)a	94.44 (9.718)a
4.	PAT4	94.44 (9.718)a	94.44 (9.718)a	94.44 (9.718)a
5.	PAT5	73.15 (8.551)cde	94.44 (9.718)a	94.44 (9.718)a
6.	PAT6	62.41 (7.900)fg	94.44 (9.718)a	94.44 (9.718)a
7.	ALT1	14.82 (3.848)k	39.82 (6.309)h	68.15 (8.249)d
8.	ALT2	58.70 (7.658)fgh	74.07 (8.606)d	94.44 (9.718)a
9.	ALT3	42.59 (6.488)j	53.52 (7.315)g	59.07 (7.686)e
10.	VYT1	67.78 (8.222)ef	94.44 (9.718)a	94.44 (9.718)a
11.	VYT2	67.41 (8.202)ef	94.44 (9.718)a	94.44 (9.718)a
12.	KUT1	55.00 (7.416)ghi	73.33 (8.563)d	85.56 (9.249)b
13.	WAT1	94.44 (9.718)a	94.44 (9.718)a	94.44 (9.718)a
14.	WAT2	83.33 (9.129)bc	94.44 (9.718)a	94.44 (9.718)a
15.	WAT3	77.59 (8.807)bcd	94.44 (9.718)a	94.44 (9.718)a
16.	WAT4	94.44 (9.718)a	94.44 (9.718)a	94.44 (9.718)a
17.	WAT5	49.44 (6.988)ij	55.19 (7.428)f	70.00 (8.360)d
18.	WAT6	59.44 (7.710)fgh	86.67 (9.309)c	94.44 (9.718)a

Sl. No.	Trichoderma isolates	*Per cent inhibition over control		
		0.2%	0.25%	0.3%
19.	WAT7	94.44 (9.718) [a]	94.44 (9.718) [a]	94.44 (9.718) [a]
20.	WAT8	85.93 (9.269) [ab]	94.44 (9.718) [a]	94.44 (9.718) [a]
21.	THT1	51.30 (7.160) [hi]	70.37 (8.388) [e]	78.52 (8.861) [c]
22.	THT2	94.44 (9.718) [a]	94.44 (9.718) [a]	94.44 (9.718) [a]
23.	THT3	94.44 (9.718) [a]	94.44 (9.718) [a]	94.44 (9.718) [a]
24.	THT4	54.59 (7.374) [ghi]	94.44 (9.718) [a]	94.44 (9.718) [a]
CD (0.05)		0.578	0.099	0.192

*Mean of three replications

In each column figures followed by same letter do not differ significantly according to DMRT.

$\sqrt{x+0.5}$ transformed values are given in parentheses.

Table 4.13 Effect of copper hydroxide 77WP on sporulation of

***Trichoderma* spp.**

Sl. No.	*Trichoderma* isolate	Sporulation of *Trichoderma* spp.		
		0.20%	0.25%	0.30%
1.	PAT1	+	-	-
2.	PAT2	-	-	-
3.	PAT3	-	-	-
4.	PAT4	-	-	-
5.	PAT5	+	-	-
6.	PAT6	++	-	-
7.	ALT1	+++	+++	+
8.	ALT2	++	+	-
9.	ALT3	+++	+++	++
10.	VYT1	+	-	-
11.	VYT2	+	-	-
12.	KUT1	+++	++	-
13.	WAT1	-	-	-
14.	WAT2	++	-	-
15.	WAT3	-	-	-
16.	WAT4	-	-	-
17.	WAT5	+++	++	-
18.	WAT6	++	-	-
19.	WAT7	-	-	-
20.	WAT8	-	-	-
21.	THT1	+	+	-
22.	THT2	-	-	-
23.	THT3	-	-	-
24.	THT4	+	-	-

-: no sporulation, +: sparse sporulation, ++: moderate sporulation, +++: high sporulation

Plate 4.7 (i)Tolerance of *Trichoderma* spp. against copper hydroxide 77 WP
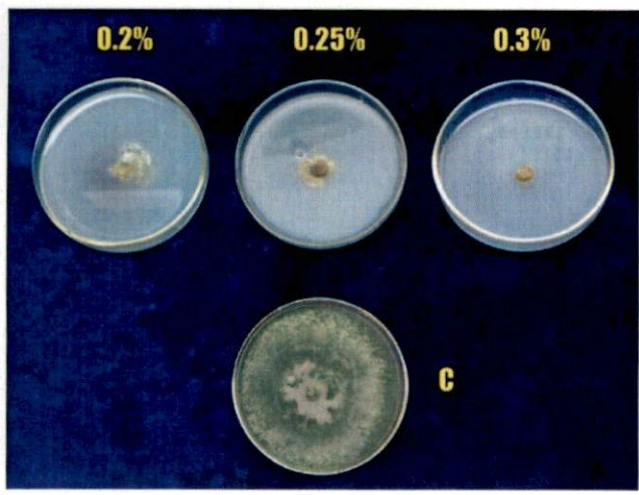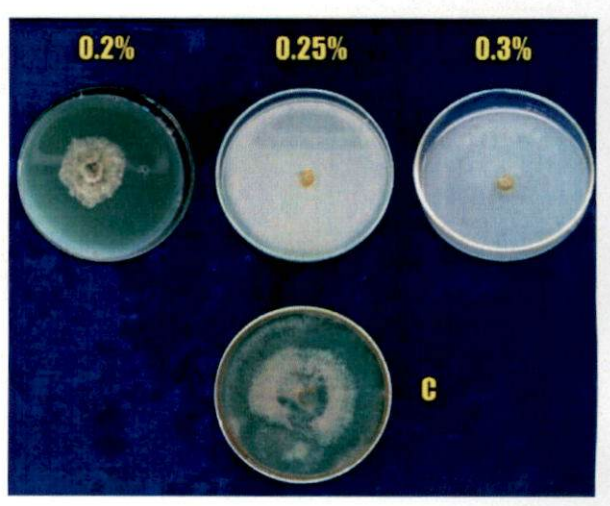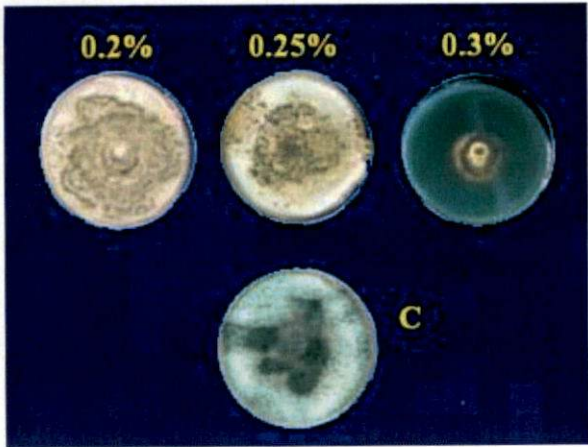

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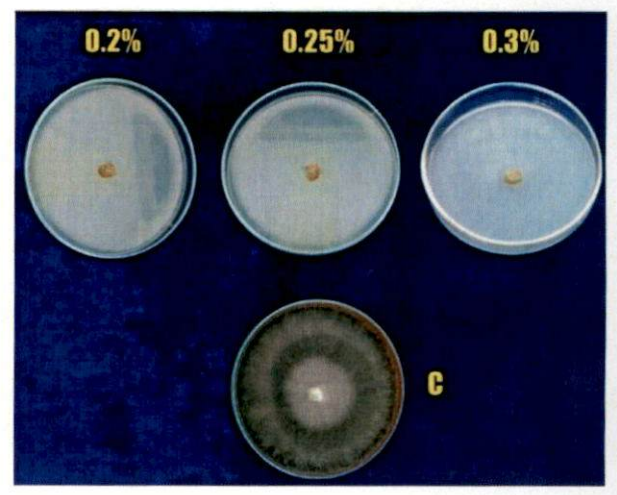
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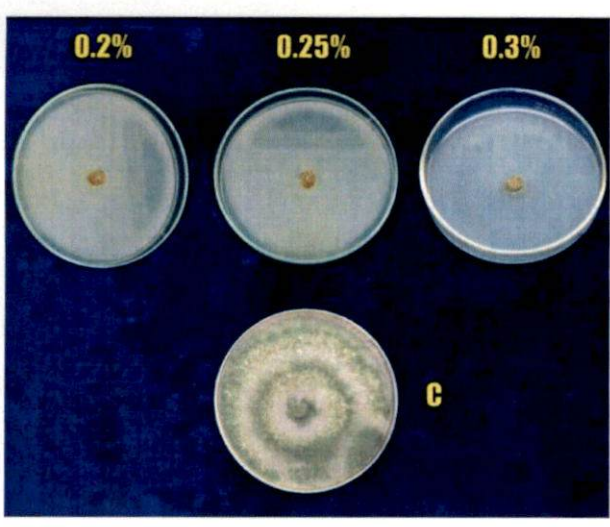
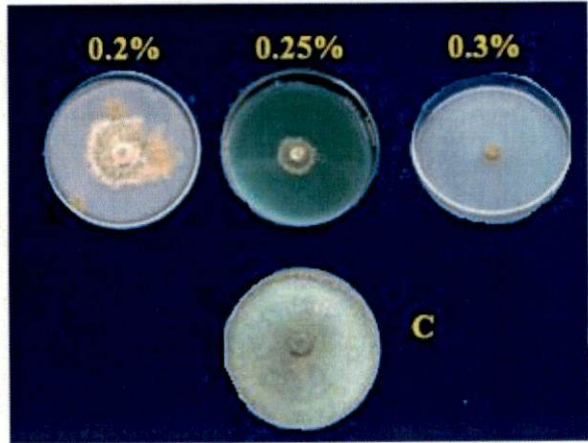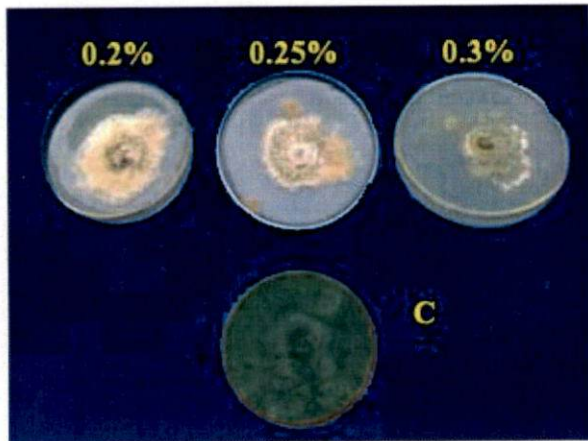3) PAT 3
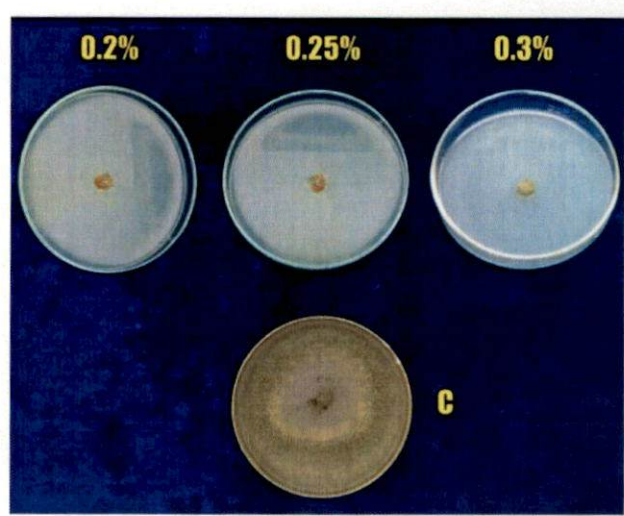
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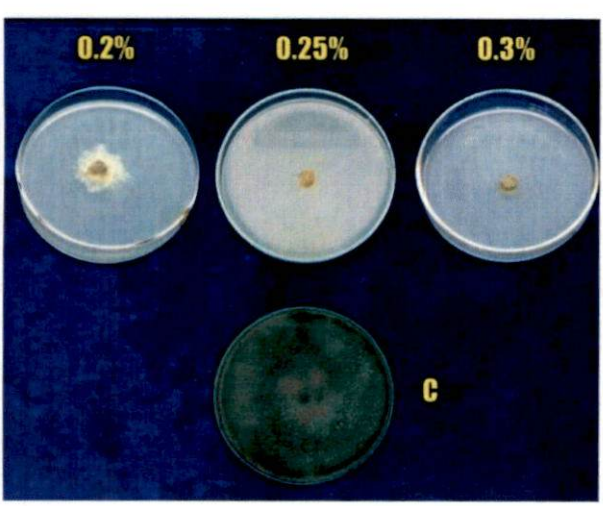
5) PAT 5

6) PAT 6

Plate 4.7 (ii)Tolerance of *Trichoderma* spp. against copper hydroxide 77 WP
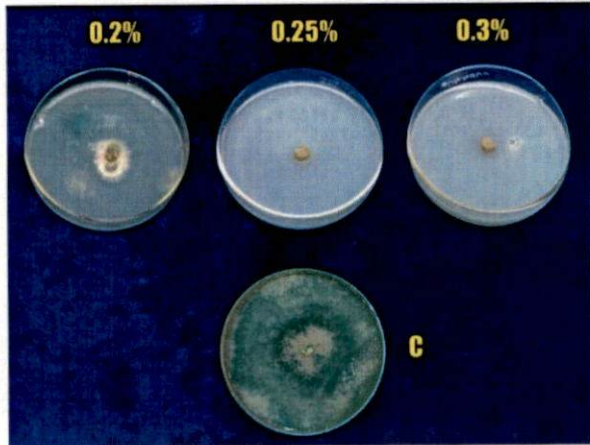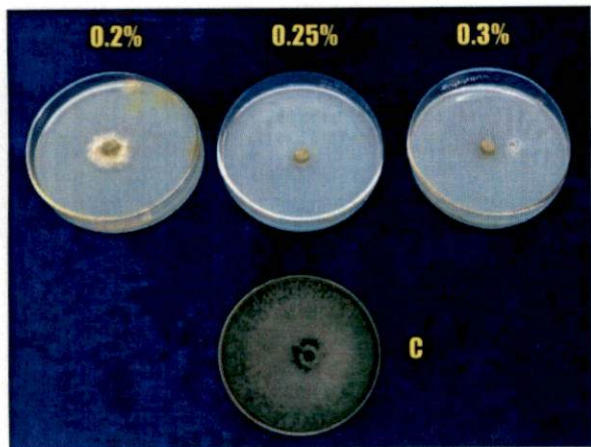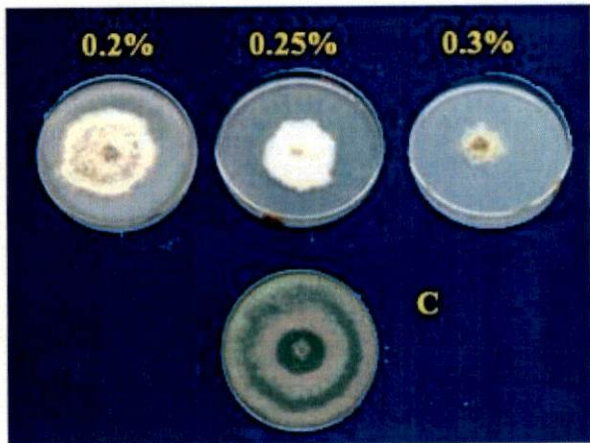

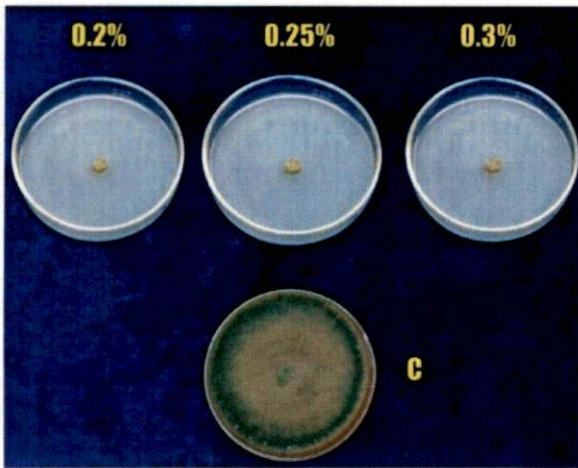7) ALT 1

8) ALT 2

9) ALT 3

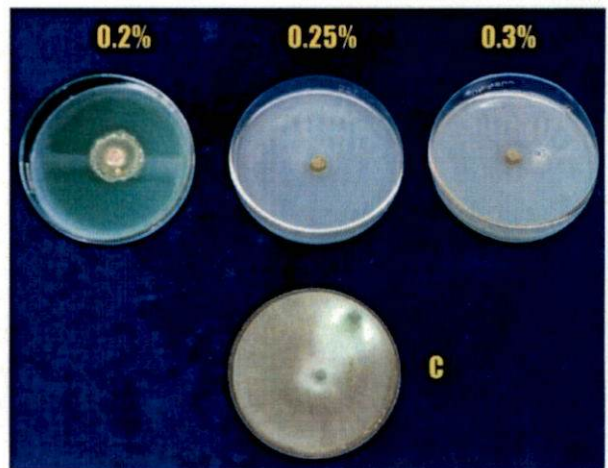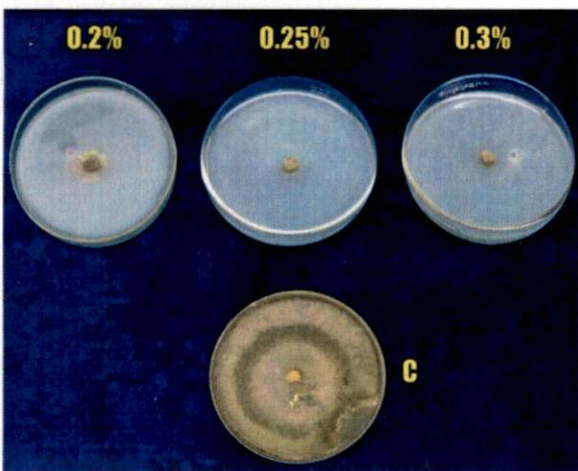
10) VYT 1

11) VYT 2

12) KUT 1

Plate 4.7 (iii)Tolerance of *Trichoderma* spp. against copper hydroxide 77 WP
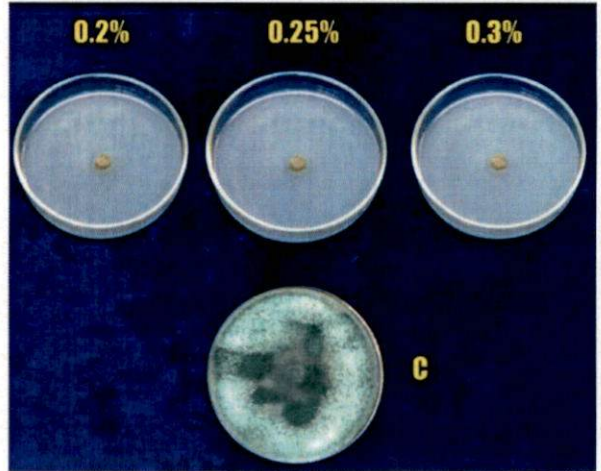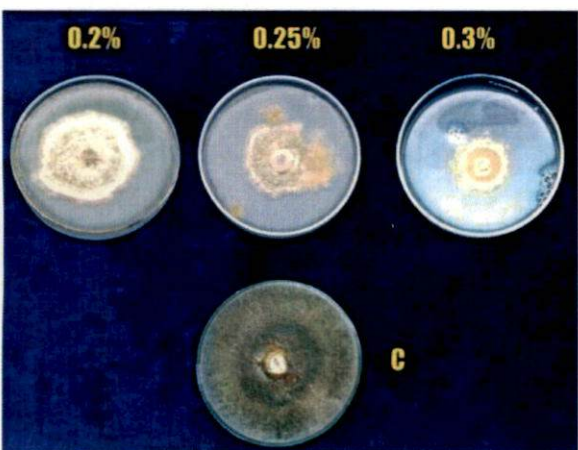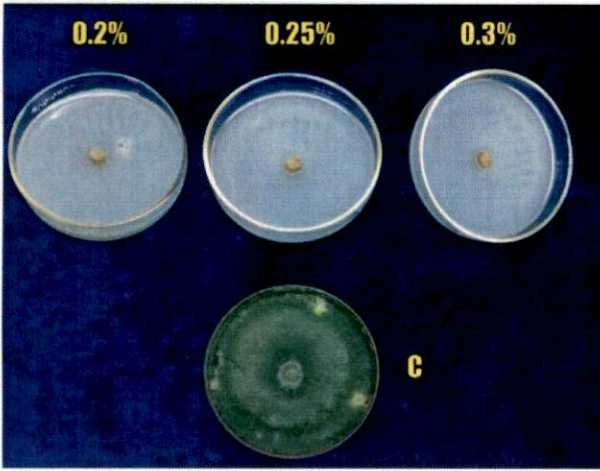

13) WAT 1

14) WAT 2

15) WAT 3
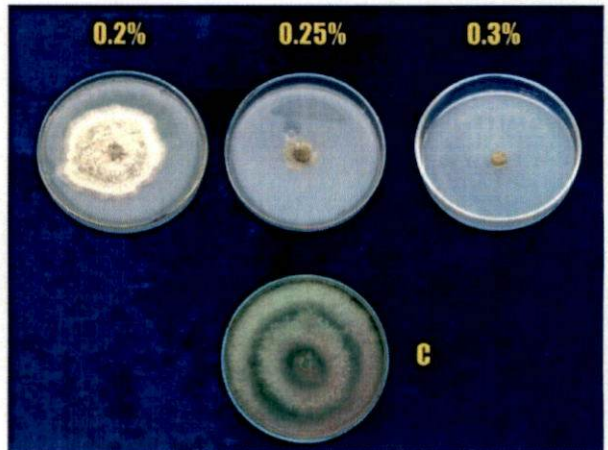
16) WAT 4

17) WAT 5
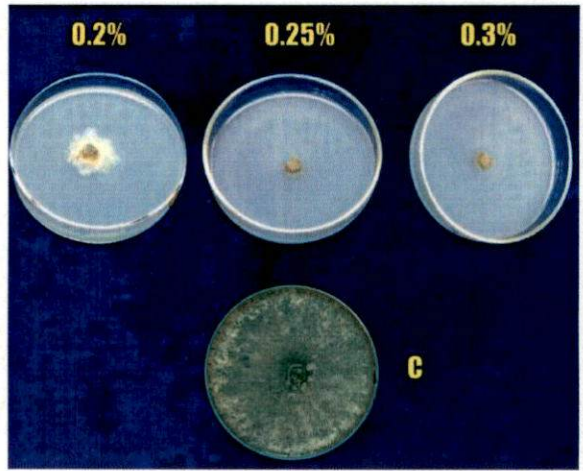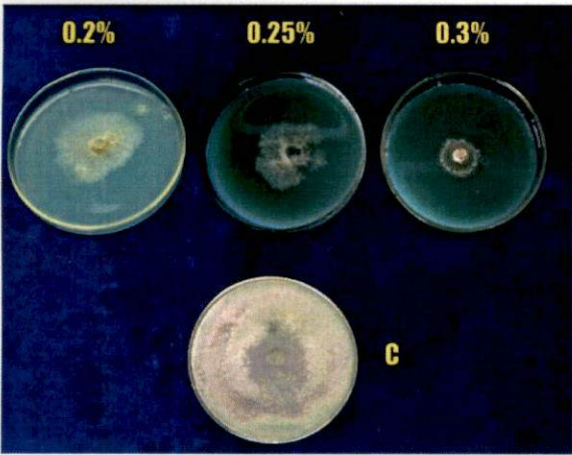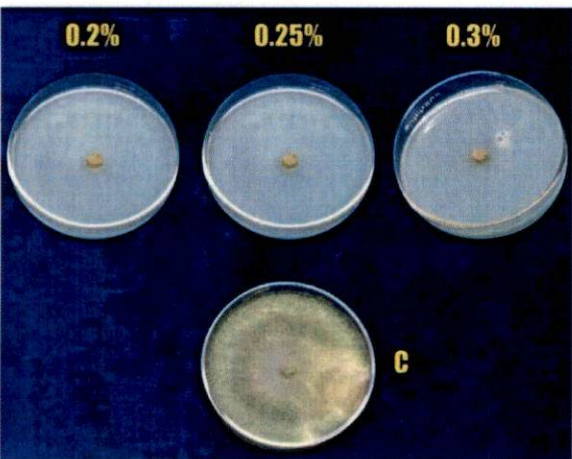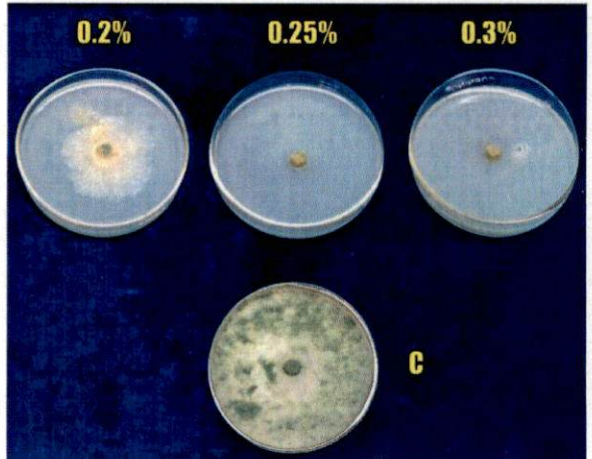
18) WAT 6
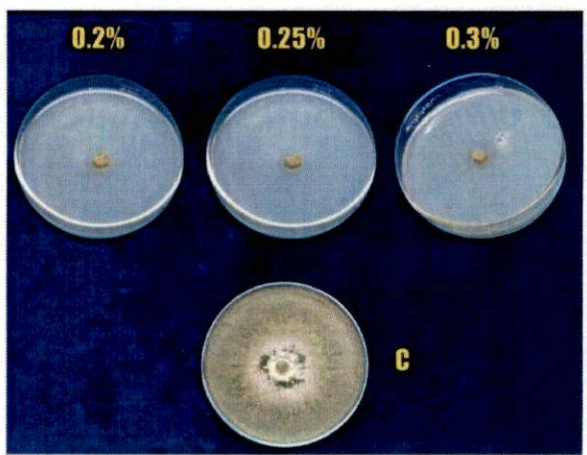
fungicides (Table 4.12) [Plate 4.7 (i), (ii), (iii) and (iv)]. At 0.2 per cent concentration of the fungicide, the per cent inhibition exhibited by the isolates ranged between 14.82 to 94.44 per cent and the isolates ALT1, ALT3 and WAT5 showed considerable growth with lower per cent inhibition of 14.82, 42.59 and 49.44 respectively. Likewise, it was noticed that all the isolates *viz.,* PAT2, PAT3, PAT4, WAT1, WAT4, WAT7, THT2, THT3 showed higher inhibition of 94.44 per cent at same concentration of copper hydroxide.

Per cent inhibition recorded in all isolates of *Trichoderma* spp. at 0.25 per cent of copper hydroxide ranged between 39.82 and 94.44 with minimum inhibition noticed with the isolate ALT1 (39.82%) followed by ALT3 (53.52%) and the maximum inhibition of 94.44 per cent was recorded in rest of the isolates. Similarly, at 0.3 per cent concentration of the fungicide also, the isolates such as ALT1, ALT3 and WAT5 showed considerable growth with minimum per cent inhibition of 68.15, 59.07 and 70.00 respectively and all other isolates except THT1 (78.52%) and KUT1 (85.56%) showed minimum growth with 94.44 per cent inhibition. The isolates showed variation in sporulation of when grown in fungicide amended medium and the observations are recorded in Table 4.13. At 0.2 per cent concentration, the isolates ALT 1, ALT 3, KUT 1 and WAT 5 showed high sporulation and the isolates PAT 6, ALT 2, WAT 2 and WAT 6 showed medium sporulation, whereas isolates such as PAT 1, PAT 5, VYT 1, VYT 2, THT 1 and THT 4 showed sparse sporulation and the rest of the isolates did not sporulate at all. On increasing the concentration to 0.25 per cent, sporulation of the isolates were also reduced and the isolates KUT 1 and WAT 5 showed medium sporulation and ALT 2 showed sparse sporulation. Nonetheless, ALT 1 and ALT 3 retained high sporulation at this concentration also. At 0.3 per cent concentration of the fungicide, ALT 3 and ALT 1 were able to produce medium and sparse sporulation.

On the basis of the growth of fungus at all three concentrations, the isolates ALT1 and ALT3 were selected as copper hydroxide tolerant isolates which was used for further studies.

4.3.5.2. Screening for copper oxychloride tolerance

The *Trichoderma* spp. obtained from different locations were screened for tolerance against copper oxychloride at 0.2, 0.25 and 0.3 per cent concentrations and it was noticed that with increase in fungicide concentration, there was a reduction in the growth of the fungus (Table 4.14) [Plate 4.8 (i), (ii), (iii) and (iv)]. Isolates showed a per cent inhibition ranging from zero to 94.44 per cent when grown at 0.2 per cent concentration of fungicide amended medium. Isolates such as PAT1, ALT1, ALT3, WAT2, WAT5, THT1 and THT2 showed good growth with zero per cent inhibition, whereas PAT2, PAT4, THT3 and THT4 showed no growth with 94.44 per cent inhibition.

Similarly at 0.25 per cent concentration of the fungicide, the isolate showed a per cent inhibition ranging from 34.07 to 94.44 per cent where isolates such as ALT3, PAT1, WAT2 and THT4 with 34.07, 45.93, 53.07 and 55.56 per cent inhibition respectively. While, minimum growth was recorded in PAT6, PAT3, PAT2, WAT1, WAT3, WAT6, WAT7, WAT8 and THT3 with 94.44 per cent inhibition in all isolates. Likewise, at 0.3 per cent concentration the isolates showed per cent inhibition ranging from 68.89 to 94.44 per cent, with maximum growth in isolate ALT3 (68.89 %) followed by PAT1 (74.82 %), WAT2 (86.30 %) and VYT1 (89.63 %) and minimum growth with higher per cent inhibition of 94.44 was noticed.

Variation in sporulation was observed among the isolates when they grown in fungicide amended medium (Table 4.15). At 0.2 per cent concentration of the fungicide, ALT 1, ALT 2, ALT 3, VYT 1, WAT 2, WAT 6, THT 1, THT 2 and THT 4 showed high sporulation and WAT 3 showed sparse sporulation whereas, PAT 2, PAT 4 and THT 4 produced no sporulation at all. Rest of the isolates showed medium sporulation at the same concentration of the fungicide.

128

Table 4.14 Effect of copper oxychloride 50 WP on growth of *Trichoderma* spp.

Sl. No.	*Trichoderma* isolates	*Per cent inhibition over control		
		0.20%	0.25%	0.30%
1.	PAT1	0.000 (0.707)[j]	45.926 (6.776)[i]	74.815 (8.649)[d]
2.	PAT2	94.444 (9.744)[a]	94.444 (9.718)[a]	94.444 (9.718)[a]
3.	PAT3	38.333 (6.215)[cde]	81.852 (9.046)[b]	94.444 (9.718)[a]
4.	PAT4	94.444 (9.744)[a]	94.444 (9.718)[a]	94.444 (9.718)[a]
5.	PAT5	27.407 (5.253)[g]	71.852 (8.476)[cd]	94.444 (9.718)[a]
6.	PAT6	10.185 (3.262)[h]	94.444 (9.718)[a]	94.444 (9.718)[a]
7.	ALT1	0.000 (0.707)[j]	62.593 (7.911)[fg]	94.444 (9.718)[a]
8.	ALT2	28.333 (5.323)[fg]	67.778 (8.233)[e]	94.444 (9.718)[a]
9.	ALT3	0.000 (0.707)[j]	34.074 (5.829)[j]	68.889 (8.300)[e]
10.	VYT1	12.963 (3.636)[h]	60.000 (7.746)[g]	89.630 (9.467)[b]
11.	VYT2	33.333 (5.817)[ef]	75.185 (8.670)[c]	94.444 (9.718)[a]
12.	KUT1	27.778 (5.317)[fg]	68.889 (8.299)[de]	94.444 (9.718)[a]
13.	WAT1	43.519 (6.634)[bcd]	94.444 (9.718)[a]	94.444 (9.718)[a]
14.	WAT2	0.000 (0.707)[j]	53.704 (7.326)[h]	86.296 (9.290)[c]
15.	WAT3	45.000 (6.743)[bc]	94.444 (9.718)[a]	94.444 (9.718)[a]
16.	WAT4	48.889 (7.020)[b]	94.444 (9.718)[a]	94.444 (9.718)[a]
17.	WAT5	0.000 (0.707)[j]	71.111 (8.430)[de]	94.444 (9.718)[a]
18.	WAT6	37.593 (6.167)[de]	94.444 (9.718)[a]	94.444 (9.718)[a]

Sl. No.	*Trichoderma* isolates	*Per cent inhibition over control		
		0.20%	0.25%	0.30%
19.	WAT7	35.185 (5.965)[e]	94.444 (9.718)[a]	94.444 (9.718)[a]
20.	WAT8	50.926 (7.167)[b]	94.444 (9.718)[a]	94.444 (9.718)[a]
21.	THT1	0.000 (0.707)[j]	63.704 (7.980)[f]	94.444 (9.718)[a]
22.	THT2	0.000 (0.707)[j]	69.259 (8.321)[de]	94.444 (9.718)[a]
23.	THT3	94.444 (9.744)[a]	94.444 (9.718)[a]	94.444 (9.718)[a]
24.	THT4	4.259 (2.159)[i]	55.556 (7.453)[h]	94.444 (9.718)[a]
	CD (0.05)	0.549	0.217	0.061

*Mean of three replications

In each column figures followed by same letter do not differsignificantly according to DMRT

$\sqrt{x+0.5}$ transformed values are given in parentheses.

130

Table 4.15 Effect of copper oxychloride 50 WP on sporulation of *Trichoderma* spp.

Sl. No.	*Trichoderma* isolate	Sporulation of *Trichoderma* spp.		
		0.20%	**0.25%**	**0.30%**
1.	PAT1	++	++	-
2.	PAT2	-	-	-
3.	PAT3	++	+	-
4.	PAT4	-	-	-
5.	PAT5	++	-	-
6.	PAT6	++	-	-
7.	ALT1	+++	+	-
8.	ALT2	+++	+	-
9.	ALT3	+++	++	-
10.	VYT1	+++	++	-
11.	VYT2	++	+	-
12.	KUT1	++	+	-
13.	WAT1	+++	-	-
14.	WAT2	+++	++	-
15.	WAT3	+	-	-
16.	WAT4	++	-	-
17.	WAT5	++	-	-
18.	WAT6	+++	-	-
19.	WAT7	++	-	-
20.	WAT8	++	-	-
21.	THT1	+++	+	-
22.	THT2	+++	+	-
23.	THT3	-	-	-
24.	THT4	+++	+	-

-: no sporulation, +: sparse sporulation, ++: moderate sporulation, +++: high sporulation

Plate 4.8 (i) Tolerance of *Trichoderma* spp. against copper oxychloride 50 WP
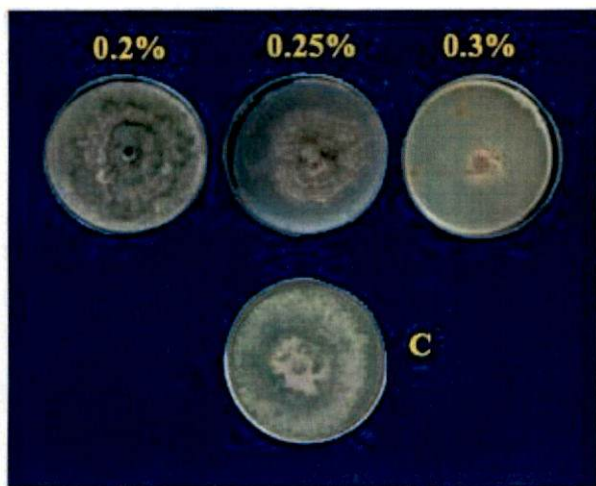
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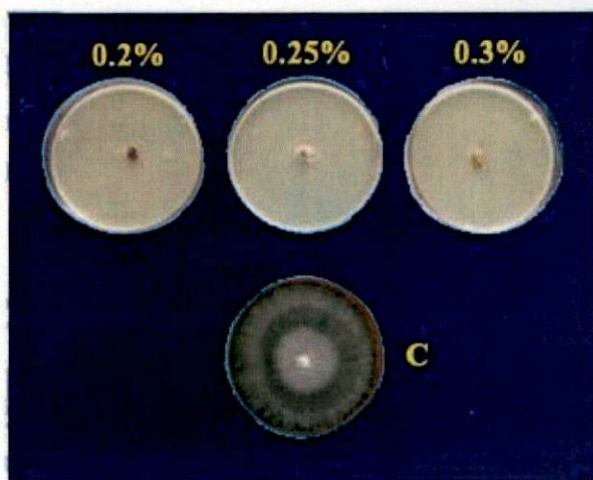
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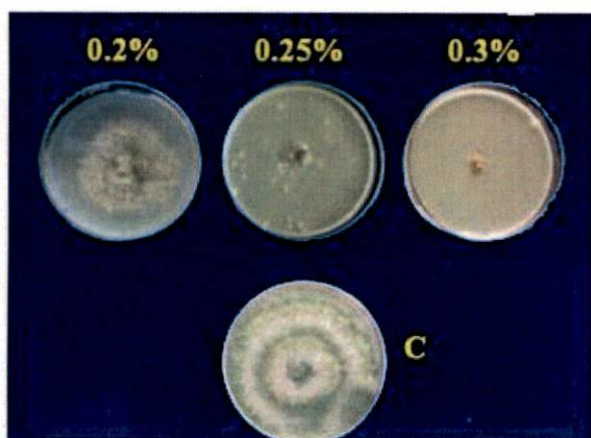
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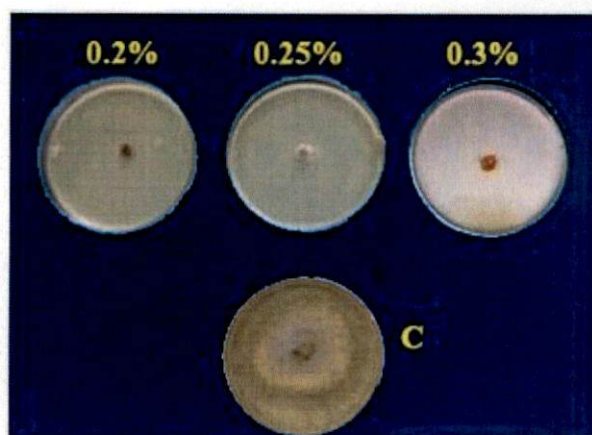
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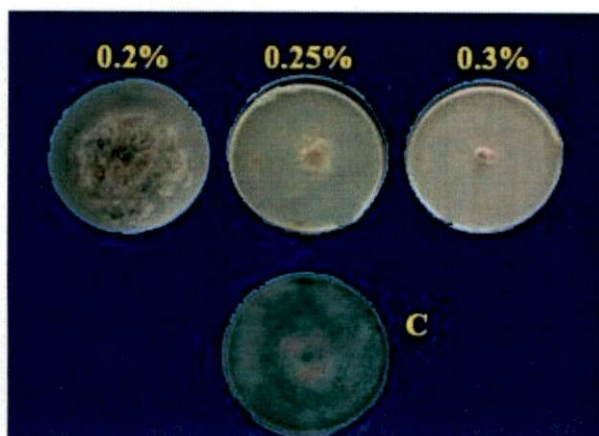
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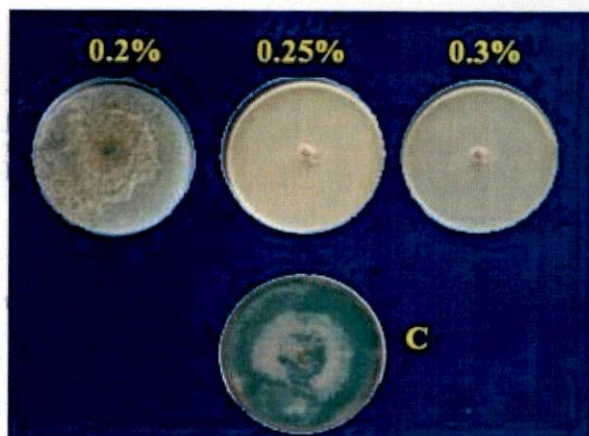
6) PAT 6

Plate 4.8 (ii) Tolerance of *Trichoderma* spp. against copper oxychloride 50 WP

7) ALT1

8) ALT 2

9) ALT 3

10) VYT 1

11) VYT 2

12) KUT 1

Plate 4.8 (iii) Tolerance of *Trichoderma* spp. against copper oxychloride 50 WP
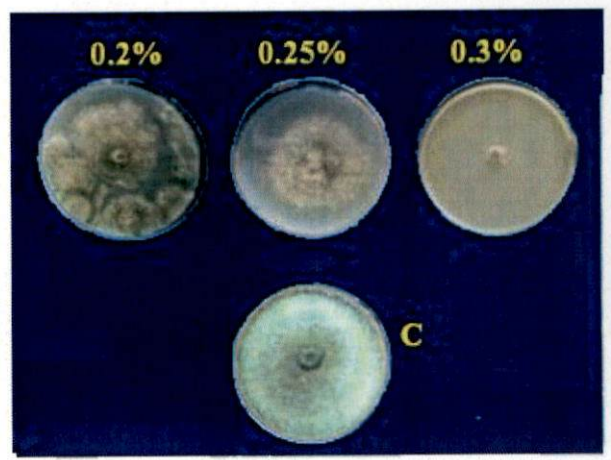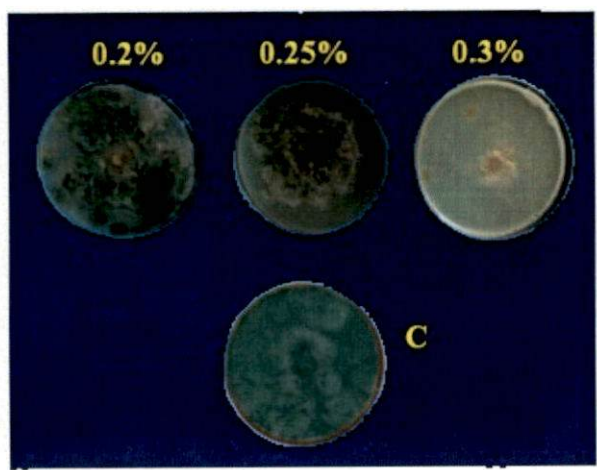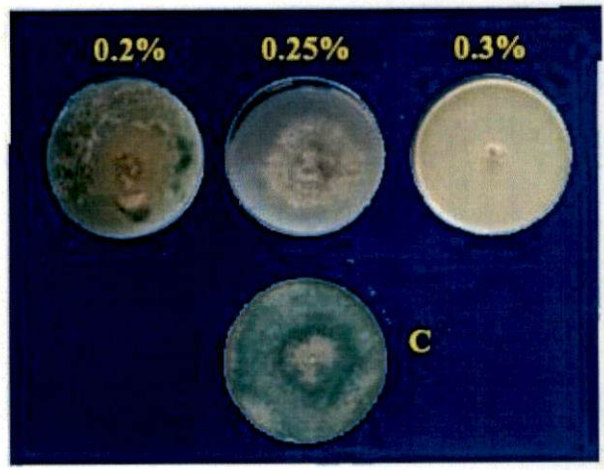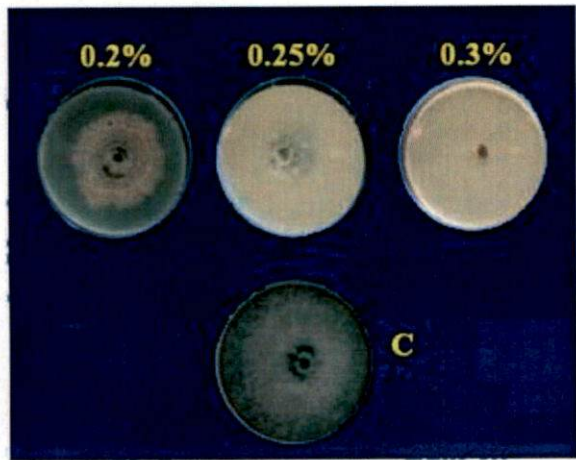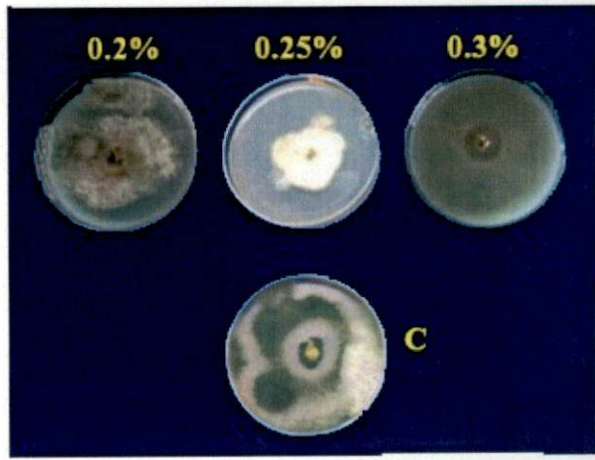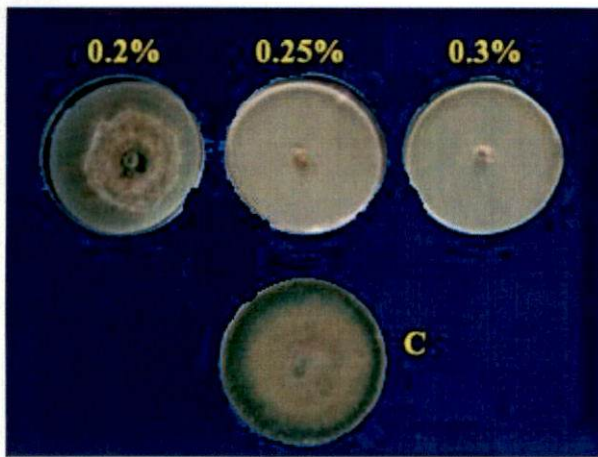
13) WAT 1
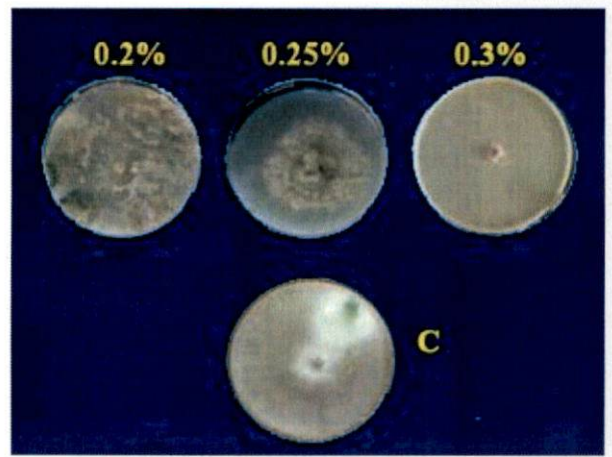
14) WAT 2
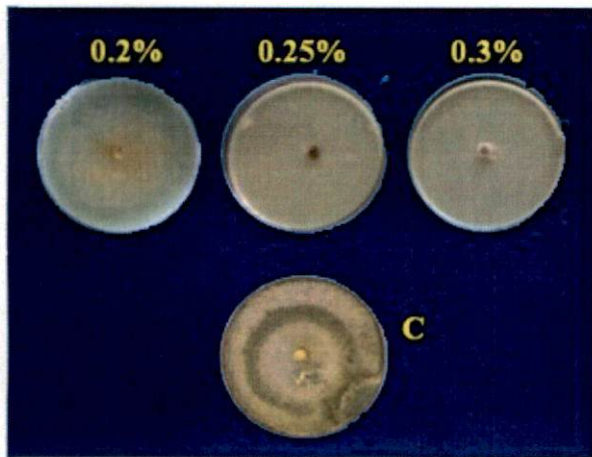
15) WAT 3
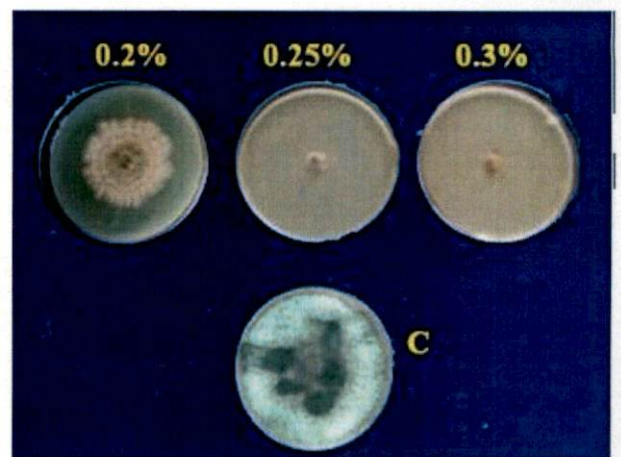
16) WAT 4
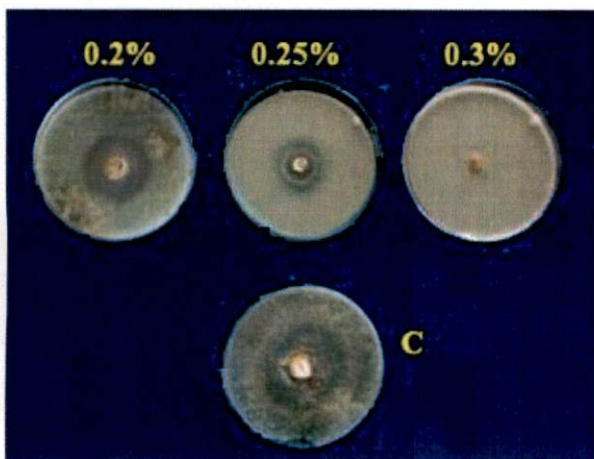
17) WAT 5
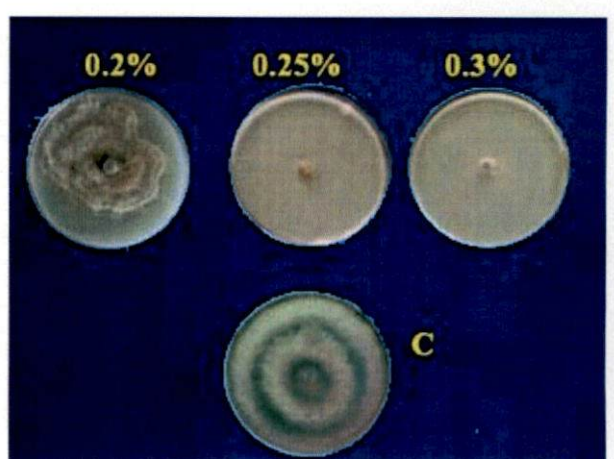
18) WAT 6

Plate 4.8 (iv) Tolerance of *Trichoderma* spp. against copper oxychloride 50 WP
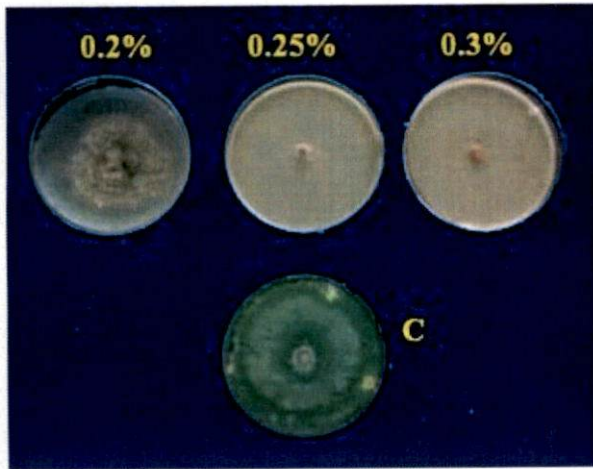
19) WAT 7
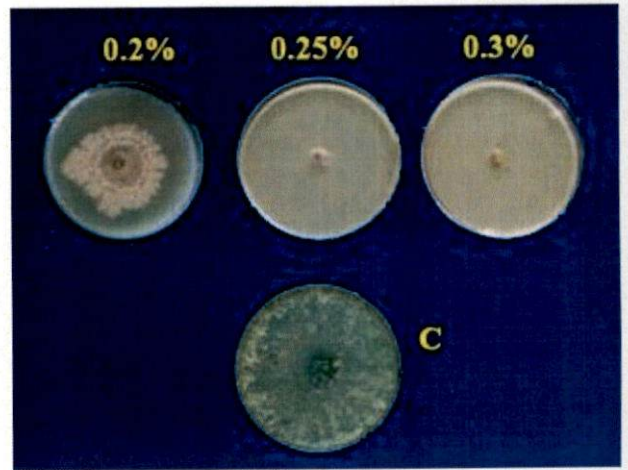
20) WAT 8
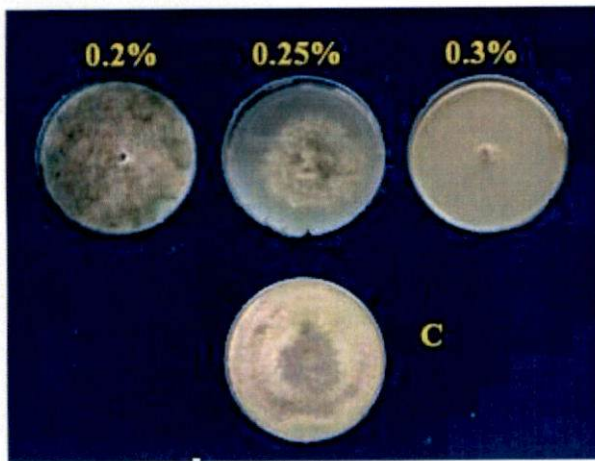
21) THT 1
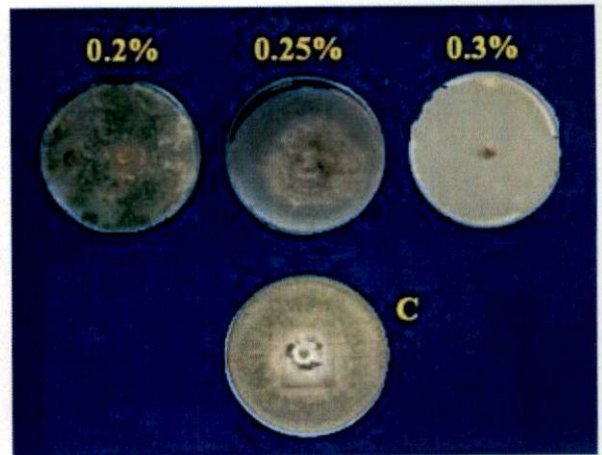
22) THT2
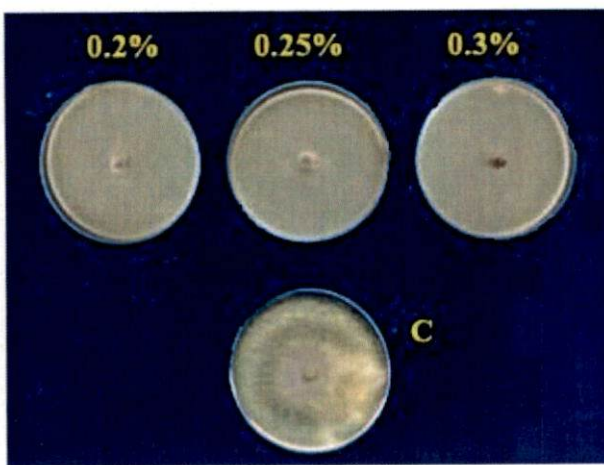
23) THT3
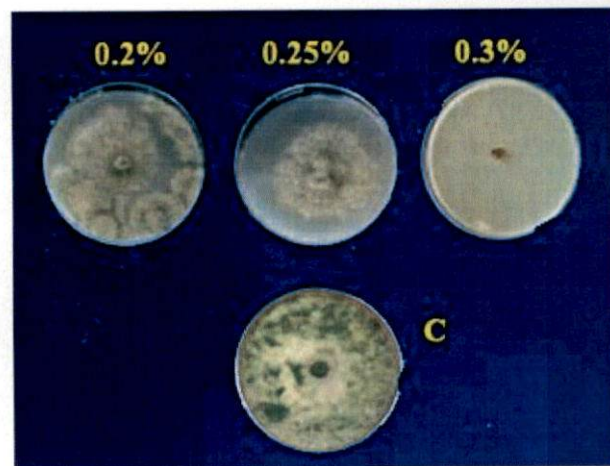
24) THT4

While increasing the concentration to 0.25 per cent, PAT 1, ALT 3, VYT 1 and WAT 2 showed medium and PAT 3, ALT 1, ALT 2, VYT 2, KUT 1, THT 1, THT 2 and THT 4 showed sparse sporulation whereas, r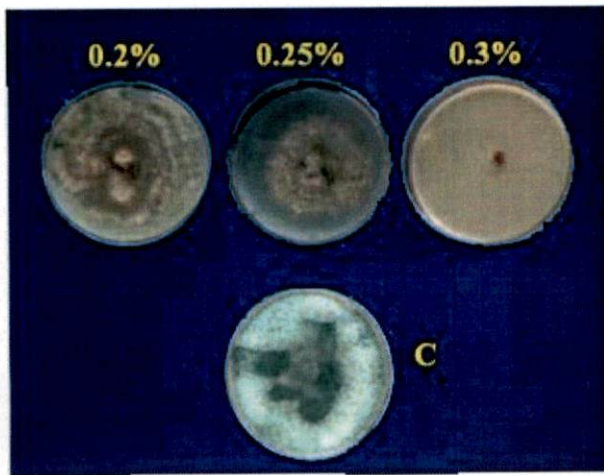est of the isolates did show sporulation. At the highest concentration of copper oxychloride (0.3 %), none of the isolates showed any sporulation at all.

After the study, it was concluded that the isolate ALT3 and PAT1 showed comparatively good growth at the highest concentration of the fungicide which can therefore be considered as fungicide tolerant isolates and hence, selected for further studies.

4.4 BIOCHEMICAL CHARACTERIZATION OF STRESS TOLERANT ISOLATES OF *Trichoderma* spp.

Out of the 24 isolates of *Trichoderma* spp., six abiotic stress tolerant isolates were selected based on the *in vitro* screening for high temperature, drought, salinity, acidity and fungicide. Two best performing isolates under each stress were selected for further studies. Isolates PAT 6 and WAT 2 from Palakkad and Wayanad respectively were selected as thermotolerant and VYT 2 and ALT1 from Ernakulam and Alappuzha as drought tolerant isolates. The isolates from Alappuzha *viz.,* ALT3 and ALT1 were selected as acidity and salinity tolerant isolates. Likewise, PAT 1 and ALT3 from Palakkad and Alappuzha were selected as copper oxychloride tolerant isolates and ALT 1 and ALT 3, as copper hydroxide tolerant isolates.

4.4.1. Assay of extracellular enzymes

The stress tolerant isolates were further subjected to biochemical tests to study the activity of enzymes such as cellulase, β- 1,3- glucanase and protease, which are responsible for the mycoparasitism exhibited by *Trichoderma* spp. The results of the enzyme activity are presented in the Table 4.16.

4.4.1.1. Cellulase assay

Six stress tolerant isolates were further used for assessment of cellulase enzyme activity which was expressed in U ml^{-1}. In general, the isolates showed cellulase activity ranging from 0.15 to 4.26 U ml^{-1} and it was observed that the isolate VYT 2 showed highest cellulase activity of 4.26 U ml^{-1} followed by ALT 1 (3.47 U ml^{-1}), whereas minimum cellulase activity was recorded with the isolate ALT 3 (0.15U ml^{-1}).

4.4.1.2. β- 1,3- glucanase assay

All the six isolates were subjected to β-1,3-glucanase assay to detect the enzyme activity. From the study, it was noticed that there was a significant difference among the isolates showing an enzyme activity ranging from 1.43 to 5.45 U ml^{-1} and the isolate ALT 3 showed highest enzyme activity of 5.45 U ml^{-1} followed by VYT 2 with 3.29 U ml^{-1}. The least activity was observed with the isolate WAT 2 with 0.62Uml^{-1}.

4.4.1.3. Protease assay

Six abiotic stress tolerant isolates were subjected to assess the protease enzyme activity under *in vitro* conditions. The protease activity exhibited by the isolates ranged between 0.98 to 8.04 U ml^{-1} and the highest enzyme activity was recorded with the isolate ALT 1 (8.04 U ml^{-1}) followed by PAT 1 (7.88 U ml^{-1}). The minimum activity was observed in the isolate WAT 2 with 0.98 U ml^{-1}.

4.4.2. Biomolecules imparting drought tolerance

All the six isolates of *Trichoderma* spp. were screened for the qualitative assay of biomolecules involved in imparting drought tolerance like ACC-deaminase (1-aminocyclopropane-1-carboxylate deaminase) and cytokinin.

Table 4.16 Enzyme activity of stress tolerant isolates of *Trichoderma* spp.

Sl. No.	*Trichoderma* isolates	*Cellulase (U ml^{-1})	*β-1,3-glucanase (U ml^{-1})	*Protease (U ml^{-1})
1.	PAT1	0.22^e	1.67^d	7.88^b
2.	PAT6	3.30^c	1.43^e	5.12^c
3.	ALT1	3.47^b	2.86^c	8.04^a
4.	ALT3	0.15^f	5.45^a	4.33^d
5.	VYT2	4.26^a	3.29^b	4.17^e
6.	WAT2	0.64^d	0.62^f	0.98^f
	CD (0.05)	0.033	0.066	0.144

*Mean of three replications

In each column figures followed by same letter do not differ significantly according to DMRT

Table 4.17 Production of ACC deaminase by various isolates of *Trichoderma* spp.

Sl. No.	*Trichoderma* isolates	*Diameter of fungal culture (cm)
1.	PAT1	9.00^a
2.	PAT6	6.90^b
3.	ALT1	9.00^a
4.	ALT3	9.00^a
5.	VYT2	7.33^b
6.	WAT2	7.00^{bc}
7.	Positive control	9.00^a
8.	Negative control	0.50^c
	CD (0.05)	0.451

*Mean of three replications

In each column figures followed by same letter do not differ significantly according to DMRT

Plate 4.9 Production of ACC deaminase by various isolates of *Trichoderma* spp.
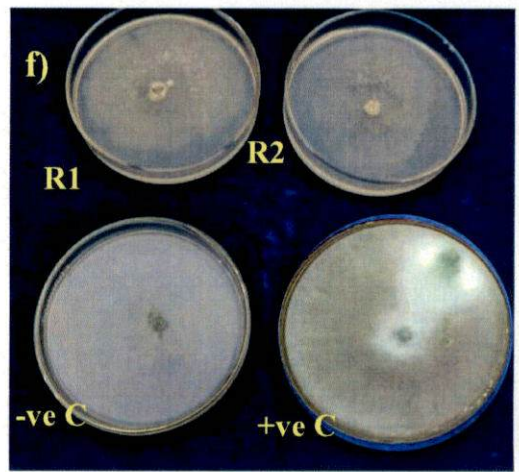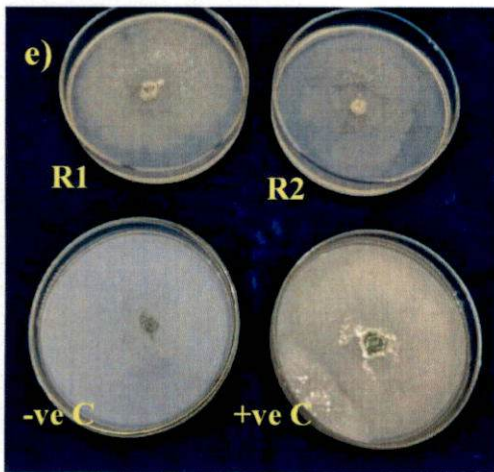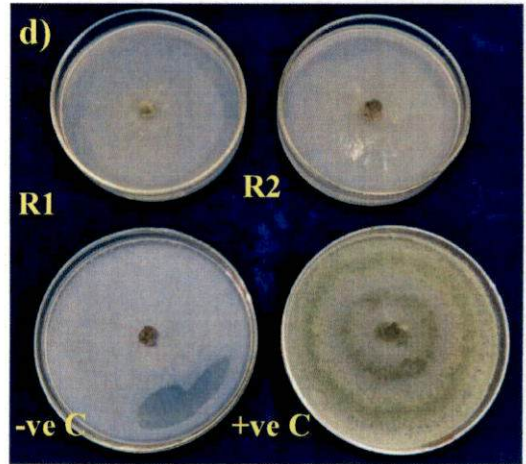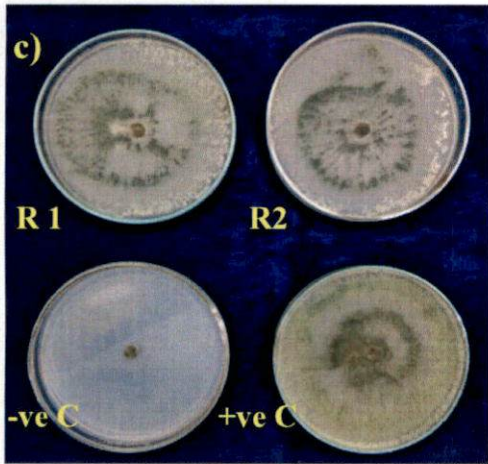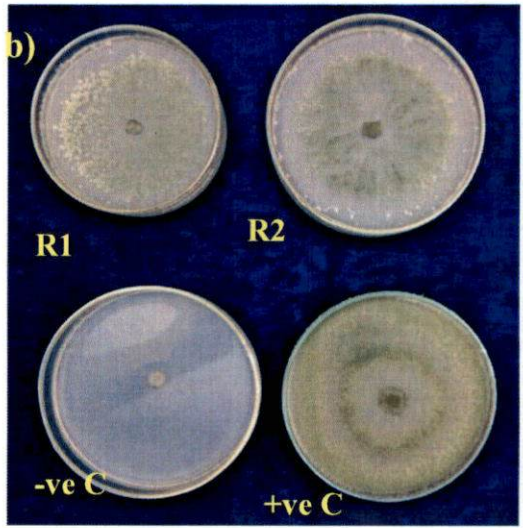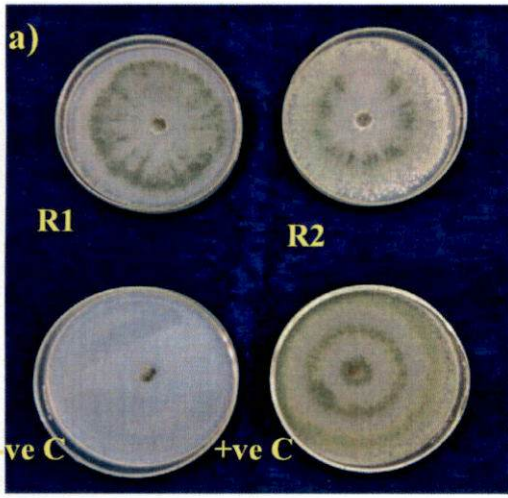
a) ALT 1; b) ALT 3; c) PAT 1; d) PAT 6; e) VYT 2; f) WAT 2

4.4.2.1. Screening for ACC deaminase production in Trichoderma spp.

All six stress tolerant isolates were screened for ACC (1-Aminocyclopropane-1-carboxylate) deaminase production. The isolates were tested for the ability to use ACC as the sole nitrogen source. A positive control with ammonium sulphate and a negative without any nitrogen source was used and the growth of each isolate was compared. Mycelial growth produced by the isolates was recorded and it ranged from 6.90 to 9.00 cm among the six different isolates (Table 4.17) (Plate 4.9). It was observed that, isolates PAT 1, ALT 1 and ALT 3 produced mycelial growth of 9 cm, which was same as that of positive control. There was no growth observed in negative control plate where no nitrogen source was provided. Thus, it was concluded that isolates PAT 1, ALT 1 and ALT 3 were better producers of the enzyme ACC- deaminase.

4.4.2.2.Screening for cytokinin production in Trichoderma spp.

Production of cytokinin by six selected isolates was carried out under *in vitro* conditions. Observations on root biomass and root length of cowpea seedlings (var. Uma) inoculated with extracts of *Trichoderma* isolates were recorded and presented in Table 4.18 (Plate 4.10). Length of roots formed after treating with extracts of isolates of *Trichoderma* spp. ranged from 9.02 to 13.60 cm with maximum root length produced by the isolate PAT 1 (13.60 cm) followed by PAT 6, WAT 2, ALT 3 and ALT 1 with root length of 10.90 cm, 10.60 cm, 10.28 cm and 10.20 cm when compared to control (8.89 cm). Minimum root length of 9.02 cm was recorded with the isolate VYT2.

Table 4.18 Effect of various isolates of *Trichoderma* spp. on root length of cowpea depicting cytokinin production

Sl. No.	*Trichoderma* isolates	*Root length (cm)
1.	ALT1	10.20^{bc}
2.	ALT3	10.28^{bc}
3.	PAT1	13.60^{a}
4.	PAT6	10.90^{b}
5.	WAT2	10.60^{b}
6.	VYT2	9.02^{c}
	Control	8.89 ^{d}
	CD (0.05)	1.513

*Mean of three replications
In each column figures followed by same letter do not differ significantly according to DMRT

Table 4.19 Effect of *Trichoderma* spp. on root biomass of cowpea seedlings indicating cytokinin production

Sl. No.	*Trichoderma* isolates	*Root biomass (g)
1.	ALT1	0.50^{a}
2.	ALT3	0.37^{bc}
3.	PAT1	0.31^{c}
4.	PAT6	0.33^{c}
5.	WAT2	0.33^{c}
6.	VYT2	0.43^{b}
7.	Control	0.30 ^{d}
	CD (0.05)	0.074

*Mean of three replications
In each column figures followed by same letter do not differ significantly according to DMRT

Plate 4. 10 Effect of various isolates of *Trichoderma* spp. on root length of cowpea depicting cytokinin production


ALT1   ALT3   PAT1   PAT6   VYT2   WAT2   CONTROL
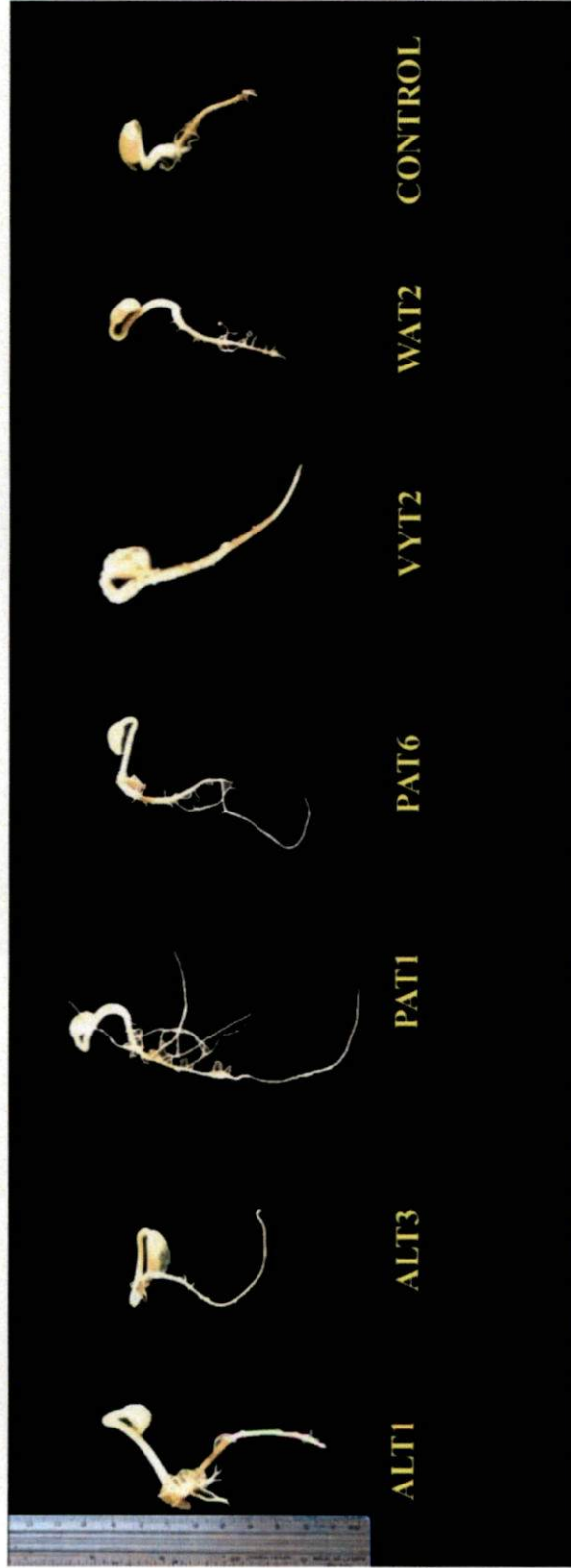
Root biomass, an indication of cytokinin production by *Trichoderma* isolates was also recorded and tabulated in Table 4.19. There was a significant difference noticed among the isolates which ranged from 0.31 to 0.5 g of root biomass. Highest root biomass was produced when the seeds were treated with ALT 1 (0.50 g) followed by VYT2 (0.43g), ALT 3 (0.37g), PAT 6 (0.33 g) and WAT 2 (0.33 g) compared to control (0.30 g). Minimum root biomass of 0.31 g was observed with PAT 1.

4.5. *In vitro* EVALUATION OF STRESS TOLERANT ISOLATES OF *Trichoderma* spp. FOR THEIR ANTAGONISTIC POTENTIAL

After the enzyme study, the isolate WAT 2 was screened out as it showed lesser activity of enzymes such as cellulase, β-1, 3- glucanase and protease. Root biomass and root length formed after the inoculation with this isolate was also found to be less which reflected its lower ability to produce cytokinin. Moreover, colony diameter formed after inoculation in DF medium with ACC as sole nitrogen source was also found minimum with this isolate. Hence, for dual culture experiment, five isolates of *Trichoderma* spp. *viz.*, ALT 1, ALT 3, PAT 1 and PAT 6 were employed to test the antagonistic potential with five major soil borne pathogens *viz., Phytophthora capsici, Pythium aphanidermatum, Scelrotium rolfsii, Fusarium solani* and *Rhizoctonia solani* collected from the Department of Plant Pathology, College of Horticulture, Vellanikkara. Antagonistic ability of the selected isolates was compared with *T. viride,* the reference cultureofKAU. The details of the experiment are given in Table 4.20.

When *Trichoderma* isolates were tested for their antagonistic ability with *Rhizoctonia solani,* a per cent inhibition ranging from 46.30 to 78.52 was noticed [Plate 4.11 (i) a].

143

Table 4.20 *In vitro* evaluation of various isolates of *Trichoderma* spp. against major soil borne fungal pathogens

Sl. No.	Trichoderma isolates	Per cent inhibition (%)				
		Rhizoctonia solani	Sclerotium rolfsii	Fusarium solani	Pythium aphanidermatum	Phytophthora capsici
1.	PAT1	75.93 $(8.71)^a$	52.22 $(7.22)^{bc}$	58.89	100.00 $(10.00)^a$	100.00
2.	PAT6	78.15 $(8.84)^a$	51.67 $(7.19)^{bc}$	57.41	79.63 $(8.923)^b$	100.00
3.	VYT2	46.30 $(6.80)^b$	55.00 $(7.42)^{ab}$	56.30	55.56 $(7.453)^d$	100.00
4.	ALT1	78.15 $(8.84)^a$	57.78 $(7.60)^a$	54.44	100.00 $(10.00)^a$	100.00
5.	ALT3	78.52 $(8.86)^a$	50.56 $(7.11)^c$	57.04	78.52 $(8.861)^{bc}$	100.00
6.	*T. viride*	78.52 $(8.86)^a$	49.26 $(7.05)^c$	57.40	100.00 $(10.00)^a$	100.00
	CD (0.05)	0.167	0.303	NS	0.099	NS

*Mean of three replications

In each column figures followed by same letter do not differ significantly according to DMRT.

$\sqrt{x+0.5}$ transformed values are given in parentheses.

Plate 4. 11(i) *In vitro* **evaluation of various isolates of** *Trichoderma* **spp. against major soil borne fungal pathogens.**
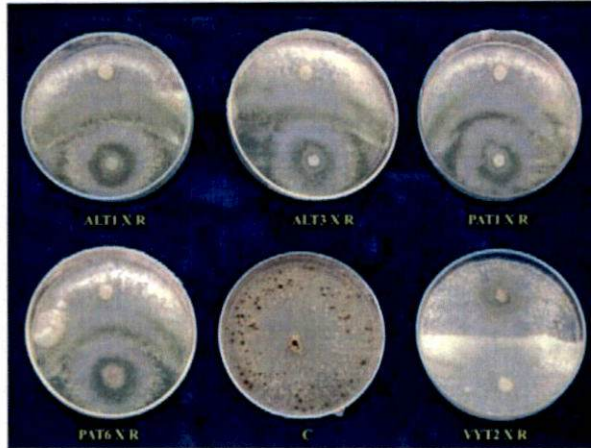
a) *Rhizoctonia solani*
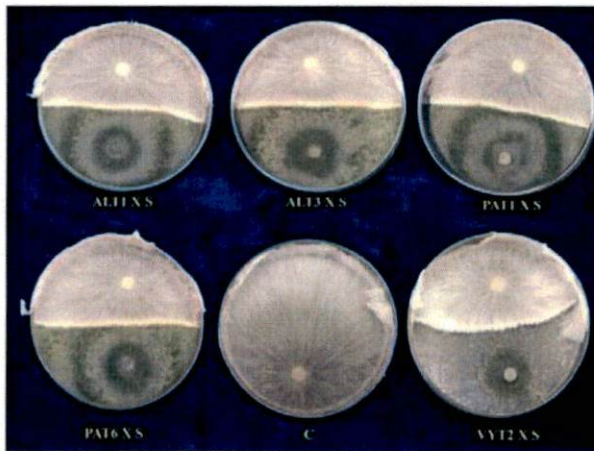
b) *Sclerotium rolfsii*

c) *Fusarium solani*

Plate 4. 11(ii) *In vitro* evaluation of various isolates of *Trichoderma* spp. against major soil borne fung
pathogens.
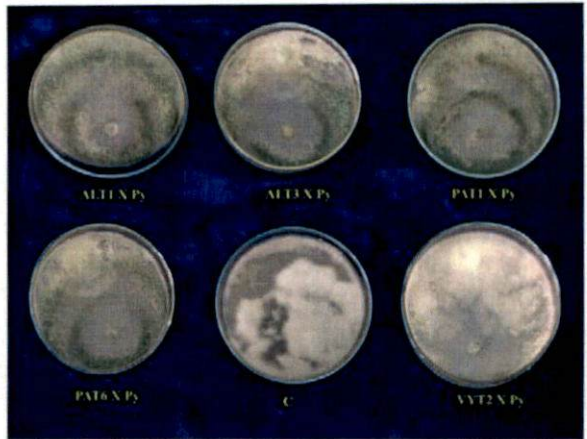
d) *Pythium aphanidermatum*

e) *Phytophthora capsici*
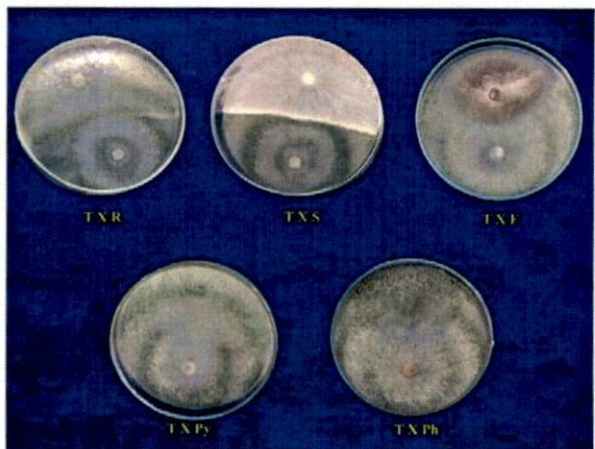
f) *T. viride*

The isolates ALT 3, ALT 1, PAT 6 and PAT 1 were on par with each other representing higher per cent inhibition of pathogen with 78.52, 78.15, 78.15 and 75.93 per cent respectively. These isolates were also on par with control, *T. viride*, which also showed 78.52 per cent inhibition of *R. solani* [Plate 4.11 (ii) e]. However, minimum per cent inhibition of the pathogen (46.30 %) was recorded with the *Trichoderma* isolate VYT 2.

Isolates such as ALT1 and VYT 2 showed 57.78 and 55 per cent inhibition of *S. rolfsii* where both these isolates were found to be on par, followed by PAT 1 and PAT 6 which showed a per cent growth inhibition of 52.22 and 51.67 of *S. rolfsii* respectively [Plate 4.11 (i) b and Plate 4.11 (ii) e]. The isolateALT 3 and control showed a minimum per cent inhibition of 50.56 and 49.26 per cent in the growth of the pathogen. Though no significant difference was noticed among the isolates when grown against *Fusarium solani* in dual culture, the highest per cent inhibition of 58.89 was noticed with PAT 1, closely followed by 57.41, 57.40 and 57.04 which were recorded with PAT 6, *T. viride* and ALT3 [Plate 4.11 (i) c and Plate 4.11 (ii) e]. Minimum per cent inhibition of 54.44 was noticed with ALT1.

Cent per cent inhibition of *Pythium aphanidermatum* was noticed when grown with *Trichoderma* isolates PAT 1 and ALT 1 [Plate 4.11 (ii) d and Plate 4.11 (ii) e]. Minimum per cent inhibition of pathogen was recorded with VYT2 (55.56%) and rest of the isolates showed an inhibition above 70 per cent. It was noticed that, all five isolates and control showed 100 per cent inhibition in the growth of pathogen *Phytophthora capsici* [Plate 4.11 (ii) d and Plate 4.11 (ii) e]. Hence, after the study only four isolates *viz.,* PAT1, PAT6, ALT1 and ALT3 were further selected for the pot culture experiment as the isolate VYT2 showed the least inhibition with *R. solani, S. rolfsii, F. solani* and *P. aphanidermatum* under *in vitro*.

4.6 MOLECULAR CHARACTERIZATION OF STRESS TOLERANT ISOLATES OF *Trichoderma* spp.

Based on the previous *in vitro* studies, four *Trichoderma* spp. showing high abiotic stress tolerance *viz.*, salinity, acidity, drought, fungicide and thermotolerance, and better enzyme activity as well as antagonistic property toward major pathogens were selected for further studies. The isolates selected for further study were ALT 1 and ALT 3 from Alappuzha and PAT 1 and PAT 6 from Palakkad district. The molecular characterization of these isolates was carried out at Rajiv Ganghi Centre for Biotechnology (RGCB), Thiruvananthapuram by ITS sequencing to identify the isolates upto species level. The PCR profile of amplified region and gel profile of DNA are given in the Plate 4.12 and Plate 4.13 respectively. The sequences retrieved were blasted in the online BLASTn programme of NCBI to analyze and to find the nucleotide homology of each of the isolates. Details of the result of sequence comparison of four isolates of *Trichoderma* spp. are given in Table 4.21.

4.6.1. Sequence comparison of ALT 1 isolate

Nucleotide sequences of ITS ofthe isolate ALT 1 (Table 4.22) was compared with other sequences and the search showed that the isolate showed 99 per cent identity with 94 per cent query coverage to *Trichoderma asperellum* strain T77 (Accession GU176467.1) and the data is depicted in Table 4.19. Yet, other sequences in NCBI with 94 per cent query coverage and 99 per cent identity was noticed with *Trichoderma asperellum* strain T 29 (Accession GU176445.1). Likewise, the isolate showed 99 per cent identity with 92 per cent query coverage to *Trichoderma asperellum* strain T 8 (Accession GU176439.1). From amongst the nucleotide database, sequence of the isolate ALT 1 when compared with nucleotide database retrieved from NCBI also revealed that it showed 95 per cent query coverage and 99 per cent identity to *Trichoderma asperellum* strain T 5 (Accession GU176437.1).

Plate 4.12 PCR amplification profile of the isolate CD1 with the universal primers of ITS 1F and ITS 4R
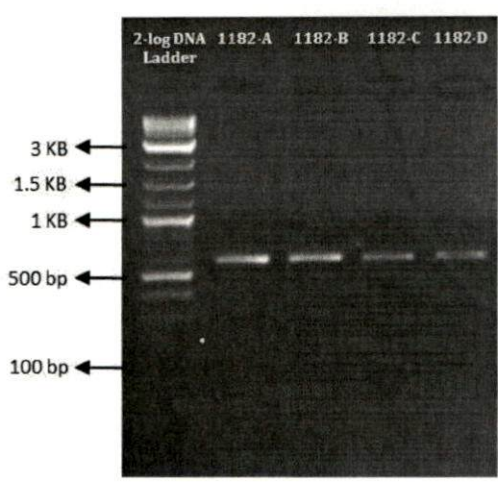
Plate 4.13 Gel profile of isolated DNA
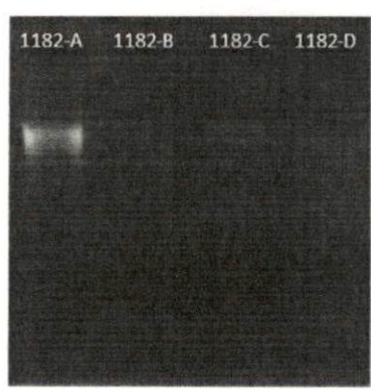
Table 4.21 Genomic sequence of *Trichoderma* spp.

Trichoderma isolates	Sequence
Trichoderma asperellum isolate ALT1	GAAAAGGACTGTCCTGATCCGAGGTCACTTTCAGAAGTTGGGTGTTTTACGGACGT GGACGCGCCGCGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTGCG GCGAGACCGCCACTGTATTTCGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCCCA ACGCCGATCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGCCC GCCAGAATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTC TGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAA GAGATCCGTTGTTGAAAGTTTTGATTCATTTTGAATTTTTGCTCAGAGCTGTAAGA AATACGTCCGCGAGGGGACTACAGAAAGAGTTTGGTTGGTTCCTCCGGCGGGCGCC TGGTTCCGGGGCTGCGACGCACCCGGGGCGTGACCCCGCCGAGGCAACAGTTTGGT AACGTTCACATGGGTGGGAAGCCAATTGCCGGGCCG
Trichoderma asperellum isolate ALT3	GGCCGGCTTTTCCTCTCTCTGCATGTGCTGTTACCGAACTGTCTGGCTCGGCGG GGTCCGCCCCGGGTGCGTCGCATCCCCGGAGCCAGGCGCCCGCCGGAGGAACCA ACCAAACTCTTTCTGTAACCCCTCGCGGACGTATTTCTTACAACTCTGAGCA AAAATTCAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCG ATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCGCAATTCAGTGAAT CATCTAATCTTTGAACGCACATTGCGCCCGCCAGGATTCTGGGGGGGATGCC TGTGCGAGCGTCATTTCACCCCTCAAACCCCTCCGG
Trichoderma asperellum isolate PAT 1	CAAAAGCACGTCCGTGAATCGAGGTCACAGTTAAGAAAGTTGGGTGTTTTACGGAC GTGGACGCGCCGCGCTCCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAGAGGCTG CGGCGAGACCGCCACTGTATTTCGGGGCCGGCACCCGTGTGAGGGGTCCCGATCCC CAACGCCGATCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCTCGGACAGGCATGC CCGCCAGAATACTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAAT TCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACC AAGAGATCCGTTGTTGAAAGTTTTGATTCATTTTGAATTTTTGCTCAGAGCTGTAA GAAATACGTCCGCGAGGGGACTACAGAAAGAGTTTGGTTGGTTCCTCCGGCGGGCG CCTGGTTCCGGGGATGCGACGCACCCGGGGCGTGACCCCGCCGAGGCAACAGTTTG GTAACGTCACATGGGGGAGGGGAAGCGAAAGCGGCCA
Trichoderma asperellum isolate PAT 6	GGGCTTCTCTTGCTGAACCTGCGGAAGGATCATTACCGAGTGCGGGCCTCGCGGCC CAACCTCCCACCCTTGTCTCTATACACCTGTTGCTTTGGCGGGCCCACCGGGGCCA CCTGGTCGCCGGGGGACGCACGTCCCCGGGCCCGCGCCCGCCGAAGCGCTCTGTGA ACCCTGATGAAGATGGGCTGTCTGAGTACTATGAAAATTGTCAAAACTTTCAACAA TGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGT GAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGC ATTCCGGGGGGCATGCCTGTCCGAGCGTCATTTCTGCCCTCAAGCACGGCTTGTGT GTTGGGTGTGGTCCCCCCGGGGACCTGCCCGAAAGGCAGCGGCGACGTCCGTCTGG TCCTCGAGCGTATGGGGCTCTGTCACTCGCTCGGGAAGGACCTGCGGGGGTTGGTC ACCACCATATTTTACCACGGTTGACCTCGGATCAGGTAGGAGTTACCCGCTGAACT TAAGCATATCAATAAGACGGAGGAAAGCATATCAGTGAGCGGAGGAACCTCCCCCC GGGTGATTCACCCTTGTGTATTG

Table 4.22 Sequence homology of *Trichoderma* isolate ALT 1 in BLASTn analysis

Sl. No.	Description	Maximum score	Query coverage (%)	E value	Identity (%)	Accession
1.	*Trichoderma asperellum* strain T77	926	94	0.0	99	GU176467.1
2.	*Trichoderma asperellum* strain T 29	926	94	0.0	99	GU176445.1
3.	*Trichoderma asperellum* strain T 8	926	92	0.0	99	GU176439.1
4.	*Trichoderma asperellum* strain T 5	950	95	0.0	99	GU176437.1

Table 4.23 Sequence homology of *Trichoderma* isolate ALT 3 in BLASTn analysis

Sl. No.	Description	Maximum score	Query coverage (%)	E value	Identity (%)	Accession
1.	*Trichoderma asperellum*	510	93	1e-140	94	KF765421.1
2.	*Trichoderma asperellum* MT02	508	94	4e-140	94	MG669099.1
3.	*Trichoderma asperellum* FZ 20	508	94	4e-140	94	KX066063.1
4.	*Trichoderma aspeellum* isolate TR1 52	508	95	4e-140	94	KX533977.1

Table 4.24 Sequence homology of *Trichoderma* isolate PAT 1 in

BLASTn   analysis

Sl. No.	Description	Maximum score	Query coverage (%)	E value	Identity (%)	Accession
1.	*Trichoderma asperellum* isolate CTCCSJ-A-GS20565	902	92	0.0	99	MF383124.1
2.	*Trichoderma asperellum* strain IIPRT-as 10	902	92	0.0	99	KX681728.1
3.	*Trichoderma asperellum* strain IIPRT-as 4	902	92	0.0	99	KX681713.1
4.	*Trichoderma asperellum* isolate TGD- 1	902	92	0.0	99	KX538809.1

Table 4.25 Sequence homology of *Trichoderma* isolate PAT 6 in

BLASTn analysis

Sl. No.	Description	Maximum score	Query coverage (%)	E value	Identity (%)	Accession
1.	*Trichoderma asperellum* isolate TV14	508	94	4e-140	94	JN104494.1
2.	*Trichoderma asperellum* isolate TV6	508	93	4e-140	94	JN104486.1
3.	*Trichoderma asperellum* isolate TV 8	508	94	4e-140	94	JX422014.1
4.	*Trichoderma asperellum* isolate T 19	507	91	1e-139	95	MF661945.1

4.6.2. Sequence comparison of ALT 3 isolate

Comparison of nucleotide sequence of the isolate ALT 3 (Table 4.23) with other sequences in NCBI showed 93 per cent query coverage and 94 per cent identity to *T. asperellum* (Accession KF765421.1). The result of sequence analysis is depicted in Table 4.20. Nucleotide sequences of ITS of the isolate ALT 3 was compared with other sequences and the search showed that the isolate showed 94 per cent identity and 94, 94 and 95 per cent query coverage to *T. asperellum* MT02 (Accession MG669099.1), *T. asperellum* FZ 20 (Accession KX066063.1) and *T. asperellum* isolate TR1 52 (Accession KX533977.1) respectively.

4.6.3 Sequence comparison of PAT 1 isolate

Sequence of the isolate PAT 1 (Table 4.24) when compared with nucleotide database retrieved from NCBI revealed that it showed 92 per cent query coverage and 99 per cent identity to *T. asperellum* isolate CTCCSJ-A-GS20565 (Accession MF383124.1). Details of sequence analysis are presented in Table 4.21. Comparison of nucleotide sequence of the isolate PAT1 with other sequences in NCBI with 92 per cent query coverage and 99 per cent identity was observed with *T. asperellum* strain IIPRT-as 10 (Accession KX681728.1), *T. asperellum* strain IIPRT-as 4 (Accession KX681713.1) and *T. asperellum* isolate TGD- 1 (Accession KX538809.1).

4.6.4 Sequence comparison of PAT 6 isolate

Sequence of the isolate PAT 6 when compared with nucleotide database retrieved from NCBI revealed that it showed 94 per cent query coverage and 94 per cent identity to *T. asperellum* isolate TV14 (Accession JN104494.1). Comparison of nucleotide sequence of the isolate PAT 6 with other sequences in NCBI with 93 per cent query coverage and 94 per cent identity to *T. asperellum*

isolate TV6 (Accession JN104486.1). Nucleotide sequence of ITS of the isolate PAT 6 was compared and verified with database retrieved from NCBI showed 94 per cent query coverage and 94 per cent identity to *T. asperellum* isolate TV 8 (Accession JX422014.1) (Table 4.25) and it also showed homology to *T. asperellum* isolate T 19 (Accession MF661945.1) with 91 per cent query coverage and 95 per cent identity.

After the molecular characterization of the four isolates of *Trichoderma* spp., they were identified as *T. asperellum* isolate ALT 1, *T. asperellum* isolate ALT 3, *T. asperellum* isolate PAT 1 and *T. asperellum* isolate PAT 6 as they were showing sequence homology towards these isolates. They were used for the assessment of growth promotion and biocontrol efficacy against *R. solani* under *in vivo* conditions.

4.7 PHYLOGENETIC ANALYSIS

A neighbor joining phylogenetic tree was constructed upon alignment of nucleotide sequences of four isolates generated from the study and 16 other sequences coding for ITS sequence of *T. asperellum* reported from India and elsewhere available in the Genbank. The phylogenetic tree consists of two groups *i.e.,* group 1 and group 2. Group 1 is further divided into two subgroups *viz.,* subgroup 1A and subgroup 1B (Fig. 4.1). Two of the isolates under present study *i.e.* ALT 3 and PAT 6 from Alappuzha and Palakkad respectively are clustered together with the isolates reported from other locations in India. This clustering is supported by a bootstrap value of 99. However, both ALT 3 and PAT 6 form a separate cluster within the subgroup and are closely related to each other. It shows little variation from other isolates in the subgroup *viz., T. asperellum* strain JSB 481 (Accession No. KC569358.1, Thrissur) *T. asperellum* strain KU-KP/RK/EF-17 (Accession No. MG203930, Thiruvananthapuram) *T. asperellum* strain MC 1(Accession No. KX712093, Thalassery), *T. asperellum* isolate NCIPMCAU-21B (Accession No.KU933478, New Delhi) and *T. asperellum*

Fig. 4.1 Phylogenetic analysis of *T. asperellum* isolates
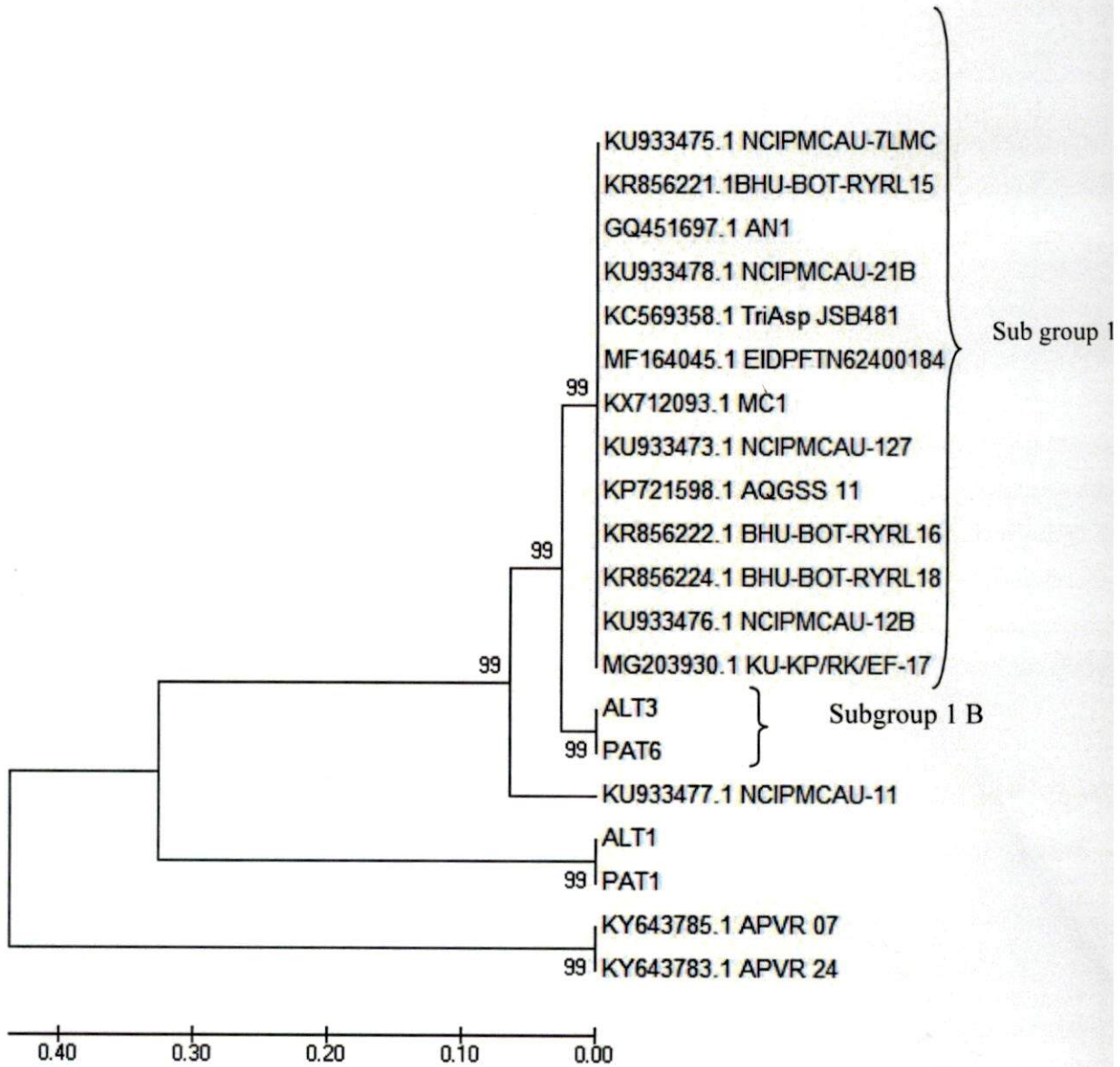
strain EIDPFTN62400184 (Accession No.MF164045, Tamil Nadu). In contrast to this, isolates ALT1 and PAT1 are clustered separately in a different subgroup. They show a considerable variation from isolates ALT 3 and PAT6 and all the other isolates from different locations of India.

4.8 *In vivo* EVALUATION OF THE PROMISING STRESS TOLERANT STRAINS FOR BIOCONTROL EFFICACY AND GROWTH PROMOTION

A pot culture experiment was laid out to test the biocontrol efficacy against *Rhizoctonia solani* and growth promotion of the promising stress tolerant isolates of *Trichoderma* spp. which showed better enzyme activity and antagonistic ability. Four isolates *viz., Trichoderma asperellum* (ALT1, ALT3, PAT1, PAT6) were selected for the *in vivo* experiment against the pathogen (Plate 4.12). These were compared with a positive control, (*Trichoderma viride*) the reference culture of KAU and a negative control, without the application of *Trichoderma* spp. The experiment was carried out during March 2018 at College of Horticulture, Vellanikkara. Observation on germination percentage, disease incidence and biometric characters were recorded at regular intervals.

4.8.1 Per cent seed germination

During this experiment, the pathogen *R. solani* was mass multiplied on rice grains (var. Uma) which was challenge inoculated in the pots used for the study. Cowpea seeds (var. Bhagyalakshmi) were treated with four selected *Trichoderma* isolates and *T. viride* (positive control) and one treatment was kept as negative control (without *Trichoderma*). Cent per cent seed germination was recorded when T 4 (*T. asperellum* isolate PAT 6) and T 5 (*Trichoderma viride*, KAU isolate) were applied in the pathogen inoculated pots followed by 80 per cent germination noticed with T2 (*T. asperellum* isolate ALT 3). Seed germination percentage of 60 per cent was only recorded with *T. asperellum* strain ALT 1 and *T. asperellum* isolate PAT 1 which was similar to the control pots (Table 4.26).

Thus, it can be inferred that T 4 (*T. asperellum* isolate PAT 6) and T 5 (*T. viride, KAU* isolate) were the best performing isolates that resulted in 100 percent germination of seeds in pathogen inoculated pots.

4.8.2. Biometric observations

Observations on height of the plant, number of leaves, number of flowers, days to flowering, number of pods and yield were recorded and compared among the treatments.

4.8.2.a. Plant height

Height of the plants were measured prior to treatment application *i.e.,* 30 days after sowing (DAS) and thereafter, the relative per cent increase in height at 30- 45 DAS and 45- 60 DAS was also recorded and the data is tabulated in Table 4.25. The height of plants at one week after sowing and 30 DAS were found to be non significant as the *Trichoderma* isolates were applied only after 30 DAS. However, there was a significant difference noticed in the relative per cent increment in height at 30- 45 DAS and 45- 60 DAS (Table 4.27). Maximum increase in height was observed in *T. asperellum* isolate PAT 6 (16.49%), followed by *T. asperellum* isolate ALT 3 (11.67 %) and *T. asperellum* isolate PAT 1 (10.86 %) at 30- 45 DAS, whereas, treatments *T. asperellum* isolate ALT 1 (8.16 %) and control plants (5.02%) showed a minimum relative per cent increase in height at the same time interval.

Table 4.26 Effect of treatments on per cent seed germination

Treatments	*Trichoderma* isolate	per cent seed germination (%)
T₁	*T. asperellum* isolate ALT 1	60
T₂	*T. asperellum* isolate ALT 3	80
T₃	*T. asperellum* isolate PAT 1	60
T₄	*T. asperellum* isolate PAT 6	100
T₅	*T. viride*	100
T₆	Control	60

Table 4.27 Effect of treatments on plant height at different intervals

Treatments	*Trichoderma* isolates	*Plant height (cm) Pretreatment (30 DAS*)	*Relative per cent increase in height 30- 45 DAS*	45- 60 DAS*
T₁	*T. asperellum* isolate ALT 1	154.26	8.16 (2.84)^c	12.39 (3.52)^b
T₂	*T. asperellum* isolate ALT 3	143.72	11.67 (3.41)^b	8.76 (2.93)^d
T₃	*T. asperellum* isolate PAT 1	161.00	10.86 (3.26)^b	8.78 (2.95)^d
T₄	*T. asperellum* isolate PAT 6	141.11	16.49 (4.03)^a	14.38 (3.78)^a
T₅	*T. viride*	149.50	10.58 (3.10)^{bc}	9.68 (3.09)^c
T₆	Control	166.33	5.02 (2.19)^d	4.64 (2.11)^e
CD (0.05)		NS	0.550	0.335

*Mean of nine replications
In each column figure followed by same letter do not differ significantly according to DMRT
√x+0.5 transformed values are given in parentheses.

At 45 to 60 DAS, *T. asperellum* isolate PAT 6 and *T. asperellum* isolate ALT 1 showed maximum relative increase in height of 14.38 and 12.39 per cent respectively, which was followed by *T. viride*, *T. asperellum* isolate PAT 1 and *T. asperellum* isolate ALT 1which recorded a per cent increase in height of 9.68, 8.78 and 8.76 per cent respectively. Control plants where no *Trichoderma* isolates were inoculated recorded the minimum increase in height of 4.64 per cent at 45-60 DAS.

4.8.4.2.b. Number of leaves

Number of leaves was measured prior to treatment application *i.e.,* 30 days after sowing (DAS) and thereafter, the relative per cent increase in number of leaves at 30- 45 DAS and 45- 60 DAS was also recorded and the data is tabulated in Table 4.28. It was observed that there was no significant difference between treatments at 1 WAS and 30 DAS. Nevertheless, there was significant difference between the number of leaves formed between treatments when observations were taken at 45 DAS and 60 DAS.

Number of leaves formed at 45 DAS ranged from 17.67 to 24.89 and the highest number of leaves of 24.89 was noticed in the plants when *T. asperellum* isolate PAT 6 was applied in the grow bags followed by *T. asperellum* isolate ALT 1 (23.67), *T. asperellum* isolate ALT 3 (23.44), *T. asperellum* isolate PAT 1 (23.00) and *T. viride* (22.33) at 45 DAS. Lowest number of leaves (17.67) was recorded in T6 (control), where *Trichoderma* was not inoculated.

Number of leaves produced by the plants treated with the six treatments at 45 to 60 DAS ranged from 25.44 to 34.11 and it was noticed that the maximum number of leaves was noticed in *T. asperellum* isolate PAT 6 (34.11) followed by *T. asperellum* isolate ALT 1 (31.89), *T. viride* (31.11), *T. asperellum* isolate ALT 3 (30.11) and *T. asperellum* isolate PAT 1 (29.67). Minimum number of leaves of 25.44 was recorded with T6 (control).

Table 4.28 Effect of treatments on relative per cent increase in number of leaves formed at different intervals

Sl. No.	*Trichoderma* isolate	*No. of leaves	*Relative increase in number of leaves	
		Pretreatment (30 DAS)	30- 45 DAS	45- 60 DAS
1.	*Trichoderma asperellum* isolate ALT 1	9.56	23.67(4.86)^{ab}	31.89(5.65)^{ab}
2.	*Trichoderma asperellum* isolate ALT 3	9.78	23.44(4.83)^{ab}	30.11(5.48)^{b}
3.	*derma asperellum* isolate PAT 1	10.56	23.00(4.78)^{ab}	29.67(5.43)^{b}
4.	*derma asperellum* isolate PAT 6	11.11	24.89(4.98)^{a}	34.11(5.83)^{a}
5.	*Trichoderma viride*	10.67	22.33(4.72)^{b}	31.11(5.57)^{b}
6.	Control	10.89	17.67(4.19)^{c}	25.44(5.03)^{c}
	CD (0.05)	NS	1.94	2.30

*Mean of nine replications

In each column figure followed by same letter do not differ significantly according to DMRT

√x+0.5 transformed values are given in parentheses.

4.8.4.2. c. *Days to first flowering*

Days taken to first flowering among plants in different treatments were recorded and there was significant difference noted. Days taken for first flowering ranged from 38.67 to 41.44. Earliest flowering was observed in *T. asperellum* isolate PAT 6 (38.67) and all other treatments were on par with each other. Maximum number of days to flowering of 41.44 was observed in control (Table 4.29).

4.8.4.2.d. *Yield*

Pods were formed after two months after planting (MAP) and the yield obtained after all pickings were compared among the treatments and the data is tabulated in Table 4.30. Yield from all six treatments varied from 52.39 to 83.39 g and the maximum yield of 83.39 g was noticed from *T. asperellum* isolate PAT 6 followed by *T. viride* (78.70 g), *T. asperellum* isolate PAT 1 (72.90 g), *T. asperellum* isolate ALT 1 (71.97 g) and *T. asperellum* isolate ALT 3 (63.95 g). Minimum yield of 52.39 g was obtained from T6 (control) where no isolate of *Trichoderma* spp. was applied. After the pot culture experiment, biometric observations such as relative per cent increase in number of leaves and plant height, days taken to first flowering and yield were recorded and *T. asperellum* isolatePAT 6 was found as the best performing isolate.

119

Table 4.29 Effect of treatments on days to flowering

Sl. No.	*Trichoderma* isolates	*Days to first flowering
1.	*T. asperellum* isolate ALT 1	40.33[a]
2.	*T. asperellum* isolate ALT 3	40.00[a]
3.	*T. asperellum* isolate PAT 1	40.00[a]
4.	*T. asperellum* isolate PAT 6	38.67[b]
5.	*T. viride*	41.00[a]
6.	Control	41.44[a]
CD (0.05)		1.241

*Mean of nine replications
In each column figure followed by same letter do not differ significantly according to DMRT.

Table 4.30 Effect of treatment on yield/ plant

Sl. No.	*Trichoderma* isolate	*Yield/ plant (g)
1.	*Trichoderma asperellum* isolate ALT 1	71.97[ab]
2.	*Trichoderma asperellum* isolate ALT 3	63.95[bc]
3.	*derma asperellum* isolate PAT 1	72.90[ab]
4.	*derma asperellum* isolate PAT 6	83.39[a]
5.	*Trichoderma viride*	78.70[a]
6.	Control	52.39[c]
CD (0.05)		12.88

*Mean of nine replications
In each column figure followed by same letter do not differ significantly according to DMRT

4.8.3. Per cent disease incidence

Mass multiplication of four isolates of *Trichoderma* spp. (ALT1, ALT3, PAT1, PAT3) and *T. asperellum* were carried out in rice seeds (var. Uma) and they were applied to the respective treatments 30 DAS. *Rhizoctonia solani* was also mass multiplied in the same manner and were then inoculated to all plants 10 days after inoculation of *Trichoderma* spp. Appearance of symptoms were observed and per cent disease incidence was taken after one week of pathogen inoculation (Plate 4.13).*Trichoderma asperellum* isolate ALT 3 and *Trichoderma asperellum* isolate PAT 6 showed the least disease incidence of 11.11 per cent when the pathogen *R. solani* was challenge inoculated in the pots (Fig. 4.2 and Plate 4.12). *Trichoderma asperellum* isolate ALT 1, *Trichoderma asperellum* isolate PAT 1 and the reference culture, *Trichoderma viride* showed disease incidence of 22.22 per cent. Cent per cent disease incidence was recorded in control plants where no isolate of *Trichoderma* spp. was inoculated. So it can be concluded that the isolates *Trichoderma asperellum* isolate ALT 3 and *Trichoderma asperellum* isolate PAT 6 were found as the best isolates with lesser per cent disease incidence.

Plate 4. 14 *In vivo* evaluation of various isolates of *Trichoderma* spp. for growth promotion and antagonistic ability
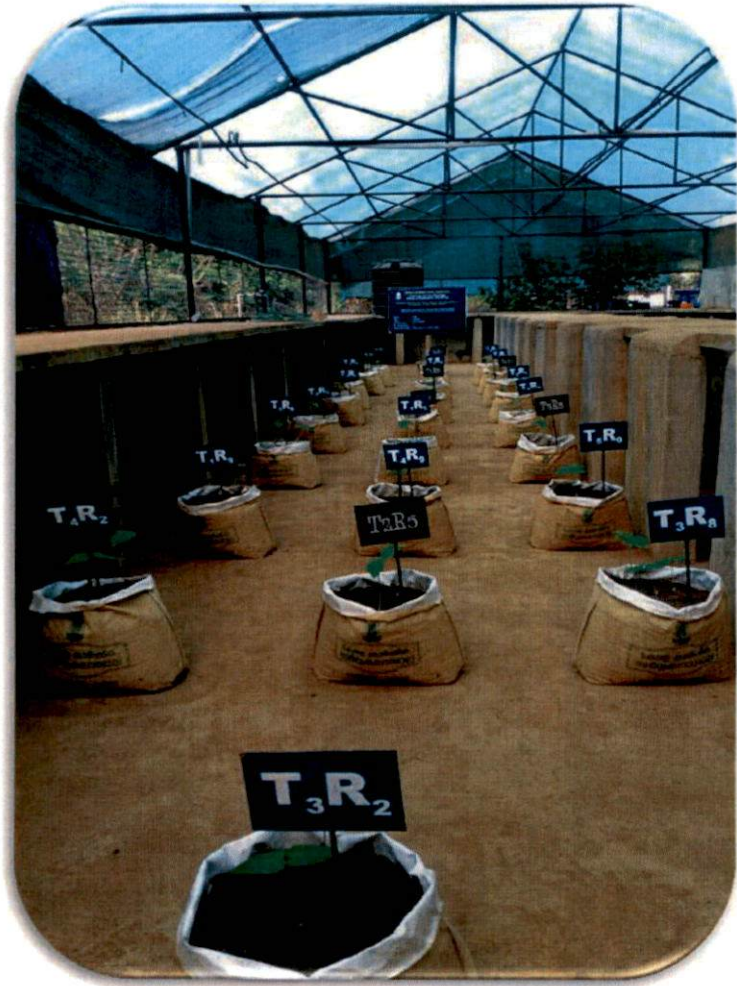
a) Experiment plot

b) Challenge inoculation with *R. solani*

c) Symptom appearence

164

Fig. 4.2 Effect of treatments on per cent disease incidence
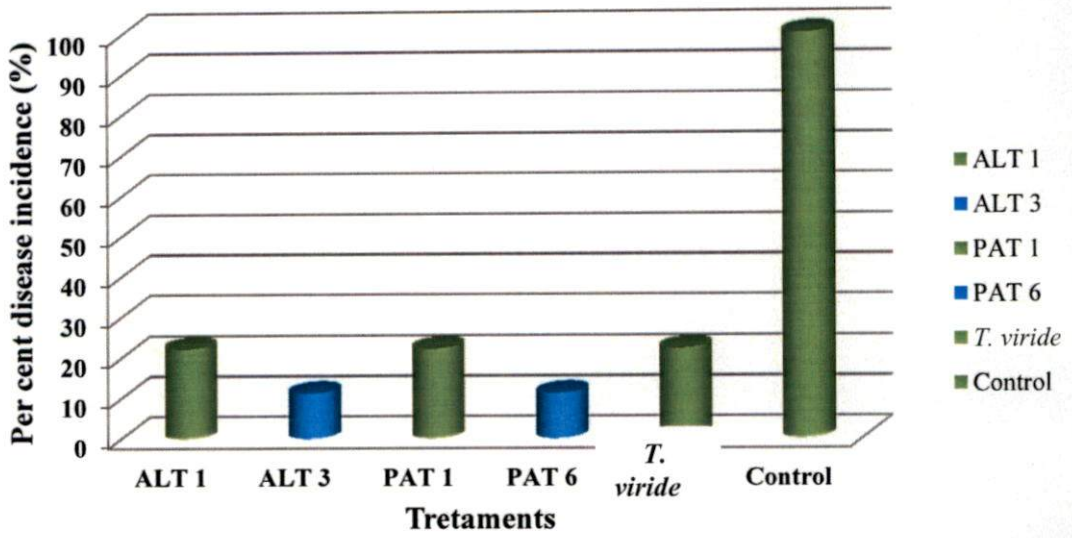
Discussion

5. DISCUSSION

The fungi belonging to the genus *Trichoderma* are one among the most exploited biocontrol agents in the field of agriculture for the management of crop diseases. *Trichoderma* species are imperfect filamentous fungi with a perfect stage/ teleomorph belonging to Order *Hypocreales* under Phylum *Ascomycota* Subphylum *Pezizomycotina*. The antagonistic properties of *Trichoderma* spp. are attributed to competition for nutrients (Elad, 2000), production of hydrolytic enzymes (Benitez *et al.,* 2004) and their ability to produce antibiotics (Vinale *et al.,* 2008). Moreover, *Trichoderma* species are known to promote plant growth and induce biotic and abiotic stress resistance in plants (Djonovic *et al.,* 2007). The genus *Trichoderma* is thus a rich source to explore potential bioagents for application across adverse climatic zones. However, in certain conditions, *Trichoderma* strains show poor inconsistent performance in fields which is attributed to their low osmotolerance and thermotolerance levels by varying environmental condition. Moreover, the formulations of *Trichoderma* spp. available in the market are only suitable for general conditions. Therefore, there is a need to identify potential strains of *Trichoderma* spp. which could sustain and survive the fluctuating temperature and which can tolerate salinity, acidity, pesticide and drought. Thus, identification of the isolates of *Trichoderma* spp., that are capable to survive such stress conditions and retain their biocontrol potential will be found very effective against pathogens that can survive such adverse soil conditions. Hence, this study is proposed to identify different isolates of *Trichoderma* spp. in Kerala showing resistance to these abiotic stresses.

5.1 SURVEY, COLLECTION AND ESTIMATION OF ELECTRO CHEMICAL PROPERTIES OF STRESSED ECOSYSTEMS OF KERALA

Intensive soil sample surveys were conducted across different stressed ecosystems of Kerala. A total of 52 soil samples were collected from the stress prone areas of Kerala *viz.,* Palakkad, Alappuzha, Ernakulam, Kumarakom,

Wayanad and Thrissur representing salinity, drought, temperature and acidity. Thereafter, soil reaction and conductivity of these samples were estimated prior to microbial enumeration. Soil properties such as pH and electrical conductivity (EC) influence the type of dominant microflora existing in that soil type (Domsch *et al.,* 1980; Gal- Hemed *et al.,* 2011; Poosapati *et al.,* 2014). This necessitates the need for estimating pH and EC of the soil so as to determine the optimum conditions for the growth and multiplication of the organism.

5.1.1. Soil reaction (pH)

pH of all 52 soil samples were estimated using standard procedures. pH of the soil samples collected from Palakkad district reflected a moderately to slightly acidic range of 5.20 to 6.20 (Fig 5.1). Likewise, Alappuzha showed a pH in the range of 2.80- 3.50 making them very strongly acidic. Vytilla and Kumarakom recorded strongly acidic pH values ranging from 4.10 - 4.30 and 3.90 - 4.70 respectively. Samples collected from Wayanad were noticed with acidic to neutral pH of 5.40- 7.10, and soil collected from *Kole* lands of Thrissur were strongly acidic to moderately acidic with pH values ranging from 4.30 to 5.40. Similar reports on pH of soils were elucidated by Bastin *et al.* (2014) where they reported a pH of 5.58 to 8.58 from Palakkad, 3.91 to 7.50 from Alappuzha with a majority of soils in the acidic range, 5.0- 5.4 from Kumarakom, 5.27 to 6.98 from Wayanad and 3.50 to 7.40 from Thrissur. Mohan and Sreelatha (2016) also observed soils from Vytilla with a pH range of 3.9 to 4.26, which also in line with the present study. Similar results were reported by Koruth *etal.* (2014) and Kavitha and Sujatha (2015).

5.1.2. Electrical conductivity (EC)

Electrical conductivity of soil samples collected from Palakkad, Alappuzha, Ernakulam, Kumarakom, Wayanad and Thrissur was estimated under laboratory conditions (Fig. 5.2).

Fig. 5.1. pH of soil samples
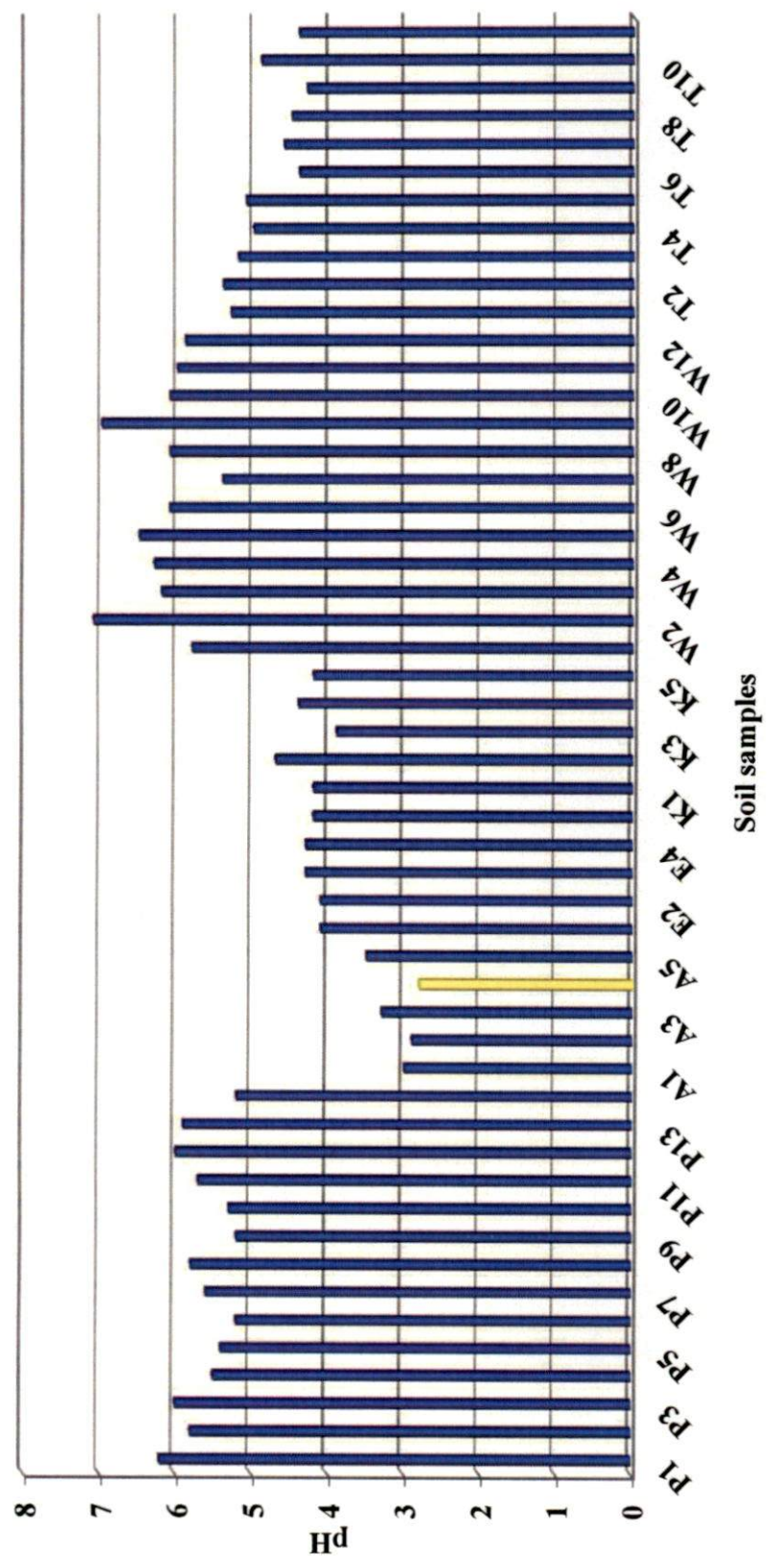
Fig. 5.2. Electrical conductivity of soil samples
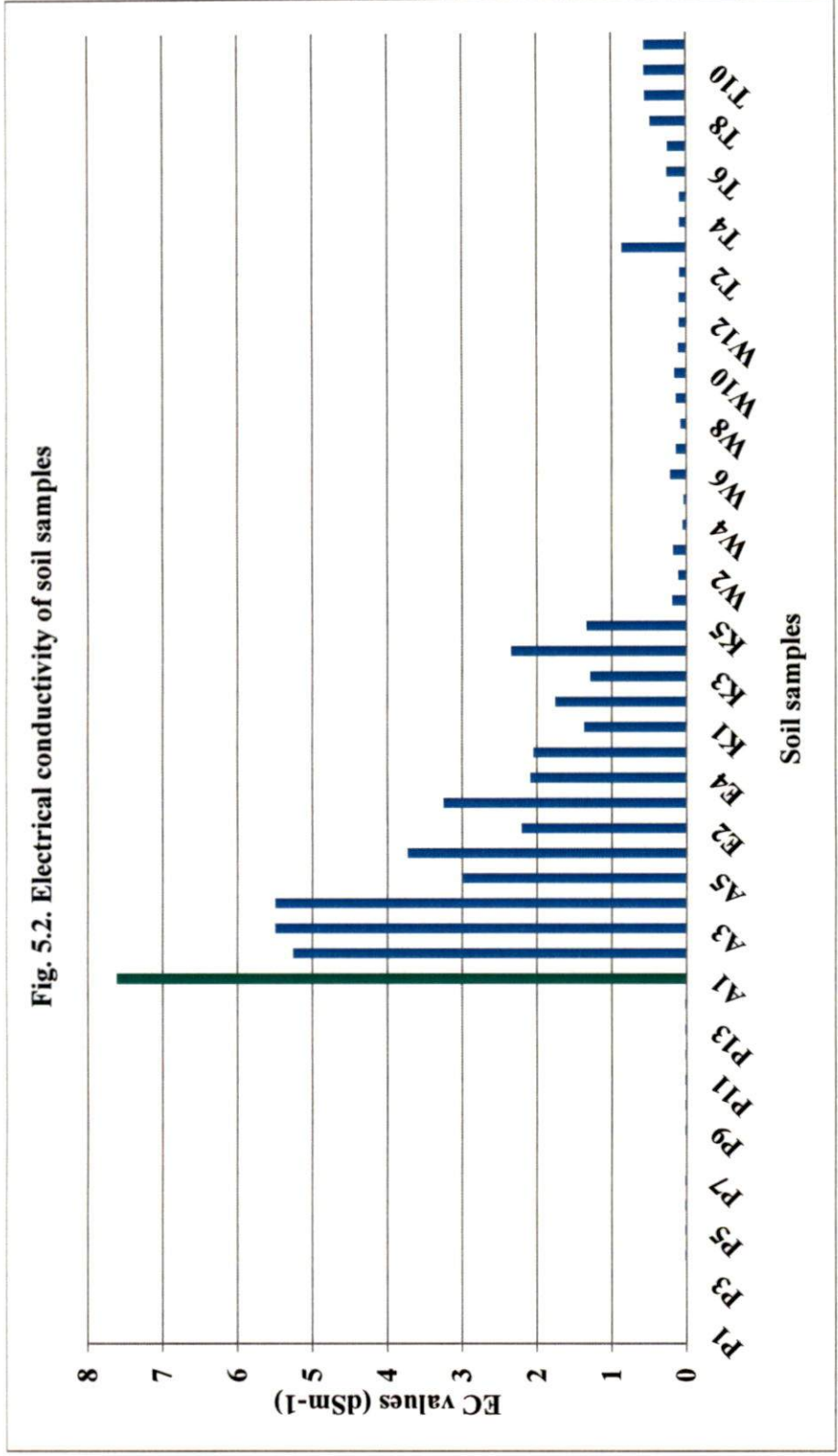
Electrical conductivity of soil samples collected from Palakkad district were non- saline with an EC ranging from 0.009 dSm^{-1} to 0.24 dSm^{-1}, while, soil samples from Alappuzha district recorded high EC value ranging between 3.00 to 7.61 dSm^{-1} rendering them slightly saline. Moreover, samples from Vytilla and Kumarakom also depicted an EC ranging from 2.05- 3.73 dS m^{-1} and 1.34- 2.35 dS m^{1} respectively which makes them very slightly saline. Soil samples collected from Wayanad district were observed with an EC ranging from 0.04 dSm^{-1} to 0.22 dSm^{-1} making them non saline. Likewise, soil samples collected from *Kole* lands revealed an EC ranging from 0.088 to 0.562 dSm^{-1}, hence considered as non saline. Similar reports were put forward by Bastin *et al.* (2014) where they recorded an EC value ranging from 0.03- 0.85 dS m^{-1} from Palakkad district and a mean EC value of 0.16 dS m^{-1} recorded from Alappuzha district. They also recorded an EC value of 0.31 dS m^{-1} from Wayanad soil samples and a mean EC value of 0.06 from Thrissur district which is also comparable with the present study.

In the present study, electrical conductivity of the soil samples collected from Vytilla of Ernakulam district reflected saline soil type and this is in agreement with the findings of Mohan and Sreelatha (2016) where the hike in EC value was noticed during mound preparation stage of rice crop.

5.2 ISOLATION AND ENUMERATION OF *Trichoderma* spp.

Native *Trichoderma* spp. was isolated from the soil samples procured from different locations by adopting serial dilution plating technique. Selective media such as *Trichoderma* Selective Media (TSM), Malt Extract Agar (MEA), Oatmeal Extract Agar (OEA), Special Nutrient Agar (SNA) and Potato Dextrose Agar (PDA) were used for the isolation and enumeration (Fig. 5.3). Elad *et al.* (1981) employed *Trichoderma* selective media for the isolation of *Trichoderma* spp.Likewise, Mustafa *et al.* (2009) also observed growth and sporulation of *Trichoderma* spp. on media such as Oatmeal Extract Agar, Potato Dextrose Agar,

and Special Nutrient Media. Similarly, Narayanan *et al.* (2006) made cultural and morphological observation of *Trichoderma* cultures grown on PDA, OMA, SNA and TSM.

A total of 24 *Trichoderma* species were obtained from all six locations selected under the current study. Number of *Trichoderma* isolates obtained from Wayanad, Palakkad, Thrissur, Vytilla, Alappuzha and Kumarakom were 8, 6, 4, 2, 3 and 1 respectively (Fig. 5.4). Among all the locations, Wayanad recorded the maximum number of eight isolates which may be due to the presence of abundant soil microflora in the forest soils of this district. This is in consistent with the results obtained by Kendrick and Burges (1962), Hayes (1965), Macauley and Thrower (1966), Danielson and Davey (1973), Okoth *et al.* (2007) and Rahman *et al.* (2011) where they confirmed that the propagules of *Trichoderma* spp. were found more in forest soils. They reported that *Trichoderma* spp. are secondary colonizers of a wide range of forest litter and are found most abundantly in them. Likewise, Chakraborty *et al.* (2010) obtained 19 isolates of *Trichoderma* spp. which included forest soils of north Bengal region. There are several reports of *Trichoderma* species being isolated from stress prone areas where as Rahman *et al.* (2011) conducted studies on the isolation of *Trichoderma* spp. from bio soild wastes. Likewise, El Kharbotly *et al.* (2016) obtained *Trichoderma* spp. from various parts of Qatar and Vithya (2017) isolated *Trichoderma* spp. from different stressed ecosystems of Thrissur district.

In the present study, *Trichoderma* species were found in almost all locations indicating the abundance of the population and they could be exploited as bioagents for the control of many plant pathogens. After enumeration of microflora, highest population of 32.67 x 10^3 cfu g^{-1} of *Trichoderma* spp. was observed in Tholpetty-1 (W7) of Wayanad district during the study. This is in congruence with the study by Rahman *et al.* (2011) where he observed a highest population of 36.67± 3.66 x 10^3 CFU g^{-1} during the isolation of the fungi from biosolid waste. However, they observed the lowest population of 13.23 ± 0.87 × 10^3 CFU g^{-1}, which was much higher when compared to the present study.

Fig. 5.3. Enumeration of *Trichoderma* spp. from soil
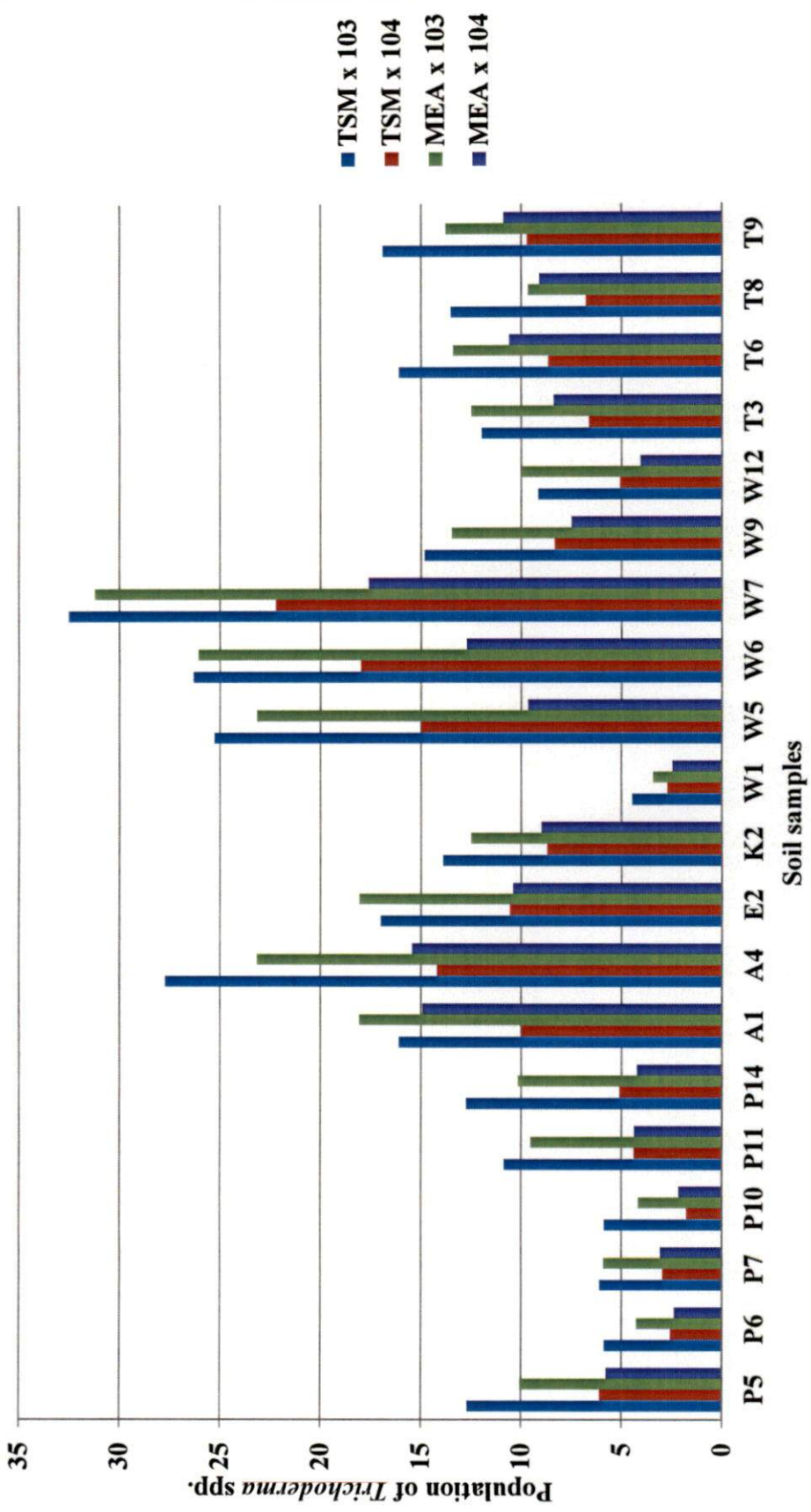
Fig. 5.4. Number of *Trichoderma* isolates
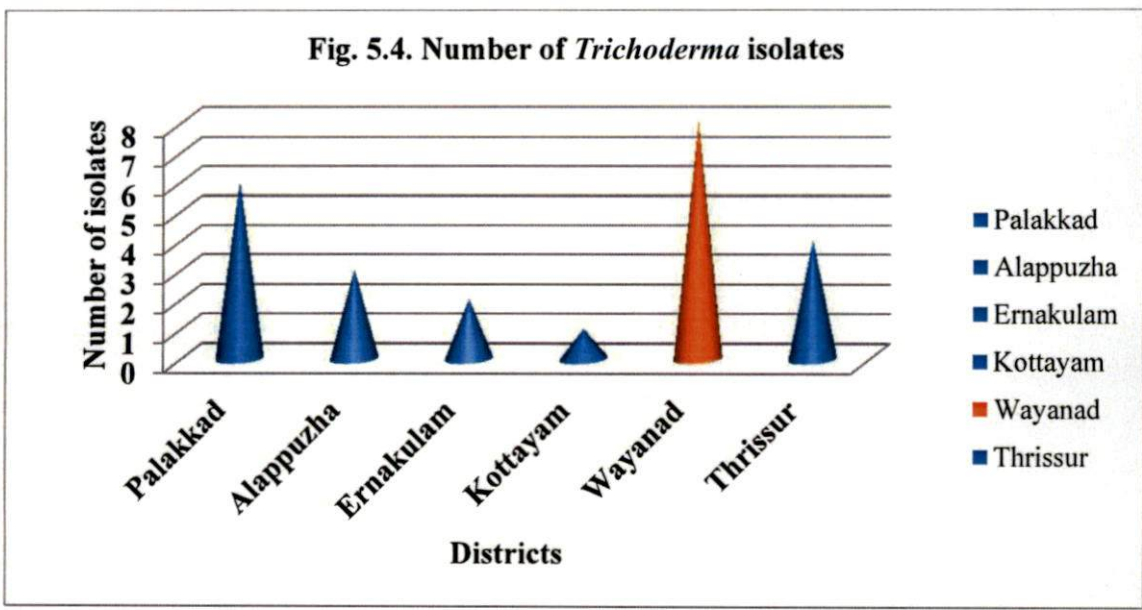
Similarly, population of *Trichoderma* spp. from Alappuzha soils in TSM medium ranged from 16.11 to 27.33 x 10^3 cfu g^{-1} and 10 to 14.20 x 10^4 cfu g^{-1} at 10^{-3} and 10^{-4} dilutions respectively. Soil sample E2 from Vytilla of Ernakulam district was only noticed with a population of 17.00 x 10^3 and 10.56 x 10^4 cfu g^{-1} in TSM medium.

Out of all locations, the lowest population of 2.17 x 10^4 cfu g^{-1} was recorded from soil sample P 10 from Palakkad district. Similar number of colonies (1.0 x 10^2 cfug^{-1}) was recorded by Rai (2017) when *Trichoderma* spp. was isolated from soil samples collected from different locations of Uttaranchal using TSM.

Likewise in the present study, population of *Trichoderma* spp. from *Kole* lands of Thrissur district ranged from 11.98 - 16.90 x 10^3 cfu g^{-1} and 6.62 - 9.72 x 10^4 cfu g^{-1} by employing TSM medium. Similar population of 12.5 x 10^3 cfu g^{-1} was obtained during the study conducted by Vithya (2017) from Panenchery area of Thrissur district.

5.2.1. Cultural and morphological characterization of *Trichoderma* isolates

Cultural and morphological characterization of *Trichoderma* spp. isolated from different locations was carried out *in vitro*. The results obtained were compared with existing reports and are discussed below.

Based on the number of *Trichoderma* spp. obtained from each district, they were serially numbered and abbreviated according to the name of the location. Accordingly, PAT 1 to PAT 6 represents number of isolates of *Trichoderma* spp. from Palakkad district, ALT 1 to ALT 3 from Alappuzha, VYT 1 and VYT 2 from Ernakulam district, KUT 1 alone from Kottayam district, WAT 1 to WAT 8 from Wayanad and THT 1 to THT 4 from Thrissur district.

Isolates of *Trichoderma* spp. PAT 1, PAT 5, PAT 6, ALT3 and VYT1 showed cream coloured sparse mycelial growth and the colony later turned into

dark green colour due to profuse sporulation. The growth was completed within four days of inoculation at room temperature. The reverse side of the plate appeared cream or buff coloured. Studies on the cultural and morphological characters of *Trichoderma* isolates were carried out by Sekhar *et al.* (2017) is in agreement with the above results. They observed that the culture of *Trichoderma viride* showed dark green to dark- bluish green sporulation with amber colour on the reverse side. Conidiophores were found to be long, less branched and with verticillate conidiophores. Phialides appeared paired, lageniform, converged or divergent.

Likewise, WAT 7 isolate showed light cream mycelium initially which turned into uniform green on sporulation with yellow patches after an incubation of four days at room temperature. Conidia appeared round to ovoid which were seen attached to ellipsoid phialides (7.74 µm). Sutton *et al.* (1998) observed similar colonies of *Trichoderma* which grows and matures quickly with an incubation period of five days on potato dextrose agar at 25°C where the colony is wooly and compact. According to them, the colony colour is initially white and as the conidia were formed, widely dispersed blue-green or yellow-green patches became visible in the culture which may form concentric rings in some cases. Similar colonies of *Trichoderma* spp. with yellow patches were reported by Sharma and Singh (2014).

ALT1 and WAT 4 showed cream coloured sparse mycelial growth initially which after five days of incubation formed undulating mycelia with green spore mass distributed throughout the media. Size of conidia of ALT1 and WAT 4 were 2.86 µm and 2.36 µm and the size of phialides were 8.261 µm and 7.674 µm. Sekhar *et al.* (2017) observed similar colonies with dull green to bluish green sporulation with a pale yellow to colourless on the reverse side which was noticed in the culture plate of *Trichoderma koningii* fungal culture. Conidiophores were broad to narrow and appeared in whorls with frequent branching. Phialides took a lageniform or ampulliform shape with the terminal phialide more elongated.

Likewise, Shah *et al.* (2012) also observed *Trichoderma* colonies with green conidia distributed throughout the media.

In ALT 2 isolate, the growth started as white fluffy mycelium and was later turned into pale green growth because of sporulation. Conidia were distributed as compact mass and limited aerial mycelium was seen in the Petri plate. Similar observations were reported by Bisset (1991) and Druzhinina and Kubicek(2005). Reverse side of the plate had an amber colour. Hyphae were hyaline and with septation. Conidia globose to ovoid with a size of 3.302 µm and were attached to the tip of elongated cylindrical phialides (13.23 µm). The above findings are comparable with the observation of Sekhar *et al.* (2017), where they with minute tufts in the colony of *T. reesei* with pale yellowish colour. They also observed pale yellow colour on the reverse side of the Petri plate. Conidiophores were verticillate with rare branching habit. Cylindrical or moderately inflated and divergent phialides were observed under microscope.

Trichoderma isolate PAT2 started its growth as white feeble mycelia which turned green due to sporulation. Concentric rings of spores were visible in the media and the fungus appeared amber coloured on the reverse side of the Petri plate. Hyphae were hyaline and septate, conidia of 2.77µm round dark green colour and arranged at the tip of penicillate phialides of length 15.73µm. Savitha and Sriram (2015) reported similar characteristics in *T. asperellum* with dark green sub globose to ovoidal conidia with a size of 3.33 µm with phialides (11.17 µm) arranged in whorls.

In the present study, isolates of *Trichoderma* spp. *viz.,* PAT3, KUT 1, WAT 6, THT2, THT3 and THT4 showed a fluffy white mycelium initially which later turned green due to the formation of conidia in two concentric rings. Sharma and Singh (2014) observed similar concentric growth pattern in *Trichoderma* spp. isolated from different locations of Uttarakhand. They possessed conidia of size ranging from 1.98 to 2.64 µm and were attached to the tip of elongated phialides of length ranging from 6.75 to 11.61 µm. Also a similar study was carried out by

Sekhar *et al.* (2017) where they noticed *Trichoderma harzianum* with dark green areas intermingled with white mycelium. They noticed dull yellowish colour on the reverse side of the culture and conidiophores appeared in whorls with numerous branching and the phialides were lageniform and convergent. Conidia appeared subglobose to obovoid shape.

In PAT4 isolate, there was a sparse white mycelial growth initially which later covered the plate and light yellowish green conidia developed after fifth day of inoculation. Hyphae were hyaline, septate, conidia light green in colour arranged in cluster at the tip of penicillate phialides. Conidia had round shape appearing at the tip of phialides in clusters with a size of 2.25 μm and phialides (7.67 μm) were subglobose to ovoidal in shape. Similar yellowish green cultures growing rapidly at 25^0C were also noticed by several workers (Samuels *et al.*, 2002), Gams and Bissett (2002), Lin and Heitman (2005) and Sharma and Singh (2014). Sekhar *et al.* (2017) also observed similar colonies of *Trichoderma aureoviride* with complete dull green colour and discoloured reverse side. They noticed conidiophores arranged in pyramidal structures with more branches and the phialides were flasks shaped and divergent.

The culture WAT 2 initiated as white fluffy growth which after five days of incubation produced no or few spores. Reverse side of the culture appeared cream colour. Conidia appeared round to ovoid with a size of 2.95 μm which were attached to short, plump phialides (size- 10.31 μm) arranged in whorls. Similar pattern of growth was observed by Shah *et al.* (2012) where they noticed white mycelia with no conidial formation in *T. pseudokoningii*.

Isolates WAT 8, WAT 5, THT1 and VYT2 initiated the growth as white sparse mycelium and later dark green spores which covered the plate with less spores in the centre. Dark green spores were distributed evenly throughout the plate. All these characters are in accordance with those reported by Savitha and Sriram (2015) where they studied similar morphological characters and according to them, the characters resembled with *T. virens*.

5.3 *In vitro* SCREENING OF *Trichoderma* spp. FOR ABIOTIC STRESS TOLERANCE

The effects of temperature, drought, pH, fungicides and salinity on the growth of all 24 *Trichoderma* isolates were studied under *in vitro* conditions. Such stress tolerant strains can be utilized for biocontrol of plant diseases under adverse climatic conditions.

5.3.1 *In vitro* screening of *Trichoderma* spp. for high temperature tolerance

All 24 isolates were grown at different levels of temperature such as 25^{0}C, 30^{0}C, 35^{0}C and 40^{0}C to study the optimum temperature required for the growth of the fungus and to screen the isolates for thermotolerance (Fig. 5.5). At all tested temperature levels, there was a variation in growth and sporulation of *Trichoderma* spp. Generally, it was noticed that there was a reduction in growth and sporulation of the fungus with increase in temperature. This is in agreement with the study conducted by Widden and Hsu (1987), where they observed that the penetration and colonization of litter by *Trichoderma* spp. decreased with increase in temperature. Santos and Linardi (2004) reported that the common incubation temperature for the growth of fungi including *Trichoderma* spp. was found to be 30^{0}C.

At 25^{0}C, mycelia produced by the isolates ranged from 0.003 to 0.44 g and maximum mycelial weight of 0.44g was noticed with the isolate PAT6 followed by ALT1 (0.34 g), whereas minimum of 0.003 g was recorded with PAT3. Meanwhile, when the temperature was increased to 30^{0}C, all the isolates showed an increase in mycelial biomass production which ranged from 0.02 to 0.78 g. Further at 35^{0}C, mycelial weight produced by the isolates showed a general reduction and it ranged from 0.002 to 0.33 g where, maximum mycelial biomass was produced by the isolate PAT 6 (0.33 g) followed by ALT 1 (0.25 g), while the minimum of 0.002 g was noticed with PAT 2 and PAT 3. Finally, at 40^{0}C, only two isolates PAT 6 and WAT 2 showed satisfactory growth and sporulation when

compared to other isolates.

This is in congruence with the study conducted by Eastburn and Butler (1991) where they evaluated the effect of soil temperature on the growth and development of *Trichoderma* species. According to them, optimum temperature required for the growth of *T. harzianum* isolate 1059 was recorded between 27^0C and 30^0C. There was no substantial growth reported beyond 37^0C. Host root colonization by the antagonist was also found maximum at temperature range of 30 - 33^0C. There are several other studies which reveal that majority of the naturally existing ascomycetes fungi show optimum growth at 30 to 35^0C which was also comparable with the present study (Begoude *et al.*, 2007; Moustafa and Abdel-Azeem, 2008). Amalraj *et al.* (2010) assessed the thermotolerance of *Trichoderma viride* under *in vitro* conditions and came to similar conclusions that there is a significant reduction in the population of the fungus when temperature was raised from 30^0C to 45^0C and it was further declined when the temperature was increased to 50^0C. The results of the present study is also in line with the findings of Poosapati *et al.* (2014), where they could prove that while increasing temperature to 37^0C, germination rates of all isolates with an exception to *T. asperellum* TaDOR673 was reduced greatly. In addition, they noticed that when the cultures were exposed to extreme temperature such as 52^0C for 4 h, only *T. asperellum* TaDOR673 was able to tolerate this temperature. In another study by Singh*et al.* (2014), they found that the most suited temperature for growth and sporulation of *T. harzianum* was found to be 30 ^{0}C followed by 25^0C, and this is in congruence with the present study. Similarly, Maurya *et al.* (2017) reported that the growth of *T. viride* continues upto 30^0C and thereafter it decreases. Singh (2016) also identified superior thermotolerant strains of *T. asperellum* Tvb1 which showed growth even at 45^0C. This showed that certain isolates of *Trichoderma* spp. have the ability to tolerate high temperature. Vithya (2017) reported that highest mycelial weight of *Trichoderma* spp. was observed at 37^0C.

Kredics *et al.* (2003) reported that, the bioagent *Trichoderma* species have

the ability to produce extracellular enzymes which are responsible for the antagonistic property of the fungus which provides scope for improving the strains for abiotic stress tolerance. According to Ruijter *et al.* (2003), thermotolerance of *Trichoderma asperellum* TaDOR673 may be attributed to accumulation of stress protectants such as trehalose, mannose and raffinose under unfavourable conditions. The part played by trehalose in the survival of fungal cell structure and proteins have also been well explained by Fillinger *et al.* (2001) and Ruijter *et al.* (2003). Moretti *et al.* (2012) observed the thermostability of endo- glucanase and cellulase produced by *T. reesi* even upto 60^0C.

5.3.2 *In vitro* screening of *Trichoderma* spp. for drought tolerance

Mechanisms adopted by *Trichoderma* spp. for drought tolerance is very less explored, however those used by plant growth promoting rhizobacteria (PGPR) include nitrogen fixation, production of plant growth regulators (auxins, gibberellins and cytokinins), iron sequestration by siderophores, solubilization of phosphate minerals (Glick *et al.*, 1999). Moreover, they are involved in the catabolism of complex molecules such as 1-aminocyclopropane-1-carboxylate (ACC) by using enzymes such as ACC deaminase which results in reduction in ethylene concentration (Mayak *et al.*, 2004; Arshad *et al.*, 2008) which also assist in drought tolerance imparted by these micro organisms.

Hence, studies were further carried out for the screening of *Trichoderma* spp. which are able to tolerate drought condition and all the isolates were exposed to different osmotic potential such as -0.15 M Pa, -0.49 M Pa and -1.03 M Pa by amending the media with 10, 20 and 30 percent polyethylene glycol (PEG). It was observed that, mycelial biomass formed by the fungus reduced when the concentration of PEG was increased from 10 to 30 per cent (Fig. 5.6). At 10 per cent PEG concentration, highest mycelial weight was recorded with WAT2 (2.61 g) followed by ALT3 (1.61 g), while the minimum biomass was recorded in the isolate PAT 3 (0.43 g). When PEG concentration was further increased to 20 per

Fig. 5.5 Effect of temperature on growth of isolates

Fig. 5.6 Effect of drought on growth of isolates

cent, the isolate WAT2 produced the maximum biomass of 1.94 g followed by VYT2 (1.40 g) and the minimum of 0.33 g was produced by PAT3. Thereafter, at 40 per cent PEG, highest mycelial weight was recorded with the isolates VYT2 and ALT 1 which were considered as drought tolerant.

Parallel results were obtained for a range of soil inhabiting fungi including *Trichoderma* spp. by Magan and Whipps (1988). They noticed that optimum growth of the fungi occurred between -0.1 to -0.7 M Pa on PDA media. Jackson *et al.* (1991) also made similar findings regarding the growth of *Trichoderma* spp. at various water potential. They observed that as water potential decreases, the hyphal growth of the fungus decreased and minimum growth of *Trichoderma* isolates were noticed under -7 M Pa to -14 M Pa.

In the present study, a gradual reduction in the mycelial weight of the fungus with increase in PEG concentration was observed. This is in accordance with the experiment conducted by Eastburn and Butler (1991). They found that activity of *T. harzianum* was found to be higher at -0.05 to 0.1 M Pa. Moreover, colonization of soil by *Trichoderma* spp. occurred maximum when the osmotic potential was at -0.05 M Pa and it decreased considerably when the osmotic potential was increased to -1.50 M Pa.

Several studies demonstrate that bioagents such as *Trichoderma* species will impart drought tolerance in plants through colonization of the root surface. Bae *et al.* (2009) described that *T. hamatum* increased tolerance to drought by increasing root growth thereby increasing water uptake. Recent evidences also suggest that higher root and shoot growth in tomato plants inoculated with *T. harzianum* exhibited higher proline and total soluble protein at normal as well as drought conditions (Mona *et al.*, 2017). In this study, they also noticed an obvious increase in flavonoid content, phenol, growth regulators such as indole acetic acid, indole butyricacid and gibberillic acid were also found higher when the plants were inoculated with *Trichoderma* spp. under drought stress. According to them,

183

these secondary metabolites play an important role in plant stress tolerance when inoculated with *T. harzianum* by protecting membranes from reactive oxygen species (ROS) and enhancing plant growth through accessing more nutrients by root system.

5.3.3. *In vitro* screening of *Trichoderma* spp. for acidity tolerance

An attempt was made to study the tolerance of *Trichoderma* spp. to varying levels of pH and select the best suitable isolate which can withstand acidity. Thus, *Trichoderma* species isolated from different stressed ecosystems were screened to study their growth at different pH levels such as 3.5, 4.5 and 5.5. A significant difference in the mycelial biomass was noticed in the isolates and it was observed that as the pH of the culture media increased, an increase in the mycelial biomass was noticed (Fig. 5.7). At pH 3.5, the maximum biomass of 0.90 g was produced by ALT3 followed by ALT1 (0.67g) and the minimum biomass of 0.001 g were produced by the isolates PAT2, PAT3 and PAT4. When the isolates were grown at pH 4.5, the maximum was recorded with the isolate WAT 3 (2.45 g) followed by PAT5 (1.98 g) and the minimum was observed in PAT3 (0.04 g) and THT1 (0.09 g). At pH 5.5, the maximum biomass was produced by WAT3 followed by PAT5 (2.48 g) and the minimum was recorded with THT1 (0.07 g). There was a general increase in the mycelial weight and sporulation of all isolates when the pH was increased from 3.5 to 5.5.

From the study, it was concluded that the isolates only ALT 1 and ALT 3 from Alappuzha showed the highest mycelial weight when grown at lowest pH of 3.5, whereas most of the other isolates showed good growth at pH 4.5 and 5.5 when compared to pH 3.5. This is in congruence with the study by Jackson *et al.* (1991) where they observed the effect of pH on the biomass production of *Trichoderma* species (*T. virens, T. pseudokoningii, T. viride*). They reported that maximum mycelial biomass was formed when the fungus was grown at pH of 4.6-6.8 and minimum was observed at two extreme pH values *viz.*, 2.5 and 7.4.

Similarly, Singh *et al.* (2014) noticed that none of the *Trichoderma* isolates employed for the study showed higher growth at a lower pH levels pH of 4.0.

A perusal through the literature revealed that there are also many other reports showing tolerance of *Trichoderma* spp. towards acidity. Przybylowicz and Donoghue (1988) confirmed that the optimum pH for the growth and development of *Trichoderma* spp. is between 4.5 and 5.5 in moist environment. Likewise, Bonilla (2006) observed the highest rate of development of *Trichoderma* colony on PDA medium at pH 5.24 and the lowest rate at pH 11.42. Romero (2007) also observed that *T. harzianum* exhibited highest development rate on PDA medium at pH 5.6, whereas minimum growth rate was observed at pH 10.8.

There are several studies which could prove the role of extracellular enzymes like cellulases, xylanases and glucanases involved in the mycoparasitism of pathogenic fungi by *Trichoderma* spp. Bailey *et al.* (1993) reported that production of cellulase enzyme usually occur at lower pH levels. Recently it has been observed by Li *et al.* (2013) that synthesis of endoglucanase enzyme by *T. reesei* was found to be highest at pH 4.5, whereas exoglucanase and β-glucanase production reached their highest values at pH 5 and 5.5, respectively which may be attributed to the survival and antagonistic ability of *Trichoderma* spp. at lower pH levels.

5.3.4. *In vitro* screening for salinity tolerance

It has been observed by Soliman *et al.* (1994) that at reduced water potential, microbes make certain changes in their physiological activity such as accumulation of specific intercellular substances as well as alteration in the biosynthetic pathways which serve as osmoprotectant. Khan *et al.* (2001) reported that at increased saline concentrations, sodium (Na) content of *Trichoderma* isolates increased, while Ca^{2+}, Mg^{2+} and K^+ concentrations decreased. Hence, an

attempt was made to study the ability of *Trichoderma* spp. which can tolerate salinity.

In the present study, all 24 isolates were screened for salinity stress under *in vitro* conditions at 0.5M, 1 M, 1.5 M and 2 M (Fig. 5.8). When the isolates were exposed to 0.5 M NaCl concentration, the per cent inhibition varied between 0 to 94.44 per cent and minimum per cent inhibition was noticed with the isolates such as PAT1, ALT1, ALT3, WAT3 and WAT8. Minimum per cent inhibition of zero was noticed at 1 M NaCl concentration with the isolate WAT3 and it was on par with PAT1 (1.85 %), WAT8 (1.85 %) and ALT3 (3.70 %). It was also found that *Trichoderma* isolates ALT 1 and ALT 3 possessed maximum ability to alleviate this condition as they were able to grow at 1.5 M NaCl concentration with 90 and 90.741 per cent inhibition respectively, whereas all other isolates showed 94.44 per cent inhibition of growth when grown under the same salt concentration. Finally at 2 M concentration, all isolates showed no growth with 94.44 per cent inhibition of growth. Results of the present study were comparable with the results putforth by Ghildiyal and Pandey (2008), Amalraj *et al.* (2010) and Rawat *et al.* (2013). However, contrary results were obtained by Kumar *et al.* (2016) where they observed that 92.8and 45.7 per cent of *Trichoderma* isolates were able to survive 5 and 10 per cent NaCl concentration respectively. Rawat *et al.* (2013) also observed a significant difference in the growth of *Trichoderma* isolates to various salt concentrations such as 4, 6, 9 and 10 dS m^{-1} and they noticed a general reduction in growth of the fungus with increase in EC. All isolates showed full growth (9 cm) at 4 dS m^{-1} after an incubation period of four days. However, there was a general reduction in the growth of *Trichoderma* spp. when the salt concentration was increased to 6 dS m^{-1} and 9 dS m^{-1}.

According to Mittler (2002), at high saline conditions, cellular accumulation of damaging superoxide radicals and their dismutation product, hydrogen peroxide (H_2O_2) may be formed and they can damage membrane lipids, proteins and nucleic acids. Rawat *et al.* (2013) reported that *Trichoderma* treated plants showed better membrane stability, lower H_2O_2 content and higher

Fig.5.7 Effect of pH on growth of isolates

Fig. 5.8 Effect of salinity on the growth of *Trichoderma* spp.
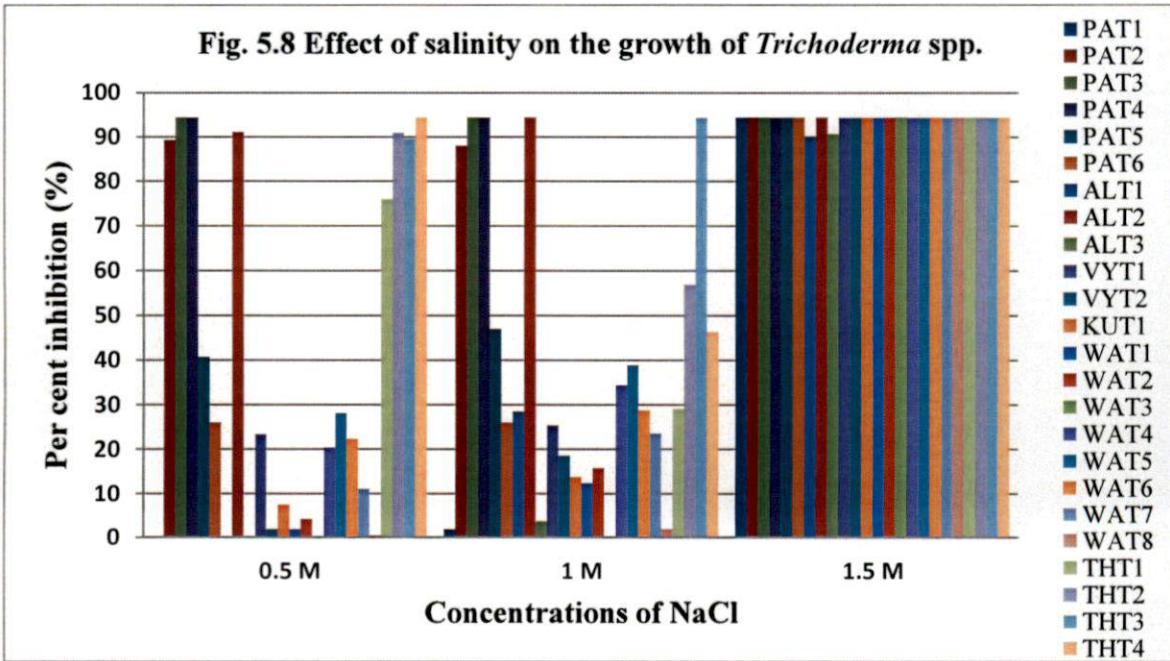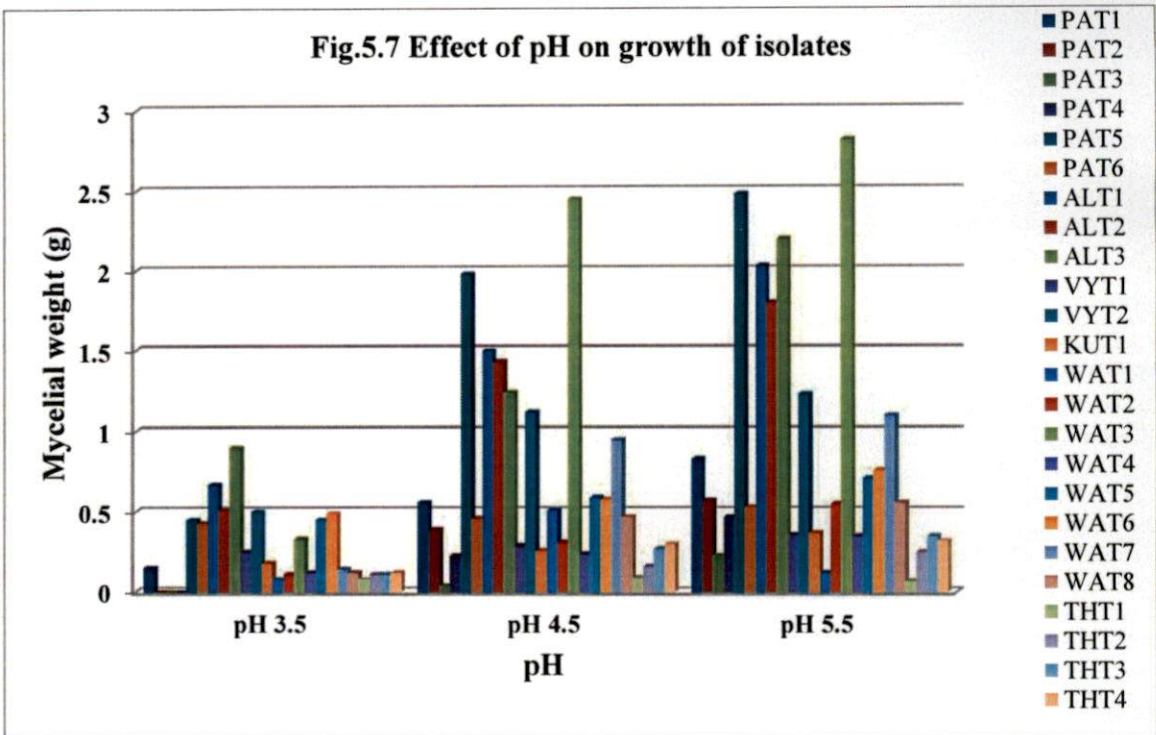
superoxide dismutase enzyme activity when compared to control plants which help in thriving salt stress. Moreover, proline is an osmolyte that help to balance the cell osmoregulation under salt stress (Rasool *et al.*, 2013) and Kumar *et al.* (2016) observed increased proline content in maize plants inoculated with *Trichoderma* isolates.

5.3.5 *In vitro* screening of *Trichoderma* isolates for fungicide tolerance

Ecofriendly management of plant diseases involving both chemical pesticides and biocontrol agents have been extensively used nowadays to combat with the disease causing pathogens. Hence, compatibilityof bioagents especially *Trichoderma* species with pesticides is imperative for disease management and also for improvement of crop yield. Moreover, studies show that addition of heavy metal containing fungicides alone cause phytotoxicity and also can impair the health of ecosystem by leaving fungicide residues leading to environmental pollution. Among the various fungicides recommended for commercial use, copper fungicides *viz.,* copper hydroxide, copper oxychloride and Bordeaux mixture play an important role in plant disease management. However, these fungicides are usually found incompatible with the bioagent *Trichoderma* spp. Hence, an attempt was made to isolate and identify strains of *Trichoderma* spp. which can tolerate to copper fungicides when used in combination in integrated disease management.

Thus, all 24 isolates isolates of *Trichoderma* species have been tested for fungicide tolerance under *in vitro* conditions.Two fungicides, *viz.,* copper oxychloride 50 WP (Fytolan) and copper hydroxide 77 WP (Kocide) at three different concentrations (0.20 %, 0.25% and 0.30 %) were used for the study. At 0.2 per cent concentration of copper hydroxide 77 WP, the isolates ALT1, ALT3 and WAT5 showed considerable growth with lower per cent inhibition of 14.82, 42.59 and 49.44 respectively (Fig. 5.9). Per cent inhibition recorded in all isolates of *Trichoderma* spp. at 0.25 per cent of copper hydroxide ranged between 53.52

and 94.44 with minimum inhibition shown by the isolate ALT3 (53.52 %) followed by ALT1 (39.82 %). Whereas, isolates ALT 1 and ALT 3 retained satisfactory growth with lower per cent inhibition (68.15% and 59.07%) when grown in 0.30 per cent copper hydroxide 77 WP. Isolates showed a per cent inhibition in the ranged from 0 to 94.44 per cent when they were grown in 0.2 per cent copper oxychloride amended medium (Fig. 5.10). However, isolates such as PAT1, ALT1, ALT3, WAT2, WAT5, THT1 and THT2 showed good growth with zero per cent inhibition, whereas PAT2, PAT4, THT3 and THT4 showed no grow that the same concentration. Similarly, at 0.25 per cent concentration of the fungicide, isolates such as ALT3, PAT1 and WAT2 recorded a per cent inhibition of 34.07, 45.93 and 53.07 per cent respectively. While, minimum growth was recorded in PAT6, PAT3, PAT2, WAT1, WAT3, WAT6, WAT7, WAT8 and THT3 with 94.44 per cent inhibition in all isolates. Isolates PAT 1 and ALT 3 showed comparatively good growth with minimum per cent inhibition of 74.82 and 68.89 when grown under 0.30 per cent copper oxychloride 50 WP. While exposing the isolates to various concentrations of both fungicides, they showed lesser per cent inhibition when grown on copper oxychloride. This may be attributed to the lower copper content of 50 per cent in this fungicide when compared to copper hydroxide (70 % copper). Moreover, the frequent use of copper oxychloride by the farmers may have increased the tolerance of the isolates against this fungicide.

A scanning through the available literature showed tolerance of *Trichoderma* spp. to copper fungicides at varying concentrations. Bagwan (2010) reported that *T. harzianum* and *T. viride* were found compatible with 0.20 per cent copper oxychloride. Several other workers also pointed out the compatibility of copper fungicides with *Trichoderma* spp. (Nallathambi *et al.* (2009), Pandya (2010), Islam *et al.* (2011), Khan and Shahzad (2007), Tapwal *et al.* (2012) and Waskale (2017).

Anand *et al.* (2006) reported that an isolate of *T. viride* has a mechanism

Fig. 5.9 Tolerance of *Trichoderma* spp. against copper hydroxide 77 WP

Fig. 5.10. Tolerance of *Trichoderma* spp. against copper oxychloride 50 WP
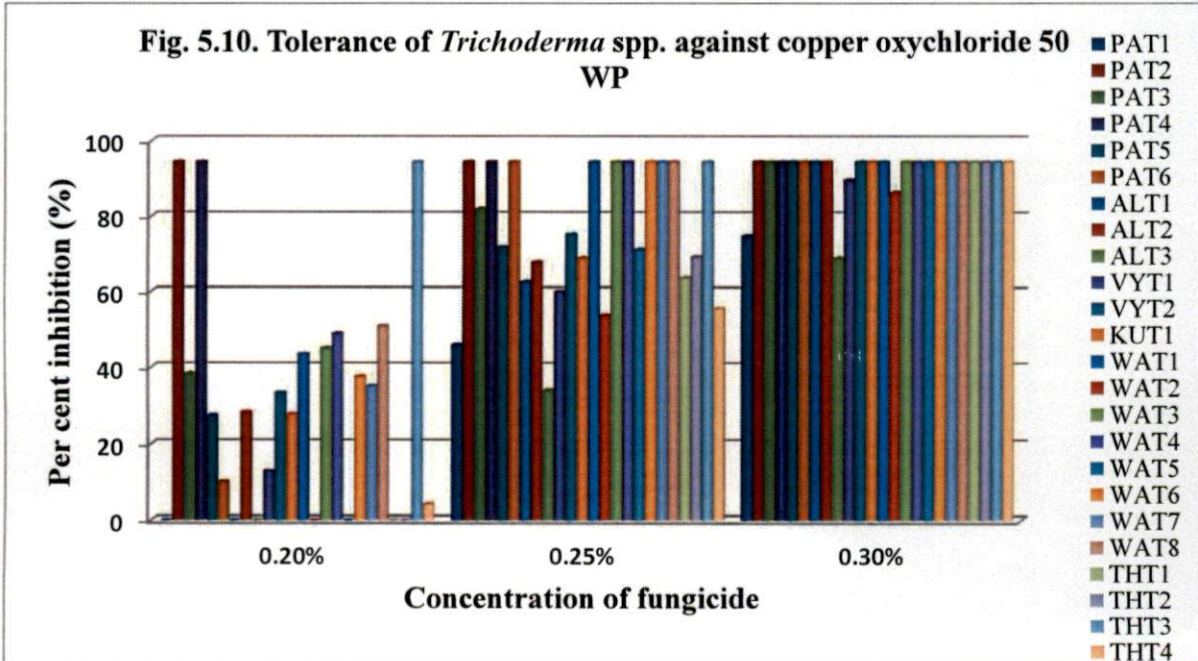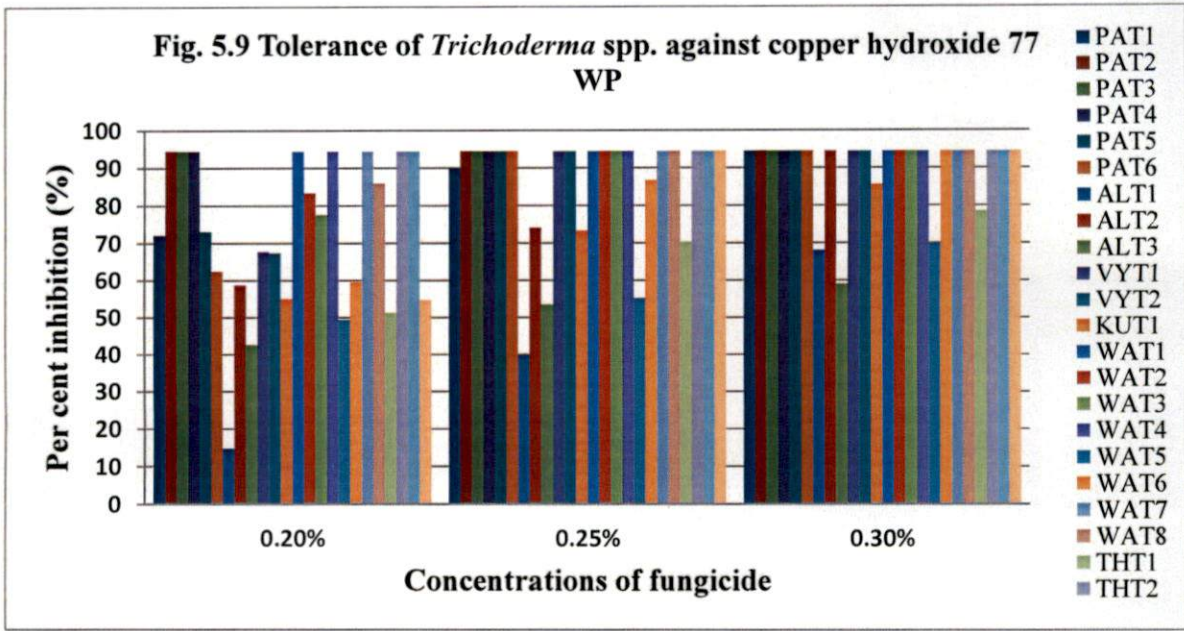
for accumulation of Cu $^{2+}$ ions in its own cell wall, thereby, decreasing the concentration of metal in the medium and hence is considered to be copper tolerant. There are numerous reports which show that microbes can remove and concentrate a variety of metals from aqueous solutions. This kind of metal uptake can be achieved by biosorption and energy dependant metal influx or bio accumulation (Cervantes and Gutierrez-Corona, 1994; Alguacil and Merino, 1998; Gomes *et al.*, 1998). Hence, to become metal resistant, microbes take up mechanisms such as cell membrane metal efflux (Kamizono *et al.*, 1989), intracellular chelation by metallothionein proteins (Presta and Stillman, 1997) and glutathione-derived-peptides called phytochelatins (Kneer *et al.*, 1992) and metal compartmentalization in vacuoles (Volesky *et al.*, 1993).

5.4 BIOCHEMICAL CHARACTERIZATION OF STRESS TOLERANT ISOLATES OF *Trichoderma* spp.

Out of the 24 isolates of *Trichoderma* spp. six were selected after abiotic stress screening for abiotic stress tolerance and were subjected to biochemical assay to determine the enzyme activity. Two best performing isolates under each stress were selected for further study. PAT 6 and WAT 2 from Palakkad and Wayanad respectively were selected as thermotolerant, VYT 2 and ALT1 from Ernakulam and Alappuzha as drought tolerant isolates. The isolates from Alappuzha *viz.,* ALT3 and ALT1 were selected as acidity and salinity tolerant isolates. PAT 1 and ALT3 from Palakkad and Alappuzha respectively also showed tolerance against copper oxychloride and the isolates, ALT 1 and ALT 3, both from Alappuzha against copper hydroxide.

Activity of extra cellular enzymes such as cellulases, *β*- 1,3 glucanases and proteases which are involved in the mycoparasitism of pathogens by *Trichoderma* spp. were estimated. Presence of biomolecules like ACC deaminase and cytokinin which imparts stress tolerance was also assessed. During the present study, isolate VYT 2 showed highest cellulase activity of 4.26 U ml^{-1} followed by ALT 1 (3.47 U ml^{-1}). Minimum cellulase activity was recorded with

the isolate ALT 3 (0.15U ml^{-1}) (Fig. 4.11). Similar results were observed by Bailey and Nevalainen (1981) where they recorded the cellulase enzyme production of *T. reesi* VTT-D-79124 by filter paper degrading activity. They noticed 3.4 and 4.4 U ml^{-1} enzyme activities after 75 h and 96 h of incubation. Likewise, Wen *et al.* (2005) reported that *T. reesei* recorded a cellulase activity of 1.74 U ml^{-1} when it was grown on agricultural waste.

Results of the present study are also in validation with the data elucidated by Montenecourt and Eveleigh (1977), Ryu and Mandels (1980), Domingues *et al.* (2001), Ahamed and Vermette (2008) and Gajera and Vakharia (2012). All six isolates were subjected to *β*-1,3-glucanase assay to detect the enzyme activity. From the study, the isolate ALT 3 showed highest enzyme activity of 5.445 U ml^{-1} followed by VYT 2 which recorded a value of 3.29 U ml^{-1}. The least activity was observed with the isolate WAT 2 (0.62U ml^{-1}). These results are in accordance with those suggested by Monteiro and Ulhoa (2006), where they studied production of *β*-1,3 glucanase enzyme in *T. koningii* by using different carbon sources. They observed that when cellulose was used as the carbon source, the fungus showed 0.5 U ml^{-1} production of the enzyme. A glance through the literature also showed similar studies conducted by El-Katatny *et al.* (2001), Bara *et al.* (2003), dos Reis Almeida *et al.* (2007), Martin *et al.*, (2007), Ahamed and Vermette (2008), Marcello *et al.* (2010). On the contrary, Wen *et al.* (2005) reported higher glucanase activity of 12.22 U ml^{-1} by *T. reesei* when grown on agricultural waste. This may be due to higher substrate concentration in the agricultural waste.

The six abiotic stress tolerant isolates were also subjected to assess the protease enzyme activity under *in vitro* conditions. The highest enzyme activity was recorded with the isolate ALT 1 (8.04 U ml^{-1}) followed by PAT 1 (7.88 U ml^{-1}). The minimum activity was observed with the isolate WAT 2 with 0.98 U ml^{-1}. These findings are in congruence with the results of De Marco and Felix(2002), where they studied the production of hydrolytic enzymes by *Trichoderma* spp.

They assayed protease activity using casein as the substrate for degradation and observed that there was a significant difference in the enzyme activity. They noticed maximum protease activity with *T. harzianum* isolate 1051 (2.0 U ml^{-1}) followed by *Trichoderma* sp. isolate TVC and *T. harzianum* 39 (1.70 U ml^{-1}, 0.98 U ml^{-1} respectively) after 72 h of incubation. Gajera and Vakharia (2012), Smitha *et al.* (2014), Parmar *et al.* (2015), Shakeri and Foster (2017) and Cherkuppally *et al.* (2017) also came to similar conclusions after their studies on protease enzyme activity. However, Elad and Kapat(1999) reported lower protease enzyme activity by *T. harzianum* T 39 (0.058 U ml^{-1}) and *T. harzianum* NCIM1185 (0.054 U ml^{-1}) on the fifth day of growth in liquid culture medium.

ACC (1- aminocyclopropane 1- carboxylate) acts as the precursor for the synthesis of plant volatile hormone ethylene engaged in plant physiological activities such as tissue differentiation, leaf and flower abscission, development of root hair, ripening of fruits and production of volatiles responsible for aroma in fruits (Abeles *et al.*, 1992). Under normal conditions, plants will produce only required levels of ethylene, nevertheless, under stressed conditions, its production may go beyond the required amount which is detrimental to the plant. The enzyme ACC deaminase will cleave the ethylene precursor ACC to ammonia and α-ketobutyrate consequently lowering ethylene levels under stressed environments (Glick *et al.*, 1998). This process may occur under any unfavourable condition such as salt stress (Cheng *et al.*, 2007), flood stress (Grichko and Glick, 2001), drought stress (Mayak *et al.*, 2004) and even in pathogen invasion (Wang *et al.*, 2000). Though only scanty literature is available about the production of ACC deaminase enzyme by *Trichoderma* spp. a study was carried out to assess the production of this enzyme on qualitative basis by *Trichoderma* spp.

In the present investigation, all six stress tolerant isolates were screened for ACC (1-aminocyclopropane-1-carboxylate) deaminase production. The isolates were tested for the ability to use ACC as the sole nitrogen source. Mycelial growth observed from isolates PAT 1, ALT 1 and ALT 3 was same as that of positive control (9 cm), whereas, isolates such as PAT 6, VYT 2 and WAT 2 showed less

mycelial growth with a diameter of 6.90, 7.33 and 7.00 cm respectively (Fig. 5.12). There was no growth observed in negative control plate where no nitrogen source was provided. This proves that out of the six *Trichoderma* isolates, three isolates *viz.*, PAT 1, ALT 1 and ALT 3 showed good growth in Dworkin and Foster (DF) salts minimal medium implying that there is a direct relationship between presence of ACC deaminase enzyme and stress tolerance. This is in congruence with the study by Gravel *et al.* (2007) where they reported that the growth promotion imparted by *T. atroviride* on tomato seedlings is attributed to the production of enzyme ACC deaminase by the fungus. Similarly, Viterbo *et al.* (2010) reported that ACC deaminase enzyme is produced by beneficial soil micro organisms such as *Trichoderma* spp. which is indirectly promoting the growth and development of plants.

According to Windham *et al.* (1986), plant growth promotion by *Trichoderma* species may be attributed to the production of growth promoting hormones such as cytokinin. Hence, a qualitative estimation of cytokinin by the six selected isolates was estimated under *in vitro* conditions. Observations on root biomass and root length of cowpea seeds (var. Uma) inoculated with extracts of *Trichoderma* isolates were recorded. The data showed that maximum root length was produced by PAT 1 followed by PAT 6, ALT 3 and ALT 1 which were all on par with each other, whereas, minimum root length was recorded with WAT 2 and uninoculated control (Fig. 5.13). Maximum root biomass was recorded by the isolate ALT 1 followed by VYT 2 and ALT 3 and minimum was also recorded with the isolate WAT 2 (Fig. 5.14). Likewise, Benitez *et al.* (2004) recorded an increase in root length and branching when tobacco seeds were inoculated with *T. harzianum* CECT2413 when compared with uninoculated control. Ortiz-Castro *et al.*, (2009) also observed that at the time of root colonization by beneficial microflora certain plant growth regulators such as auxins or cytokinins are produced. Similar results were obtained by Ross and Salisbury (1992) and Resende *et al.* (2014).

Fig 5.11 Enzyme activity of selected isolates of *Trichoderma* spp.
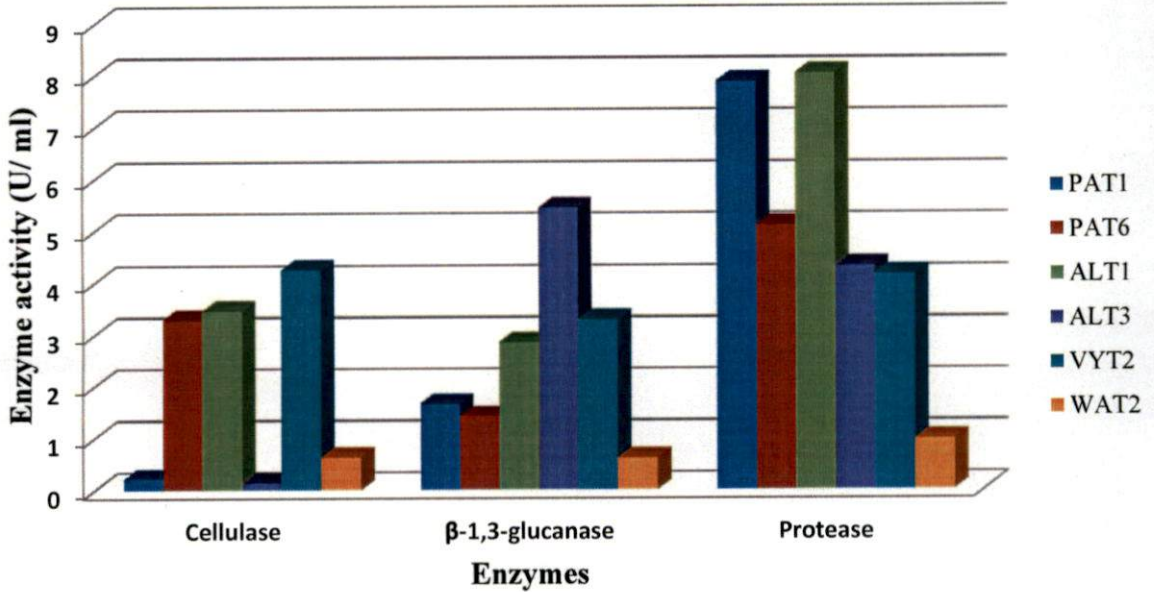
Fig 5.12 ACC deaminase production by *Trichoderma* spp.
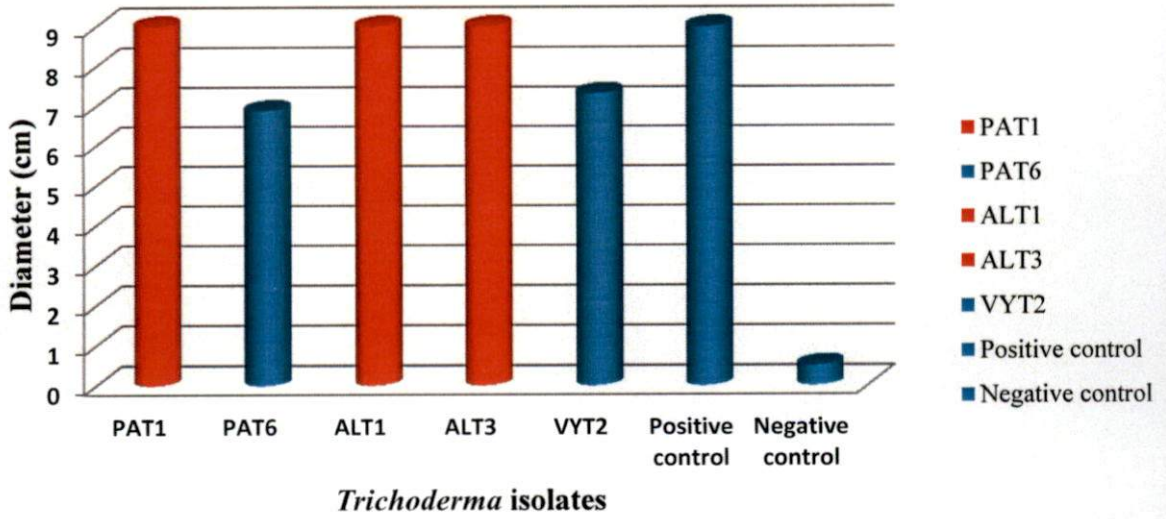
Fig. 5.13 Effect of *Trichoderma* isolates on root length of cowpea seedlings
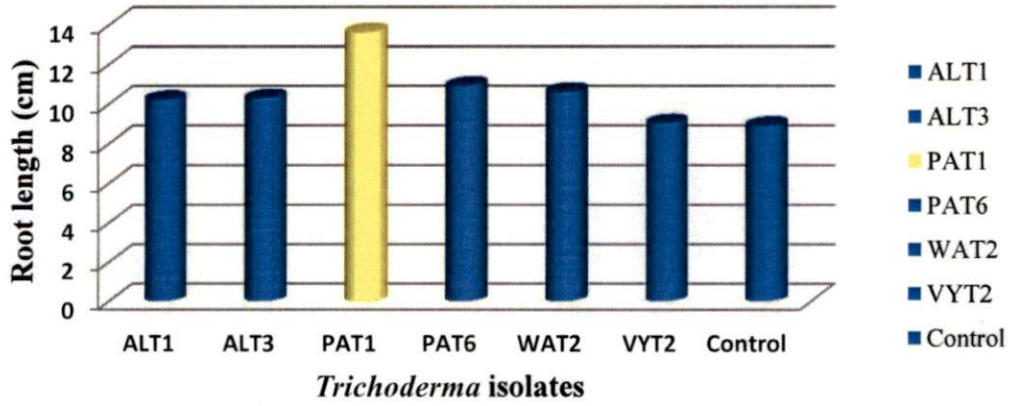
Fig. 5.14 Effect of *Trichoderma* isolates on root biomass of cowpea seedlings
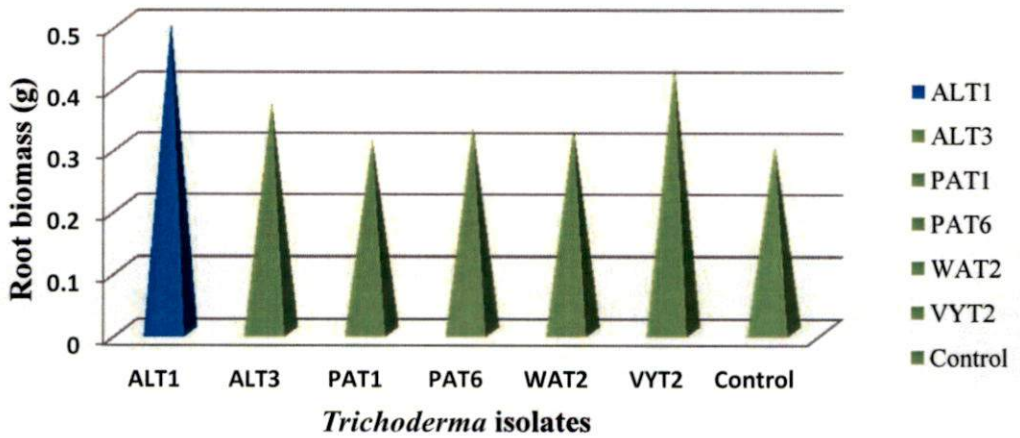
5.5 *In vitro* EVALUATION OF STRESS TOLERANT ISOLATES OF *Trichoderma* spp. FOR THEIR ANTAGONISTIC POTENTIAL

Trichoderma species, one of the most exploited bioagent against plant pathogens usually have higher metabolic rates, produce antimicrobial compounds, show better colonization and are mycoparasitizers with spatial and nutrient competition. Moreover, production of lytic enzymes such as cellulases, hemicellulases, proteases and β-1,3-glucanase also contribute their biocontrol potential. Keeping all this in mind, the stress tolerant *Trichoderma* isolates with superior biochemical properties were finally selected and tested against five major soil borne pathogens *viz.*, *Phytophthora capsici*, *Pythium aphanidermatum*, *Scelrotium rolfsii*, *Fusarium solani* and *Rhizoctonia solani* so as to evaluate their biocontrol efficiency against these pathogens under *in vitro* conditions. Hence, only five isolates, ALT 1, ALT 3, PAT 1, PAT 6 and VYT 2 were selected and thereafter employed for dual culture experiment with major soil borne pathogens. Antagonism towards the pathogens was evaluated for *T. asperellum*, which was kept as control (Fig. 5.15). *Trichoderma* isolates *viz.*, ALT 1, ALT 3, PAT 1, PAT 6 and *T. asperellum* tested for their antagonistic ability with *Rhizoctonia solani*, a percent inhibition ranging from 75.93 to 78.52 per cent was observed. It was noticed that all the isolates except VYT 2 overgrew the pathogen whereas, this isolate showed a clear inhibition zone at the junction where both the mycelia met. The present study is comparable with the findings of Bhale *et al.* (2013) who observed that *R. solani* showed 67.07 per cent inhibition when grown with *Trichoderma koningii*. Parallel results were also put forward by Kumar *et al.* (2012) where they recorded 55.56 per cent inhibition of the pathogen when grown against *Trichoderma* spp. Several other studies are also in line with the antagonism exhibited by *Trichoderma* spp. against *R. solani.* (Lewis and Lumsden, 2001; dos Reis Almeida *et al.*, 2007).

There are reports showing that *Trichoderma* spp. uses extracellular metabolites such as inorganic phosphates, IAA and siderophores for the control of the pathogen *Rhizoctonia solani* (Abbas *et al.*, 2017). Mayo *et al.* (2015) reported the induction of plant defense related genes and thereby consequent production of

Fig. 5.15Per cent inhibition of fungal pathogens by isolates *Trichoderma* spp.

198
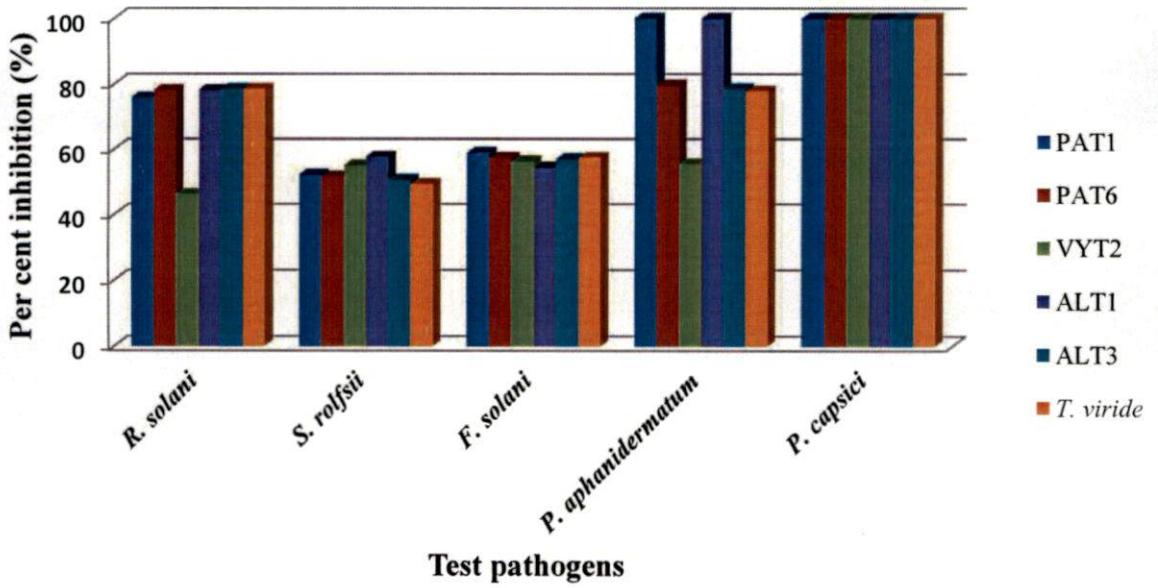
ergosterol by *T. harzianum* in the presence of *R. solani*. Similar findings of antagonism by *Trichoderma* spp. with *R. solani* were recorded by Elad *et al.* (1980), Chet and Baker(1981), Bell *et al.* (1982), Baek *et al.* (1999), Melo and Faull (2000), Yaquab and Shahzad (2005), Sreedevi *et al.* (2011), Gajera and Vakharia (2012), Gawade *et al.* (2012), Bhale *et al.* (2013) and Sab *et al.*(2014).

In the present study, all five *Trichoderma* isolates ALT1, ALT 3, PAT 1, PAT 6, VYT 2 and control showed per cent growth inhibition ranging from 49.26 - 57.78 per cent when grown in dual culture with *S. rolfsii*. Out of the six *Trichoderma* spp., *T. asperellum* showed minimum per cent inhibition of 49.26 per cent followed by ALT 3 with 50.56% per cent. Out of the five pathogens tested, *S. rolfsii* showed maximum tolerance against the bioagent *Trichoderma* spp when grown under *in vitro* conditions. This is in agreement with the studies conducted by Henis *et al.* (1984) where they observed a faster growth of *S. rolfsii* in the presence of *Trichoderma* spp. and by 15 days it had covered almost the entire plate. Similar results were putforth by Hima (2017) where the per cent inhibition on growth of *S. rolfsii* by *Trichoderma* isolates was found to be 55. However, studies conducted by Biswas and Sen (2000), Barakat *et al.* (2006) and Jegathambigai *et al.* (2010) recorded more than 60 per cent inhibition in the growth of the pathogen when grown against *Trichoderma* isolates. This may be due to the difference in inherent biocontrol efficacy towards *S. rolfsii* by isolates of *Trichoderma* spp. (Elad *et al.,* 1980; Maityand Sen, 1985). Upadhyay and Mukhopadhyay (1986) also observed that *Trichoderma harzianum* coiled around the aerial mycelium of *S.rolfsii*. The bioagent also produced haustoria like structures which entered hyphae of the pathogen and destroyed the protoplasmic contents. Metcalf and Wilson (2001) reported that hyphae of *Trichoderma koningii* penetrated into roots infected by *Sclerotium cepivorum* resulting in the destruction of the pathogen hyphae. This process was facilitated by the secretion of extracellular enzymes such as endo and exo chitinases.

When all six *Trichoderma* isolates were grown in dual culture with *Fusarium solani,* they showed similar inhibition in the growth of the pathogen. Per cent inhibition in the growth of pathogen caused by the isolates PAT 1, PAT

6, ALT 3, VYT 2, ALT 1 and *T. asperellum* ranged from 54.44 – 58.89 per cent. In all isolates, a zone of inhibition was observed when grown against *Fusarium solani*. Zhang *et al.* (1996) came to a similar conclusion where they observed that *T. virens* inhibited *F. oxysporum* f. sp. *vasinfectum* under *in vitro* conditions. Sundaramoorthy and Balabaskar (2013) also came across similar findings and he observed a per cent inhibition of 53 when *Trichoderma harzianum* was grown together with *Fusarium oxysporum* f. sp. *lycopersici*. Similar reports on antagonism of *Trichoderma* spp. against *Fusarium* spp. were put forward by many workers [Cole and Zvenyika (1988), McAllister *et al.* (1994), Rojo *et al.* (2007), Kumar *et al.* (2016) and Tomar *et al.* (2017). Moreover, Lorito *et al.* (1996)] reported the secretion of antifungal compounds and lytic enzymes produced by *Trichoderma* spp. against *Fusarium oxysporum*. According to Altomare *et al.* (1999), Howell (2003) and Kumar*et al.* (2016) *Trichoderma* species has various mode of action against *Fusarium* spp. such as production of enzymes like chitinases and glucanases, production of antibiotics and competition.

In the present study, *Pythium aphanidermatum* showed cent per cent of growth inhibition when grown against *Trichoderma* isolates PAT 1, ALT 1 and *T. asperellum*. Minimum per cent growth inhibition of 55.56 per cent of the pathogen was recorded when it was grown against the isolate VYT 2. The results were in congruence with the studies by Fajola and Alasoadura (1975), Mukherjee *et al.* (1989), Aerts *et al.* (2002), Muthukumar *et al.* (2011) and John *et al.* (2010). Howell and Stipanovic (1983) described an antibiotic gliovirin responsible for the antagonistic property of *Trichoderma virens* against *Pythium ultimum* and *Phytophthora* spp. Biological control of *Pythium* spp. by *T. harzianum* and *T. koningii* was due to the production of volatile antimicrobial compounds (Lifshitz *et al.*, 1986). According to them, zoospore germination, germ tube elongation and mycelial growth of *P. aphanidermatum* were arrested when the pathogen was treated with the culture filtrate of *T. harzianum*.

In the present study it was noticed that, all five isolates and control showed 100 per cent inhibition in the growth of pathogen *Phytophthora capsici*. Whereas, Ahamed *et al.* (1999) elucidated that *T. harzianum* showed only 53 % per cent

inhibition on the growth of *P. capsici.* Likewise, Etebarian *et al.* (2000) observed *T. harzianum* T39 and *T. virens* DAR 74290 showed per cent growth inhibition of 49- 71 and 49- 54% of *Phytophthora erythroseptica.* Howell and Stipanovic (1983) described the presence of an antibiotic in *T. virens* which is inhibitor towards the growth and development of *Phytophthora* species.In the present study, all the tested isolates of *Trichoderma* species showed 100 per cent inhibition in the growth of pathogen *Phytophthora capsici.* Smith *et al.* (1990) also reported similar results when *Trichoderma* spp. was grown against *Phytophthora* spp. Bae *et al.* (2011) reported coiling and penetration of *Trichoderma* hyphae around *Phytophthora capsici* and this also resulted in cent per cent inhibition of pathogen.

Based on the *in vitro* studies on stress tolerance, biochemical studies and dual culture technique four isolates *viz.,* ALT1, ALT3, PAT1 and PAT6 were subjected to molecular characterization at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram to confirm its identity upto species level prior to the pot culture experiment. It was observed that the isolate ALT 1, ALT 3, PAT 1 and PAT 6 showed homology with *Trichoderma asperellum.*Hence, the isolates were named as *Trichoderma asperellum* isolate ALT 1, *Trichoderma asperellum* isolate ALT 3, *Trichoderma asperellum* isolate PAT 1 and *Trichoderma asperellum* isolate PAT 6 respectively.

5.6 *In vivo* EVALUATION OF THE PROMISING STRESS TOLERANT STRAINS FOR BIOCONTROL EFFICACY AND GROWTH PROMOTION

Results of the *in vitro* studies conducted for antagonistic properties of the *Trichoderma* isolates might always not hold true under field conditions as they might show a drop in inhibition of pathogen when the study is shifted from *in vitro* to field conditions. Hence, a pot culture experiment was laid out to evaluate the biocontrol efficacy and growth promotion of four selected isolates *viz.,* ALT1, ALT3, PAT1 and PAT6 against *R. solani* using cowpea (var. Bhagyalakshmi) as the test crop.

In the present study, cent per cent seed germination was recorded when T 4 (*T. asperellum* isolate PAT 6) and T 5 (*T. viride,* KAU isolate) were applied in the pathogen inoculated pots followed by 80 per cent germination in T2 (*T. asperellum* isolate ALT 3) (Fig. 5.16). Seed germination percentage of 60 per cent was recorded with T1 (*T. asperellum* isolate ALT 1), T 3 (*T. asperellum* isolate PAT 1) and T 4 (*T. asperellum* isolate PAT 6) which were challenge inoculated pots and this was similar to the control pots. Harman *et al.* (1980) noticed that *T. hamatum* protected pea seeds against pathogens like *R. solani* more than *T. harzianum.* Moreover, the concentration of *Trichoderma* propagules were higher in the pots when compared to the pathogens, which was reduced upto 40 per cent. According to them, *T. hamatum* exhibited control of the disease nearly equivalent to chemical fungicides such as Captan and it was also higher than *T. harzianum.* Also the activity of *T. hamatum* retained for a period of time after planting showed that it can be efficiently used for disease control. Nagar (2017) noticed that germination per cent of chickpea seeds ranged between 67.73 and 80.09 when it was treated with *Trichoderma* isolates.

Various biometric observations such as plant height, number of leaves, days to first flowering and yield of the cowpea plants were recorded in order to study the growth promotion by different *Trichoderma* isolates. It was observed that, *Trichoderma asperellum* isolate PAT 6 recorded highest values with respect to all biometric characters implying that this particular isolate has the maximum growth promotion ability on host plants (Fig. 5.16, 5.17, 5.18, 5.19 and 5.20). The results of the experiment are in accordance with Singh *et al.* (2014), where they observed that application of *T. harzianum* resulted in the production of maximum number of leaves, shoot and root length, shoot and dry weight and total plant dry weight. Likewise, Hicks *et al.* (2014) reported that *T. harzianum* LU1491, *T. rossicum* LU1492 and *T. atroviride* LU132 increased the number of tubers in potato by 75, 60 and 43 per cent. *T. barbatum* LU1489 caused an increase in tuber weight by 265 per cent whereas, *T. rossicum* LU1492. Also *T. harzianum* LU1491, *T. barbatum* LU1489 and *Trichoderma* sp. 792 LU1483 recorded maximum number of tubers, total tuber weight and average tuber weight

Fig. 5.16 Effect of treatments on seed germination per cent
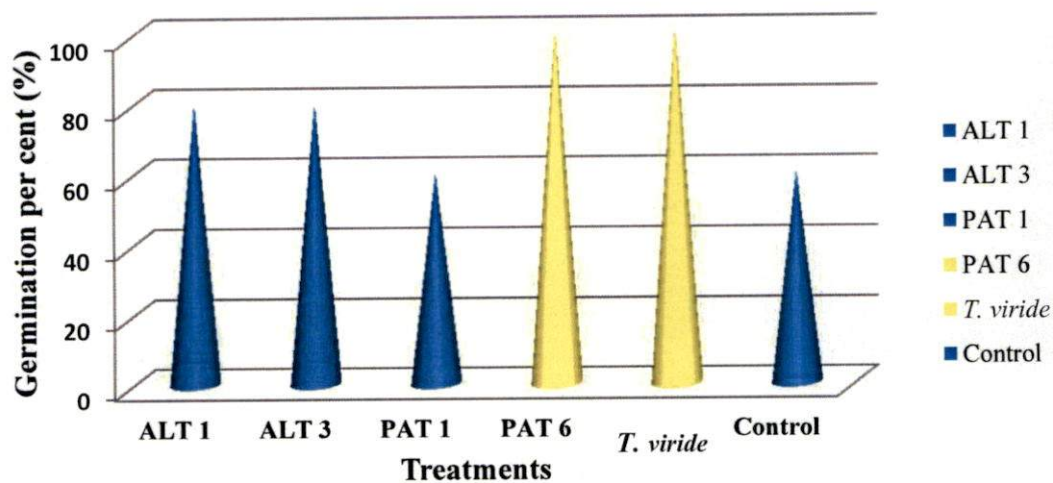
Fig. 5.17 Relative increase in no. of leaves after treatment application
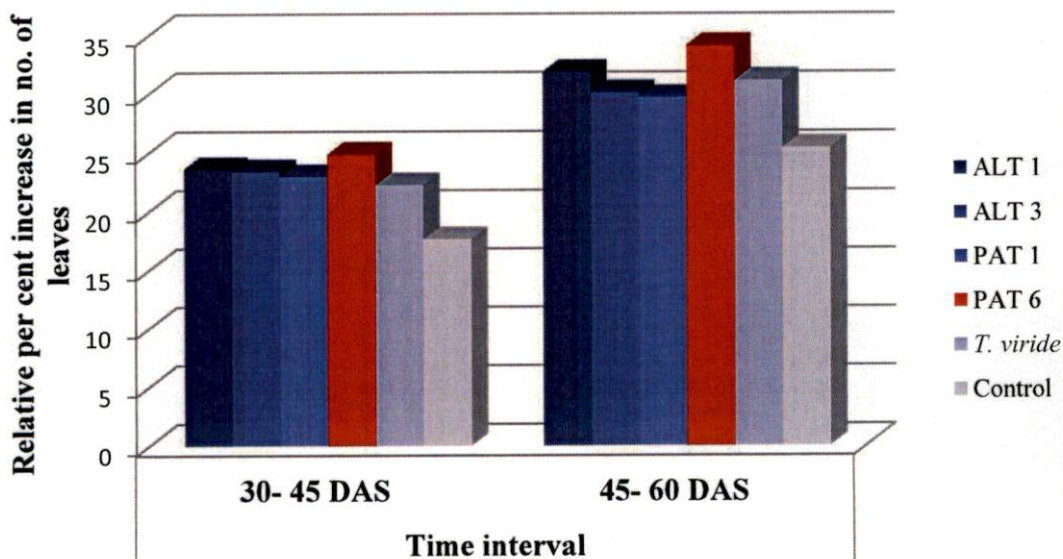
respectively. Mayo *et al.* (2015) noted that *T. harzianum* showed the highest dry weight of shoot and root when compared to control. Nagar (2017) observed that among different *Trichoderma* isolates, *Trichoderma* strain TRSH-S6 achieved significantly higher shoot length (33 cm), fresh weight (27.07g), number of nodules (30.33), dry weight (11.30g) and yield (2138.89 kg ha^{-1}) which was comparable with TRJ-S1, TRSH-S5 treatments.

In the pot culture experiment, *T. asperellum* isolate ALT 3 and *T. asperellum* isolate PAT 6 showed the least disease incidence of 11.11 per cent when the pathogen *R. solani* was challenge inoculated in the pots. However, *T. asperellum* isolate ALT 1, *T. asperellum* isolate PAT 1 and *T. viride* (KAU isolate) showed 22.22 per cent disease incidence. Cent per cent disease incidence was recorded in the control plants where no isolate of *Trichoderma* spp. was inoculated.

According to Harman *et al.* (2004), one of the most common strategies used by *Trichoderma* spp. is mycoparasitism. Detection, attachment, penetration and secretion of fungitoxic enzymes also results in the death of pathogen. Eziashi *et al.* (2007) reported that *Trichoderma* showed an affinity towards host cell wall which results in chemical bonding between functional sites of carbohydrates of antagonist and pathogen and finally leading to host cell wall penetration.

Lu *et al.* (2004) also observed that on application of *T. atroviride* in cucumber seeds sown in *R. solani* infested soil, 71 per cent survival was recorded. Wilson *et al.* (2008) and Rahman *et al.* (2011) also noticed that *T. harzianum* and *T. virens* were effective against *R. solani*. These findings are in line with the reports of many workers who studied the effect of *T. asperellum, T. harzianum, T. virens* and *T. hamatum* in controlling the growth of *R. solani* [(Beagle-Ristanio and Papavizas (1985), Lewis and Larkin (1997) and Aydin and Turhan (2009)]. There are reports of cent per cent inhibition of *R. solani* by *Trichoderma* isolates in tomato plants coupled with plant fresh and dry weight (Montealegre *et al.*, 2010). Asad *et al.* (2014) also reached to a similar conclusion, where they observed a reduction in disease incidence to 19.3 per cent when *T. asperellum* was inoculated in plants, whereas control plants showed 54.3 per cent disease

Fig. 5.18 Relative per cent increase in plant height after treatment application
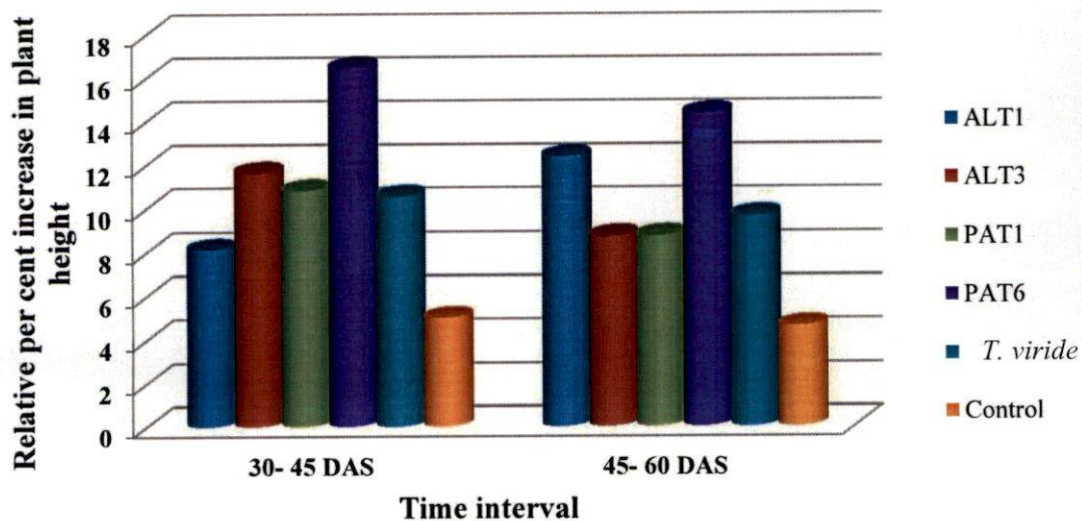
Fig. 5.19 Effect of treatments on days to flowering
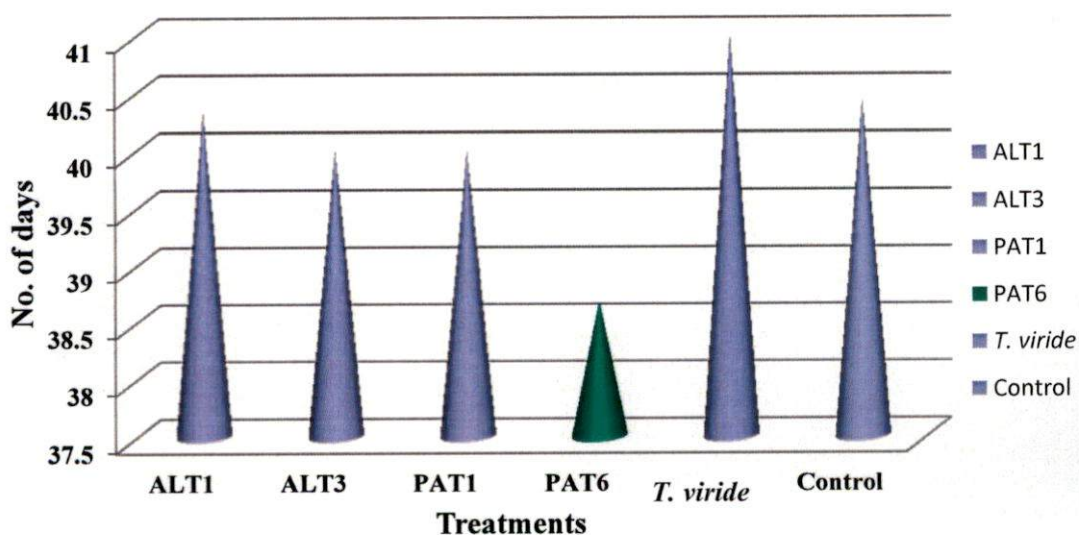
Fig. 5.20 Effect of treatments on yield
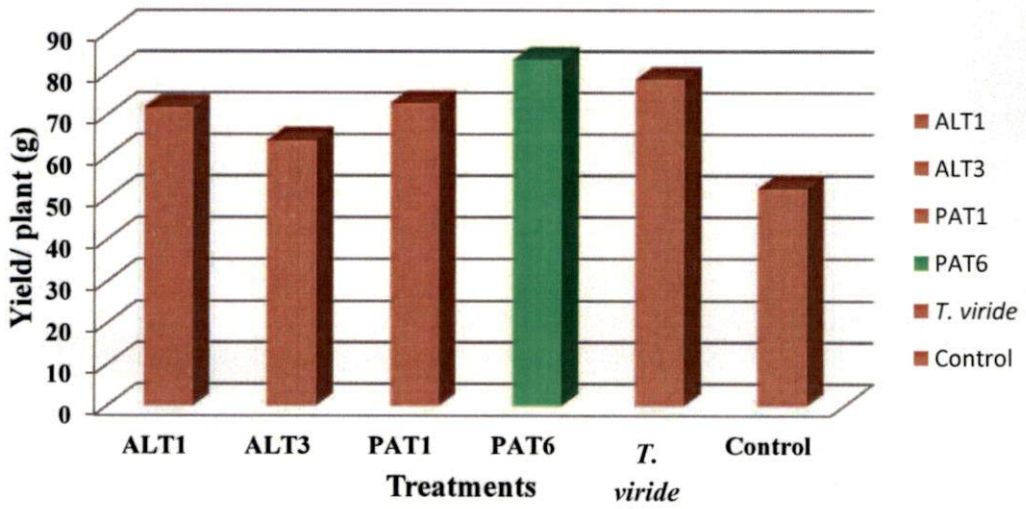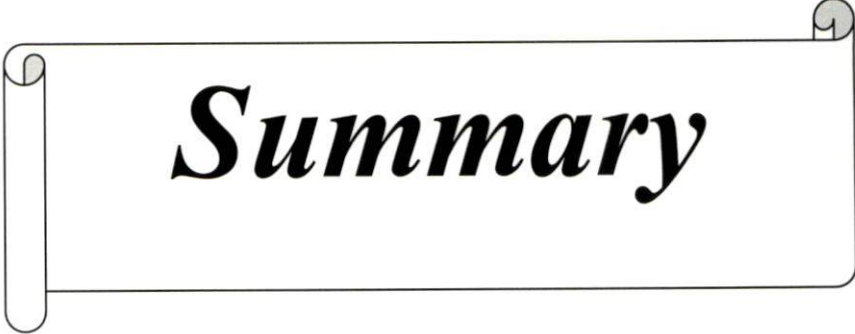
incidence. However, in the present study there was 100 per cent disease incidence in control plants without *Trichoderma* inoculation. Durak (2016) observed that *T. harzianum* and *T. virens* were found very effective against *R. solani.*

Hence, after recalling back the results of the study, a total of 24 *Trichoderma* spp.were obtained from 52 soil samples collected from six locations of Kerala where the highest population of *Trichoderma* spp. was obtained from Wayanad district and the lowest from Palakkad. Out of the 24 isolates, six abiotic stress tolerant isolates were selected and these were further subjected to biochemical tests to study the activity of enzymes such ascellulase, *β*- 1,3-glucanase and protease, which are responsible for the mycoparasitism exhibited by *Trichoderma* spp. and these were also screened for the qualitative assay of biomolecules involved in imparting drought tolerance like ACC- deaminase (1-aminocyclopropane-1-carboxylate deaminase) and cytokinin. Thereafter, five isolates of *Trichoderma* spp. were selected and later employed to test the antagonistic potential with five major soil borne pathogens.

After the dual culture study, only four isolates *viz.,* PAT1, PAT6, ALT1 and ALT3 showing better antagonism against the tested pathogens were further selected for the pot culture experiment two each from Palakkad and Alappuzha district. Molecular characterization of these four isolates *viz.,* PAT1, PAT6, ALT1 and ALT3 isolates revealed that these showed homology with sequence *Trichoderma asperellum.* These were then used for the assessment of growth promotion in cowpea and biocontrol efficacy against *R. solani* under *in vivo* conditions where the results showed that *Trichoderma asperellum* isolate PAT 6 from Palakkad was found to be the best performing isolate with both plant growth promotion and antagonistic ability. However, these observations made through *in vitro* and *in vivo* experiments may not always reflect the same under field conditions. Hence, the study should be complimented with multi-locational field trials to prove the effectiveness of these isolates under various stressed areas of Kerala. Thus it can be concluded that this study has enlightened our knowledge on the prevalence of such stress tolerant isolates of *Trichoderma* spp. in various

ecosystems of Kerala which can be exploited and thereby used as efficient biocontrol agents for the management of the major soil borne pathogens infecting various crops of Kerala.

Summary

209

6. SUMMARY

Biological control is an ecofriendly approach in the effective management of crop diseases. The fungi belonging to the genus *Trichoderma* are one among the most exploited biocontrol agents in the field of agriculture for the management of crop diseases. However, in certain conditions *Trichoderma* strains show poor or inconsistent performance in fields which may be attributed to their low tolerance towards abiotic stresses such as high temperature, drought, acidity, salinity and fungicides. Hence, the present research on "Exploitation of abiotic stress tolerant strains of *Trichoderma* spp. for the management of soil borne fungal pathogens" was conducted in order to identify different abiotic stress tolerant isolates of *Trichoderma* spp. and to study its antagonistic ability and plant growth promotion under *in vitro* and *in vivo* conditions.

1. Intensive soil sampling surveys were conducted across different abiotic stressed ecosystems of Kerala *viz.,* Palakkad, Alappuzha, Vytilla, Kumarakom, Wayanad and Thrissur for the isolation and enumeration of native *Trichoderma* spp.

    *   Electrochemical properties of the soil samples were recorded as they influence the type and amount of soil microflora. Among the 52 soil samples collected, the lowest pH of 2.9 was recorded from the sample Cherthala- 2 collected from Alappuzha district and the highest EC value of 7.61 d Sm^{-1} was observed from Cherthala-1 sample collected from Alappuzha district.

    *   After enumeration of *Trichoderma* spp., the highest population of the fungus was obtained from soil sample Tholpetty- 1 (W 7) from Wayanad district with a cfu of 32.52 x 10^3 and 22.22 x 10^4 in TSM and 31.22 x 10^3 and 17.61x 10^4 in MEA media. Lowest population of 4.18 x 10^3 and 2.17 x 10^4 cfu g^{-1} was obtained from Athipotta- 2 (P 10) of Palakkad district in TSM and MEA respectively.

- A total of 24 isolates were obtained from 52 soil samples collected from different locations, out of which maximum number of eight isolates were retrieved from Wayanad district.

- Based on the number of *Trichoderma* spp. obtained from each district, they were serially numbered and abbreviated according to the name of the location. Accordingly, PAT 1 to PAT 6 represents number of isolates of *Trichoderma* spp. from Palakkad district, ALT 1 to ALT 3 from Alappuzha, VYT 1 and VYT 2 from Ernakulam district, KUT 1 from Kottayam district, WAT 1 to WAT 8 from Wayanad and THT 1 to THT 4 from Thrissur district. Cultural and morphological identification of these isolates were done under *in vitro* conditions.

2. All the 24 isolates of *Trichoderma* spp. were subjected to *in vitro* screening for abiotic stress tolerance such as high temperature, drought, acidity, salinity and fungicides.

- The isolates were subjected to different temperature levels of 25^0C, 30^0C, 35^0C and 40^0C and it was observed that PAT6 and WAT2 produced the maximum mycelial biomass of 0.03 g and were able to sporulate at the highest temperature of 40^0C. Hence, the two isolates were considered as thermotolerant.

- At higher concentration of PEG (30 %), the isolates VYT2 (2.43 g) and ALT1 (0.090 g) produced higher mycelial biomass and showed sparse to moderate sporulation in the media and thus the two isolates were considered as drought tolerant.

- The isolates ALT 3 and ALT 1 from Alappuzha showed the highest mycelial weight of 0.90 g and 0.67 g which were also observed to produce high sporulation when grown at a low pH of 3.5. Thus, these two isolates of *Trichoderma* spp. were selected as acid tolerant isolates.

- Isolates ALT1 and ALT3 showed good growth under 0.50 M, 1.0 M and 1.50 M concentration of NaCl and ALT3 also produced spores at 0.5 and

1 M concentrations of NaCl also. Thus, the two isolates were selected as salinity tolerant *Trichoderma* spp.

- The isolates ALT1 and ALT3 were selected as copper hydroxide tolerant isolates as they showed lower per cent inhibition of 68.15 and 59.07 per cent at 0.3 per cent concentration of the fungicide.

- Likewise, ALT3 and PAT1 showed comparatively good growth at the highest concentration of the fungicide with lower inhibition per cent of 74.82 and 68.89 per cent respectively, hence considered as copper oxychloride tolerant isolates.

3. Two best performing isolates under each stress were selected for further studies and they were further subjected to biochemical tests to study the activity of enzymes such as cellulase, β- 1,3- glucanase and protease, which are responsible for the mycoparasitism exhibited by *Trichoderma* spp. The isolates were also screened for qualitative assay of biomolecules involved in imparting drought tolerance like ACC-deaminase (1-aminocyclopropane-1-carboxylate deaminase) and cytokinin.

- The isolate VYT 2 showed highest cellulase activity of 4.26 U ml^{-1} followed by ALT 1 (3.47 U ml^{-1}), whereas minimum cellulase activity was recorded with the isolate ALT 3 (0.15U ml^{-1}).

- ALT 3 showed highest β- 1,3 glucanase enzyme activity of 5.45 U ml^{-1} followed by VYT 2 with 3.29 U ml^{-1}. The least activity was observed with the isolate WAT 2 with 0.62Uml^{-1}.

- The highest protease activity was recorded with the isolate ALT 1 (8.04 U ml^{-1}) followed by PAT 1 (7.88 U ml^{-1}). The minimum activity was observed in the isolate WAT 2 with 0.98 U ml^{-1}.

- Isolates PAT 1, ALT 1 and ALT 3 produced mycelial growth of 9 cm, which was same as that of positive control when grown on Dworfkin and Foster salts minimal medium with ACC as sole nitrogen source.

- Maximum root length was produced when cowpea seeds were treated with the isolate PAT 1 (13.60 cm) and highest root biomass was produced when the seeds were treated with ALT 1 (0.50 g) and thus these were considered as better cytokinin producers.

4. After the enzyme study, the isolate WAT 2 was screened out as it showed lesser activity of enzymes and was also found to be poor producer of ACC deaminase and cytokinin. Hence, five isolates of *Trichoderma* spp. *viz.*, ALT 1, ALT 3, PAT 1 and PAT 6 were employed to test the antagonistic potential with five major soil borne pathogens *viz.*, *Phytophthora capsici*, *Pythium aphanidermatum*, *Sclerotium rolfsii*, *Fusarium solani* and *Rhizoctonia solani*. The efficacy of these cultures was evaluated in comparison with reference culture of KAU *Trichoderma viride*.

- The isolates ALT 3, ALT 1, PAT 6 and PAT 1 were on par with each other representing higher per cent inhibition of pathogen with 78.52, 78.15, 78.15 and 75.93 per cent respectively against *R. solani*. These isolates were also on par with the control, *T. viride,* which also showed 78.52 per cent inhibition of *R. solani*.
- Isolates such as ALT1 and VYT 2 showed 57.78 and 55 per cent inhibition of *S. rolfsii* where both these isolates were found to be on par, followed by PAT 1 and PAT 6 which showed a per cent growth inhibition of 52.22 and 51.67 of *S. rolfsii* respectively.
- There was no significant difference noticed among the isolates when grown against *Fusarium solani* in dual culture, though the highest per cent inhibition of 58.89 was noticed with PAT 1.
- Cent per cent inhibition of *Pythium aphanidermatum* was noticed when grown with *Trichoderma* isolates PAT 1 and ALT 1. Minimum per cent growth inhibition of pathogen was recorded with VYT2 (55.56%) and rest of the isolates showed an inhibition above 70 per cent.

- All five isolates and control showed 100 per cent inhibition in the growth of pathogen, *Phytophthora capsici.*

5. Molecular characterization of four *Trichoderma* isolates was carried out at Rajiv Ganghi Centre for Biotechnology (RGCB), Thiruvananthapuram by ITS sequencing to identify the isolates upto species level and the isolates ALT 1, ALT 3, PAT 1 and PAT 6 showed identity with *T. asperellum.*

6. A pot culture experiment was later laid out with cowpea as the test crop to test the biocontrol efficacy against *Rhizoctonia solani* and growth promotion of the promising stress tolerant isolates of *Trichoderma* spp. Four isolates *Trichoderma asperellum* (ALT1, ALT3, PAT1 and PAT 6) were selected for the *in vivo* experiment against the pathogen.

- *Trichoderma asperellum* isolate PAT 6 and *Trichoderma viride* showed cent per cent seed germination even in pathogen inoculated pots.
- Maximum per cent relative increase in height was observed in *T. asperellum* isolate PAT 6 (16.49%), followed by *T. asperellum* isolate ALT 3 (11.67 %).
- The highest per cent increase in number of leaves of 24.89 was found in the plants when *T. asperellum* isolate PAT 6 was applied in the grow bags followed by *T. asperellum* isolate ALT 1 (23.67%).
- Earliness in flowering was observed in *Trichoderma asperellum* isolate PAT 6 (38.67 days) and all other treatments except control were on par with each other.
- Maximum yield of 83.39 g per plant was obtained from *T. asperellum* isolate PAT 6 followed by the reference culture, *T. viride* (78.70 g/ plant).
- *T. asperellum* isolate ALT 3 and *T. asperellum* isolate PAT 6 showed the least disease incidence of 11.11 per cent when the pathogen *R. solani* was challenge inoculated in the pots.

References

REFERENCES

Abbas, A., Jiang, D., and Fu, Y., 2017. *Trichoderma* spp. as antagonist of *Rhizoctonia solani*. *J. Plant Pathol. Microbiol*. 8(402):2.

Abeles, F. B., Morgan, P. W., and Saltveit, M. E. 1992. *Ethylene in plant biology* (2nd Ed.). Academic Press, New York, 297p.

Aerts, R., De, B. S., and Rombouts, L. 2002. Suppression of *Pythium* spp. by *Trichoderma* spp. during germination of tomato seeds in soilless growing media. *Mededelingen (Rijksuniversiteit te Gent. Fakulteit van de Landbouwkundige en Toegepaste Biologische Wetenschappen)* 67(2): 343-351.

Ahamed, A. and Vermette, P. 2008. Culture-based strategies to enhance cellulase enzyme production from *Trichoderma reesei* RUT-C30 in bioreactor culture conditions. *Biochemical Eng. J*. 40(3): 399-407.

Ahmad, P., Hashem, A., Abd-Allah, E. F., Alqarawi, A. A., John, R., Egamberdieva, D., and Gucel, S. 2015. Role of *Trichoderma harzianum* in mitigating NaCl stress in Indian mustard (*Brassica juncea* L) through antioxidative defense system. *Front. Plant Sci*. 6: 868.

Ahmed, S. A., Perez-Sanchez, C., Egea, C., and Candela, M. E. 1999. Evaluation of *Trichoderma harzianum* for controlling root rot caused by *Phytophthora capsici* in pepper plants. *Plant Pathol*. 48(1): 58-65.

Alguacil, F. J. and Merino, Y. 1998. Biotreatment of pollutants of inorganic origin. *Metallurgy Mag*. 34 (5): 428-436.

Ali, H. Z., Aboud, H. M., Dheyab, N. S., Musa, N. K., and Gasam, F. H. 2015. Effects of pH and ECw on growth and sporulation of indigenous *Trichoderma* spp. *Int. J. Phytopathol.* 4(1):15-20.

Ali, S., Charles, T. C., and Glick, B. R. 2012. Delay of flower senescence by bacterial endophytes expressing 1-aminocyclopropane-1-carboxylate deaminase. *J. Appl. Microbiol.* 113(5): 1139-1144.

Ali, S. Z., Sandhya, V., and Rao, L.V. 2014. Isolation and characterization of drought-tolerant ACC deaminase and exopolysaccharide-producing fluorescent *Pseudomonas* sp. *Ann. Microbiol.* 64(2): 493-502.

Aly, A. A., Abdel-Sattar, M. A., Omar, M. R., and Abd-Elsalam, K. A. 2007. Differential antagonism of *Trichoderma* sp. against *Macrophomina phaseolina*. *J. Plant Prot. Res*. 47(2): 91-102.

Altomare, C., Norvell, W. A., Bjorkman, T., and Harman, G. E. 1999. Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus *Trichoderma harzianum* Rifai. *Appl. Environ. Microbio*. l65: 2926–2933.

Amalraj, L. D. E., Kumar, P.G., Desai, S., and Ahmed, M. H. S. K. 2010. *In vitro* characterization of *Trichoderma viride* for abiotic stress tolerance and field evaluation against root rot disease in *Vigna mungo* LJ *Biofertil. Biopestici.* 2(3): 1-5.

Anand, P., Isar, J., Saran, S., and Saxena, R. K. 2006. Bioaccumulation of copper by *Trichoderma viride*. *Bioresour. Technol.* 97(8): 1018-1025.

Arshad, M., Shaharoona, B., and Mahmood, T. 2008. Inoculation with *Pseudomonas* spp. containing ACC-deaminase partially eliminates the

217

effects of drought stress on growth, yield, and ripening of pea (*Pisum sativum* L.). *Pedosphere* 18(5): 611-620.

Asad, S. A., Ali, N. Hameed, A., Khan, S. A., Ahmad, R., Bilal, M., Shahzad, M., and Tabassum, A. 2014. Biocontrol efficacy of different isolates of *Trichoderma* against soil borne pathogen *Rhizoctonia solani*. *Polish J Microbiol.* 63(1): 95-103.

Aydın, M. H. and Turhan, G. 2009. Studies on determination of fungal antagonists of *Rhizoctonia solani*. *Anadolu*. 19(2): 49-72.

Bae, H., Roberts, D. P., Lim, H. S., Strem, M. D., Park, S. C., Ryu, C. M., Melnick, R. L., and Bailey, B. A. 2011. Endophytic *Trichoderma* isolates from tropical environments delay disease onset and induce resistance against *Phytophthora capsici* in hot pepper using multiple mechanisms. *Mol. Plant-Microbe Interact.* 24(3): 336-351.

Bae, H., Sicher, R. C., Kim, M. S., Kim, S. H., Strem, M. D., Melnick, R. L., and Bailey, B. A. 2009. The beneficial endophyte *Trichoderma hamatum* isolate DIS 219b promotes growth and delays the onset of the drought response in *Theobroma cacao*. *J. Exp. Bot.* 60(11): 3279-3295.

Baek, J. M., Howell, C. R., and Kenerley, C. M. 1999. The role of an extracellular chitinase from *Trichoderma virens* Gv29-8 in the biocontrol of *Rhizoctonia solani*. *Curr. Genet.* 35: 41–50.

Bagwan, N. B. 2010. Evaluation of *Trichoderma* compatibility with fungicides, pesticides, organic cakes and botanicals for integrated management of soil borne disease of soybean [*Glycine max* (L.) Merril]. *Int. J. Plant Prot.* 3(2): 206-209.

218

Bailey, M. J. and Nevalainen, K. M. H. 1981. Induction, isolation and testing of stable *Trichoderma reesei* mutants with improved production of solubilizing cellulase. *Enzyme Microbial Technol.* 3(2): 153-157.

Bailey, M. J., Buchert, J., and Viikari, L. 1993. Effect of pH on production of xylanase by *Trichoderma reesei* on xylan-and cellulose-based media. *Appl. Microbiol. Biotechnol.* 40(2-3): 224-229.

Bailey, M. J. and Tahtiharju, J. 2003. Efficient cellulase production by *Trichoderma reesei* in continuous cultivation on lactose medium with a computer-controlled feeding strategy. *Appl. Microbiol. Biotechnol.* 62(2-3): 156-162.

Bankole, S. A. and Adebanjo, A. 1996. Biocontrol of brown blotch of cowpea caused by *Colletotrichum truncatum* with *Trichoderma viride*. *Crop Prot.* 15(7): 633-636.

Bara, M. T. F., Lima, A. L., and Ulhoa, C. J. 2003. Purification and characterization of an exo-β-1, 3-glucanase produced by *Trichoderma asperellum*. *FEMS Microbiol. Lett.* 219(1): 81-85.

Barka, E. A., Nowak, J., and Clement, C. 2006. Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth-promoting rhizobacterium, *Burkholderia phytofirmans* strain PsJN. *Appl. Environ. Microbiol.* 72(11): 7246-7252.

Barakat, R. M., Al-Mahareeq, F., and Al-Masri, M. I. 2006. Biological control of *Sclerotium rolfsii* by using indigenous *Trichoderma* spp. isolates from Palestine. *Hebron Univ. Res. J.* 2(2): 27-47.

Bastin, B., Beena, V. I., Sreelatha, A. K., and Dey, P. 2014. *GPS and GIS based modelmsoil fertility maps for precise fertilizer recommendations to the farmers of Kerala.* Kerala Agricultural University, Thrissur, Kerala, 161p.

Beagle-Ristaino, J.E. and Papavizas, G.C. 1985. Biological control of *Rhizoctonia* stem canker and black scurf of potato. *Phytopathol.* 75(5): 560-564.

Beevi, N., S, Ancy Salim, M., Priya Mohan Deepti, S., and Naseema, A. 2005. Compatability of *Trichoderma harzianum* with botanicals and synthetic pesticides. *Geobios*, 32(3): 80-82.

Begoude, B. A. D., Lahlali, R., Friel, D., Tondje, P. R., and Jijakli, M. H. 2007. Response surface methodology study of the combined effects of temperature, pH, and aw on the growth rate of *Trichoderma asperellum. J. Appl. Microbiol.* 103(4): 845-854.

Belete, E., Ayalew, A., and Ahmed, S. 2015. Evaluation of local isolates of *Trichoderma* spp. against black root rot (*Fusarium solani*) on Faba bean. *J. Plant Pathol. Microbiol.* 32: 101-108.

Bell, D. K., Wells, H. D., and Markham, C. R. 1982. *In vitro* antagonism of *Trichoderma* species against six fungal plant pathogens. *Phytopathol.* 72(4): 379-382.

Benitez, T., Rincon, A. M., Limon, M. C., and Codon, A. C. 2004. Biocontrol mechanisms of *Trichoderma* strains. *Int. Microbiol.* 7(4): 249-260.

Berber, F., Touhami, O., Badoc, A., and Douira, A. 2009. *In vitro* and *in vivo* antagonism of two *Trichoderma* with respect to four pathogenic species of *Bipolaris* on sorghum. *Bull. Soc. Pharm. Bordeaux* 148: 93-114.

Bhagat, S. and Pan, S. 2006. Mass multiplication of *Trichoderma harzianum* on agricultural byproducts and their evaluation against seedling blight (*Rhizoctonia solani*) of mungbean and collar rot (*Sclerotium rolfsii*) of groundnut. *Indian J. Agric. Sci.* 77(9): 583.

Bhai, R. and Thomas, J. 2010. Compatibility of *Trichoderma harzianum* (Rifai.) with fungicides, insecticides and fertilizers. *Indian Phytopathol.* 63(2): 145-148.

Bhale, U. N., Wagh, P. M., and Rajkonda, J. N. 2013. Antagonistic confrontation of *Trichoderma* spp. against fruit rot pathogens on Sapodilla (*Manilkara zapota* L.). *J. Yeast Fungal Res.* 4(1): 5-11.

Bisset, J. 1991. A revision of the genus *Trichoderma*. II. Infrageneric classification. *Can. J. Bot.* 69: 2357-2372.

Biswas, K. K. and Sen, C. 2000. Management of stem rot of groundnut caused by *Sclerotium rolfsii* through *Trichoderma harzianum*. *Indian Phytopathol.* 53(3): 290-295.

Bonilla, Q. M. 2006. Technological innovation to control the "Green mold" (*Trichoderma* spp.) during the cultivation of edible mushrooms in the central region of Mexico. M.Sc.(Ag.) thesis, Post graduate college,Puebla, 69p.

Butt, T. M., Jackson, C. W., and Magan, N. 2001. *Fungi as biocontrol agents. Progress, Problems and Potential.* CABI publishing, UK, 390p.

Catcheside, D. E. A. and Catcheside, D. G. 1979. Survival of *Neurospora* conidia on silica gel. *Neurospora Newslett.* 26:24-25.

221

Cattelan, A. J., Hartel, P. G. and Fuhrmann, J. J., 1999. Screening for plant growth–promoting rhizobacteria to promote early soybean growth. *Soil Sci. Soc. Am. J.* 63(6): 1670-1680.

Cervantes, C. and Gutierrez-Corona, F. 1994. Copper resistance mechanisms in bacteria and fungi. *FEMS Microbiol. Rev.* 14(2): 121-137.

Chakraborty, B. N., Chakraborty, U., Saha, A., Dey, P. L., and Sunar, K. 2010. Molecular characterization of *Trichoderma viride* and *Trichoderma harzianum* isolated from soils of North Bengal based on rDNA markers and analysis of their PCR-RAPD profiles. *Glob. J. Biotechnol. Biochem.* 5(1):.55-61.

Chandra, A., Pathak, P. S., Bhatt, R. K. and Dubey, A., 2004. Variation in drought tolerance of different Stylosanthes accessions. *Biologia Plantarum.* 48(3): 457-460.

Cheng, Z., Park, E., and Glick, B. R. 2007. 1-Aminocyclopropane-1-carboxylate deaminase from *Pseudomonas putida* UW4 facilitates the growth of canola in the presence of salt. *Can. J. Microbiol.* 53:912-918.

Cherkupally, R., Amballa, H., and Bhoomi, N. R. 2017. *In vitro* screening for enzymatic activity of *Trichoderma* species for biocontrol potential. *Ann. Plant Sci.* 6(11): 1784-1789.

Chet, I. and Baker, R. 1981. Soil Naturally Suppressive to *Rhizoctonia solani. Phytopathol.* 71: 286-290.

Chung, Y. R. and Hoitink, H. A. J. 1990. Interactions between thermophilic fungi and *Trichoderma hamatum* in suppression of *Rhizoctonia* damping-off in a bark compost-amended container medium. *Phytopathol.* 80(1): 73-77.

222

Cole, J. S. and Zvenyika, Z. 1988. Integrated control of *Rhizoctonia solani* and *Fusarium solani* in tobacco transplants with *Trichoderma harziunum* and triadimenol. *Plant Pathol.* 37(2): 271-277.

Danielson, R. M. and Davey, C. B. 1973. Non nutritional factors affecting the growth of *Trichoderma* in culture. *Soil Biol. Biochem.* 5(5): 495-504.

Daryaei, A., Jones, E. E., Glare, T. R., and Falloon, R. E. 2016. pH and water activity in culture media affect biological control activity of *Trichoderma atroviride* against *Rhizoctonia solani*. *Biological Control*, 92: 24-30.

De Hoog, G. S., Guarro, J., Gene, J. and Figueras, M. J. 2000. *Atlas of clinical fungi* (2nd Ed.) CBS/ Rovira University, Reus, 988p.

De La Cruz, J., Pintor-Toro, J. A., Benitez, T., Llobell, A., and Romero, L. C. 1995. A novel endo-beta-1, 3-glucanase, BGN13. 1, involved in the mycoparasitism of *Trichoderma harzianum*. *J. Bacteriol.* 177(23): 6937-6945.

Dell'Amico, E., Cavalca, L. and Andreoni, V., 2008. Improvement of *Brassica napus* growth under cadmium stress by cadmium-resistant rhizobacteria. *Soil Biol. Biochem.* 40(1): 74-84.

De Marco, J. L. and Felix, C. R., 2002. Characterization of a protease produced by a *Trichoderma harzianum* isolate which controls cocoa plant witches' broom disease. *BMC Biochem.* 3(1):3.

De marco, J. L., Valadares- Inglis, M. C., and Felix, C. R. 2003. Production of hydrolytic enzymes by *Trichoderma* isolates with antagonistic activity

against *Crinipellis perniciosa*, the causal agent of witches broom of cocoa. *Brazilian J. Microbiol.* 34: 33-38.

Dennis, C. and Webster, J. 1971. Antagonistic properties of species-groups of *Trichoderma*: III. Hyphal interaction. *Trans. Br. Mycol. Soc.* 57(3): 363-369.

Deshmukh, P. S., Sairam, R. K., and Shukla, D. S. 1991. Measurement of ion leakage as a screening technique for drought resistance in wheat genotypes. *Indian J. Plant Physiol.* 35:89-91.

Djonovic, S., Vargas, W. A., Kolomiets, M. V., Homdeski, M., Wiest, A., and Kenerley, C.M. 2007. A proteinaceous elicitor Sm1 from the beneficial fungus *Trichoderma virens* is required for induced systemic resistance in maize. *Plant Physiol.* 145:875–889.

Domingues, F. C., Queiroz, J.A., Cabral, J. M. S., and Fonseca, L. P. 2001. Production of cellulases in batch culture using a mutant strain of *Trichoderma reesei* growing on soluble carbon source. *Biotechnol. Lett.* 23(10): 771-775.

Domsch, K. H., Gams, W., and Anderson, T. H. 1980. *Compendium of soil fungi*: Vol. 1. Academic Press, London, United Kingdom, 860p.

dos Reis Almeida, F. B., Cerqueira, F. M., do Nascimento Silva, R., Ulhoa, C. J., and Lima, A. L. 2007. Mycoparasitism studies of *Trichoderma harzianum* strains against *Rhizoctonia solani*: evaluation of coiling and hydrolytic enzyme production. *Biotechnol. Lett.* 29(8): 1189-1193.

224

Druzhinina, I. S. and Kubicek, C. P. 2005. Species concept and biodiversity in *Trichoderma* and *Hypocrea*: from aggregate species to species clusters. *J. Zhejiang University Sci.* 6(2): 100-112.

Durak, E. D. 2016. April. Biological control of *Rhizoctonia solani* on potato by using indigenous *Trichoderma* spp. In: *AIP Conference Proceedings*; 1726 Apr. 2016, (Vol. 1726, No. 1, p. 020020). AIP Publishing.

Dworkin, M. and Foster, J. 1958. Experiments with some microorganisms which utilize ethane and hydrogen. *J. Bacteriol.* 75:592-601

Eastburn, D. M. and Butler, E. E. 1991. Effects of soil moisture and temperature on the saprophytic ability of *Trichoderma harzianum*. Mycologia 6:257-263.

El Badawy, N., El Kharbotly, A., Hassanein, M., and Al-Marri, M. J. 2016. Selection of *Trichoderma* spp. tolerant to abiotic stresses with antagonistic activities against date palm leaf spot diseases in Qatar. *Am. J. Res. Commun.* 4(12): 85-101.

Elad, Y., Chet, I., and Katan, J. 1980. *Trichoderma harzianum*: A biocontrol agent effective against *Sclerotium rolfsii* and *Rhizoctonia solani*. *Phytopathol.* 70(2): 119-121.

Elad, Y., Chet, I. and Henis, Y. 1981. A selective medium for improving quantitative isolation of *Trichoderma* spp. from soil. *Phytoparasitica* 9(1): 59-67.

Elad, Y., Chet, I. and Henis, Y. 1982. Degradation of plant pathogenic fungi by *Trichoderma harzianum*. *Can. J. Microbiol.* 28(7): 719-725.

225

Elad, Y. and Kapat, A. 1999. The role of *Trichoderma harzianum* protease in the biocontrol of *Botrytis cinerea*. *Eur. J. Plant Pathol.* 105(2): 177-189.

Elad, Y. 2000. Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. *Crop Prot.* 19: 709–714.

El-Katatny, M., Gudelj, M., Robra, K. H., Elnaghy, M., and Gübitz, G. 2001. Characterization of a chitinase and an endo-β-1, 3-glucanase from *Trichoderma harzianum* Rifai T24 involved in control of the phytopathogen *Sclerotium rolfsii*. *Appl. Microbiol. Biotechnol.* 56(1-2): 137-143.

El Kharbotly, A., El Badawy, N., and Mazen, M. 2016. Biodiversity in *Trichoderma* species isolated from Qatar. *QSci. Proc.* (4): 38.

Etebarian, H. R., Scott, E. S., and Wicks, T. J. 2000. *Trichoderma harzianum* T39 and *T. virens* DAR 74290 as potential biological control agents for *Phytophthora erythroseptica*. *Eur. J. Plant Pathol.* 106(4): 329-337.

Eziashi, E. I., Omamor, I. B., and Odigie, E. E. 2007. Antagonism of *Trichoderma viride* and effects of extracted water soluble compounds from *Trichoderma* species and benlate solution on *Ceratocystis paradoxa*. *Afr. J. Biotechnol.* 6(4): 101-107.

Fajola, A. O. and Alasoadura, S. O. 1975. Antagonistic effects of *Trichoderma harzianum* on *Pythium aphanidermatum* causing the damping-off disease of tobacco in Nigeria. *Mycopathologia* 57(1): 47-52.

Fillinger, S., Chaveroce, M. K., Van Dijck, P., De Vries, R., Ruijter, G., Thevelein, J., and D'Enfert, C. 2001. Trehalose is required for the

226

acquisition of tolerance to a variety of stresses in the filamentous fungus *Aspergillus nidulans*. *Microbiol.* 147: 1851–1862.

Gajera, H. P., Patel, S. V., Khatrani, T. J., and Goalkiya, B. A. 2012. Antagonism of *Trichoderma* spp. against *Macrophomina phaseolina*: evaluation of coiling and cell wall degrading enzymatic activities. *J. Plant Pathol. Microbiol.* 44: 36-38.

Gajera, H. P. and Vakharia, D. N. 2012. Production of lytic enzymes by *Trichoderma* isolates during in vitro antagonism with *Aspergillus niger*, the causal agent of collar rot of peanut. *Brazilian J. Microbiol.* 43(1): 43-52.

Gal-Hemed, I., Atanasova, L., Komon-Zelazowska, M., and Yarden, O. 2011. Marine isolates of *Trichoderma* as potential halotolerant agents of biological control for arid-zone agriculture. *Appl. Environ. Microbiol.*, 77(15):5100–5109.

Gams, W. and Bissett, J. 2002. Morphology and identification of *Trichoderma*. In: Kubicek, C. P. and Harman, G. E. (eds.). *Trichoderma and Gliocladium: Basic biology, taxonomy and genetics*. Taylor & Francis Ltd, London, pp. 3-31.

Gawade, D. B., Pawar, B. H., Gawande, S. J., and Vasekar, V. C. 2012. Antagonistic Effect of *Trichoderma* against *Fusarium moniliformae* the causal of sugarcane wilt. *Am. J. Agric. Environ. Sci.* 12(9): 1236-1241.

Geremia, R. A., Goldman, G. H., Jacobs, D., Ardrtes, W., Vila, S. B., Van Montagu, M., and Herrera-Estrella, A. 1993. Molecular characterization of the proteinase-encoding gene, prb1, related to mycoparasitism by *Trichoderma harzianum*. *Mol. Microbiol.* 8(3): 603-613.

227

Ghildiyal, A. and Pandey, A. 2008. Isolation of cold tolerant antifungal strains of *Trichoderma* sp. from glacial sites of Indian Himalayan region. *Res. J. Microbiol*. 3(8): 559-564.

Glick, B., Patten, C., Holguin, G., and Penrose, D. 1999. *Biochemical and genetic mechanisms used by plant growth promoting bacteria*. World scientific, Singapore, 267 p.

Glick, B. R., Penrose, D. M., and Li, J. 1998. A model for the lowering of plant ethylene concentrations by plant growth promoting bacteria. *J. Theor. Biol*. 190: 63-68.

Gomes, N. C. M., Mendonca-Hagler, L., and Savvaidis, I. 1998. Metal bioremediation by microorganisms. *Rev. Microbiol*. 29: 85–92.

Gomez, K. A. and Gomez, A. A. 1984. *Statistical procedures for agricultural research*. John Wiley & Sons, Australia, 639p.

Gravel, V., Antoun, H., and Tweddell, R. J. 2007. Growth stimulation and fruit yield improvement of greenhouse tomato plants by inoculation with *Pseudomonas putida* or *Trichoderma atroviride*: possible role of indole acetic acid (IAA). *Soil Biol. Biochem*.39(8): 1968-1977.

Grichko, V. P. and Glick, B. R. 2001. Amelioration of flooding stress by ACC deaminase containing plant growth-promoting bacteria. *Plant Physiol. Biochem*. 39: 11-17.

Grove, S. N., Bracker, C. E., and Morre, D. J. 1970. An ultrastructural basis for hyphal tip growth in *Pythium ultimum*. *Am. J. Bot*. 57(3): 245-266.

228

Grover, R. K. and Moore, J. D. 1962. Toximetric studies of fungicides against brown rot organism *Sclerotina fruticola*. *Phytopathol*. 52: 876-880.

Guevara, C. and Zambrano, M. M. 2006. Sugarcane cellulose utilization by a defined microbial consortium. *Fems. Microbiol. Lett.* 255: 52-58.

Gusain, Y. S., Singh, U. S., and Sharma, A. K. 2014. Enhance activity of stress related enzymes in rice (*Oryza sativa* L.) induced by plant growth promoting fungi under drought stress. *Afr. J. Agric. Res.* 9(19): 1430-1434.

Han, H. S. and Lee, K. D. 2005. Plant growth promoting rhizobacteria effect on antioxidant status, photosynthesis, mineral uptake and growth of lettuce under soil salinity. *Res. J. Agric. Biol. Sci.* 1(3): 210-215.

Haritha, V. 2003. Studies on the management of damping off disease incited by *Pythiuma aphanidermatum* (Edson) Fitzp in Tobacco nurseries. MSc. thesis Acharya N. G. Ranga Agricultural University, Rajendranagar, Hyderabad, 109p.

Harman, G. E. 2000. Myths and dogmas of biocontrol changes in perceptions derived from research on *Trichoderma harzinum* T-22. *Plant Dis.* 84(4): 377-393.

Harman, G. E., Chet, I., and Baker, R. 1980. *Trichoderma hamatum* effects on seed and seedling disease induced in radish and pea by *Pythium* spp. or *Rhizoctonia solani*. *Phytopathol*. 70(12): 1167-1172.

Harman, G. E., Jin, X., Stasz, T. E., Peruzzotti, G., Leopold, A. C., and Taylor, A. G. 1991. Production of conidial biomass of *Trichoderma harzianum* for biological control. *Biological Control* 1(1): 23-28.

229

Harman, G. E., Howell, C. R., Viterbo, A., Chet, I., and Lorito, M. 2004. *Trichoderma* species- opportunistic, avirulent plant symbionts. *Nat. Rev. Microbiol.* 2(1): 43.

Hayes A. J. 1965. Some microfungi from Scots pine litter. *Trans. Brit. Mycol. Sot.* 48: 179-185.

Henis, Y., Lewis, J. A., and Papavizas, G. C. 1984. Interactions between *Sclerotium rolfsii* and *Trichoderma* spp: Relationship between antagonism and disease control. *Soil Biol. Biochem.* 16(4): 391-395.

Hicks, E., Bienkowski, D., Braithwaite, M., McLEAN, K., Falloon, R., and Stewart, A. 2014. Trichoderma strains suppress Rhizoctonia diseases and promote growth of potato. *Phytopathologia Mediterranea*, pp.502-514.

Hima, V. V. 2017. Enhancing bio- efficiency of *Trichoderma* spp. for the management of soil borne fungal pathogens. Ph. D (Ag) thesis, Kerala Agricultural University, Thrissur, 195p.

Howell, C. R. and Stipanovic, R. D. 1983. Gliovirin, a new antibiotic from *Gliocladium virens*, and its role in the biological control of *Pythium ultimum*. *Can. J. Microbiol.* 29(3): 321-324.

Howell, C. R. 2003. Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: history and evolution of current concepts. *Plant Dis.* 89(11): 1195-1200.

Islam, M. S., Ali, M., and Rahman, M. S. 2011. *In vitro* studies on the fungicidal effect on *Trichoderma* species in tea plantation. *Bangladesh J. Agric. Res.* 36(4): 677-683.

230

Iqbal, S. 2017. Isolation, preservation and revival of *Trichoderma Viride* in culture media. *J. Entomol. Zoology Stud.* 5(3): 1640-1646.

Jackson, M. L. 1958. *Soil chemical analysis: advanced course.* UW-Madison Libraries Parallel Press London, 925p.

Jackson, A. M., Whipps, J. M., and Lynch, J. M. 1991. Effects of temperature, pH and water potential on growth of four fungi with disease biocontrol potential. *World J. Microbiol. Biotechnol.* 7(4): 494-501.

Jegathambigai, V., Wijeratnam, R. W., and Wijesundera, R. L. C. 2010. Effect of *Trichoderma* sp. on *Sclerotium rolfsii*, the Causative Agent of Collar Rot on *Zamioculcas zamiifolia* and an on Farm Method to Mass Produce *Trichoderma* species. *Plant Pathol. J.* 9(2): 47-55.

John, R. P., Tyagi, R. D., Prévost, D., Brar, S. K., Pouleur, S., and Surampalli, R.Y. 2010. Mycoparasitic *Trichoderma viride* as a biocontrol agent against *Fusarium oxysporum* f. sp. *adzuki* and *Pythium arrhenomanes* and as a growth promoter of soybean. *Crop Prot.* 29(12): 1452-1459.

Kamizono, A., Nishizawa, M., Teranishi, Y., Murata, K., and Kimura, A. 1989. Identification of a gene conferring resistance to zinc and cadmium ions in the yeast *Saccharomyces cerevisiae. Mol. Gen. Genet.* 219(1-2): 161-167.

Karaoglu, S. A., Bozdeveci, A., and Pehlivan, N. 2018. Characterization of local *Trichoderma* spp. as potential bio-control agents, screening of in vitro antagonistic activities and fungicide tolerance. *Hacettepe J. Biol. Chem.* 46(2): 247-261.

Karpagavalli, S. 1997. Effect of different fungicides on the growth of *Trichoderma*. *Indian J. Plant Prot.* 25(1): 82-83.

Kavitha, C. and Sujatha, M. P. 2015. Evaluation of soil fertility status in various agro ecosystems of Thrissur District, Kerala, India. *Int. J. Agric Crop Sci.* 8(3): 328.

Kendrick W. B. and Burges A. 1962. Biological aspects of the decay of *Pinus syluestris* leaf litter. *Norla Hedwigia.* 4(3): 15-344.

Khan, M. A., Gul, B., and Weber, D. J. 2001. Effect of salinity on the growth and ion content of *Salicornia rubra. Commun. Soil Sci. Plant Anal.* 32(17-18): 2965-2977.

Khan, M.O. and Shahzad, S. 2007. Screening of *Trichoderma* species for tolerance to fungicides. *Pak. J. Bot.* 39(3): 945-951.

Khandelwal, M., Datta, S., Mehta, J., Naruka, R., Makhijani, K., Sharma, G., Kumar, R., and Chandra, S. 2012. Isolation, characterization and biomass production of *Trichoderma viride* using various agro products-A biocontrol agent. *Adv. Appl. Sci. Res.* 3(6): 3950-3955.

Kitamoto, Y., Suzuki, A., Shimada, S. and Yamanaka, K. 2002. A new method for the preservation of fungus stock cultures by deep-freezing. *Mycosci.* 43(2): 0143-0149.

Kneer, R., Kutchan, T. M., Hochberger, A., and Zenk, M. H. 1992. *Saccharomyces cerevisiae* and *Neurospora crassa* contain heavy metal sequestering phytochelatin. *Arch. Microbiol.* 157: 305–310.

Kolmark, H. G. 1979. Preservation of *Neurospora* stock cultures with the silica gel method for extended periods of time. *Neurospora Newslett.* 26: 26.

Koruth, A., Kumary, S. L., and Sreekumaran, V. 2014. *Three decades of soil science research in Kuttanad and Pokkali.* Kerala agricultural university press, Mannuthy, 72P.

Kredics, L., Antal, Z., Manczinger, L., Szerkeres, A., Kevei, F., and Nagy, E. 2003. Influence of environmental parameters on *Trichoderma* strains with biocontrol potential. *Food Technol. Biotech.* 41: 37–42.

Kucuk, C. and Kivanc, M. 2004. Isolation of *Trichoderma* spp. and determination of their antifungal, biochemical and physiological features. *Turkish J. Biol.* 27(4): 247-253.

Kumar, K., Amaresan, N., Bhagat, S., Madhuri, K., and Srivastava, R. C. 2012. Isolation and characterization of *Trichoderma* spp. for antagonistic activity against root rot and foliar pathogens. *Indian J. Microbiol.* 52(2): 137-144.

Kumar, K., Manigundan, K., and Amaresan, N. 2017. Influence of salt tolerant *Trichoderma* spp. on growth of maize (*Zea mays*) under different salinity conditions. *J. Basic Microbiol.* 57(2): 141-150.

Kumar, V., Kumar, A., Srivastava, M., Pandey, S., Shahid, M., Srivastava, Y. K., and Trivedi, S. 2016. *Trichoderma harzianum* (Th. azad) as a Mycoparasite of *Fusarium* and growth enhancer of Tomato in Glass house Conditions. *J. Pure Appl. Microbiol.* 10(2): 1463-1468.

Lewis, J. A. and Larkin, R. P. 1997. Extruded granular formulation with biomass of biocontrol *Gliocladium virens* and *Trichoderma* spp. to reduce damping-off of eggplant caused by *Rhizoctonia solani* and saprophytic

growth of the pathogen in soil-less mix. *Biocontrol Sci. Technol.* 7(1): 49-60.

Lewis, J. A., Fravel, D. R., Lumsden, R. D., and Shasha, B. S. 1991. Application of biocontrol fungi in granular formulations of pregelatinized starch-flour to control damping-off diseases caused by *Rhizoctonia solani*. *Biological control.* 5(3): 397-404.

Lewis, J. A. and Lumsden, R. D. 2001. Biocontrol of damping-off of greenhouse-grown crops caused by *Rhizoctonia solani* with a formulation of *Trichoderma* spp. *Crop Prot.* 20(1): 49-56.

Li, C., Yang, Z., Zhang, R.H.C., Zhang, D., Chen, S., and Ma, L. 2013. Effect of pH on cellulase production and morphology of *Trichoderma reesei* and the application in cellulosic material hydrolysis. *J. Biotechnol.* 168(4): 470-477.

Lieckfeldt, E., Samuels, G. J., Nirenberg, H. I., and Petrini, O. 1999. A morphological and molecular perspective of *Trichoderma viride*: is it one or two species?. *Appl. Environ. Microbiol.* 65(6): 2418-2428.

Lifshitz, R., Windham, M. T., and Baker, R. 1986. Mechanism of biological control of pre emergence damping-off of pea by seed treatment with *Trichoderma* spp. *Phytopathol.* 76(7): 720-725.

Lin, X. and Heitman, J. 2005. Chlamydospore formation during hyphal growth in *Cryptococcus neoformans*. *Eukaryot. cell* 4(10): 1746-1754.

Lorito, M., Woo, S. L., D'ambrosio, M., Harman, G. E., Hayes, C. K., Kubicek, C. P., and Scala, F. 1996. Synergistic interaction between cell wall degrading

234

enzymes and membrane affecting compounds. *Mol. Plant Microbe Interact.* 9(3): 206-213.

Lu, Z., Tombolini, R., Woo, S., Zeilinger, S., Lorito, M., and Jansson, J. K. 2004. *In vivo* study of *Trichoderma*-pathogen-plant interactions, using constitutive and inducible green fluorescent protein reporter systems. *Appl. Environ. Microbiol.* 70(5): 3073-3081.

Macauley, B. J. and Thrower, L. B. 1966. Succession of fungi in leaf litter of Eucalyptus regnans. *Trans. Br. Mycol. Soc.* 49(3): 509-520.

Magan, N. and Whipps, J. M. 1988. Growth of *Coniothyrium minitans*, *Gliocladium roseum*, *Trichoderma harzianum* and *T. viride* from alginate pellets and interaction with water availability 1. *EPPO Bulletin*, 18(1): 37-45.

Maity, D. and Sen, C. 1985. Integrated biocontrol of *Sclerotium rolfsii* with nitrogenous fertilizers and *Trichoderma harzianum*. *Indian J. Agric. Sci.* 9: 112-115.

Mandhare, V. K. and Suryawanshi, A.V. 2005. Standarization of storage conditions to increase the shelf life of *Trichoderma* formulations. *Agric. Sci. Digest.* 25 (1):71-73.

Marcello, C. M., Steindorff, A. S., da Silva, S. P., do Nascimento Silva, R., Bataus, L. A. M., and Ulhoa, C. J. 2010. Expression analysis of the exo-β-1, 3-glucanase from the mycoparasitic fungus *Trichoderma asperellum*. *Microbiol. Res.* 165(1): 75-81.

235

Martin, K., McDougall, B. M., McIlroy, S., Chen, J., and Seviour, R. J. 2007. Biochemistry and molecular biology of exocellular fungal β-(1, 3)-and β-(1, 6)-glucanases. *FEMS Microbiol. Rev.* 31(2): 168-192.

Marulanda, A., Porcel, R., Barea, J. M. and Azcón, R. 2007. Drought tolerance and antioxidant activities in lavender plants colonized by native drought-tolerant or drought-sensitive *Glomus* species. *Microbial Ecol.* 54(3): 543.

Maurya, M. K., Srivastava, M., Singh, A., Pandey, S., and Ratan, V., 2017. Effect of different temperature and culture media on the mycelia growth of *Trichoderma viride* isolates. *Int. J. Curr. Microbiol. App. Sci.* 6(2): 266-269.

Mayak, S., Tirosh, T., and Glick, B. 2004. Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. *Plant Physiol. Biochem.* 42: 565–572.

Mayo, S., Gutierrez, S., Malmierca, M. G., Lorenzana, A., Campelo, M. P., Hermosa, R., and Casquero, P. A. 2015. Influence of *Rhizoctonia solani* and *Trichoderma* spp. in growth of bean (*Phaseolus vulgaris* L.) and in the induction of plant defense-related genes. *Front. Plant Sci.* 6: 685.

McAllister, C. B., Garcia-Romera, I., Godeas, A., and Ocampo, J. A. 1994. *In vitro* interactions between *Trichoderma koningii*, *Fusarium solani* and *Glomus mosseae*. *Soil Biol. Biochem.* 26(10): 1369-1374.

Melo, I. S. D. and Faull, J. L. 2000. Parasitism of *Rhizoctonia solani* by strains of *Trichoderma* spp. *Scientia Agricola.* 57(1): 55-59.

Metcalf, D. A. and Wilson, C. R. 2001. The process of antagonism of *Sclerotium cepivorum* in white rot affected onion roots by *Trichoderma koningii*. *Plant Pathol.* 50(2): 249-257.

Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* 7(9): 405-410.

Mohan, A. and Sreelatha, A. K. 2016. Nutrient dynamics in *Pokkali* soils. *Int. J. Appl.Pure Sci. Agric.* 2 (9): 119-123.

Mokhtar, H. and Dehimat, L. 2015. *In vitro* and *in vivo* efficiency of *Trichoderma harzianum* against *Phoma* and *Glocladium* Soft Rot Occurred on Tomato Fruits (*Lycopersicon esculentum*). *Int. J. Curr. Microbiol. Appl. Sci.* 4(8): 141-147.

Mona, S. A., Hashem, A., Abd_Allah, E. F., Alqarawi, A. A., Soliman, D. W. K., Wirth, S., and Egamberdieva, D. 2017. Increased resistance of drought by *Trichoderma harzianum* fungal treatment correlates with increased secondary metabolites and proline content. *J. Integrative Agric.* 16(8): 1751-1757.

Montealegre, J., Valderrama, L., Sanchez, S., Herrera, R., Besoain, X., and Perez, L. M., 2010. Biological control of *Rhizoctonia solani* in tomatoes with *Trichoderma harzianum* mutants. *Electr. J. Biotechnol.* 13(2): 1-2.

Monteiro, V. N. and Ulhoa, C. J. 2006. Biochemical characterization of a β-1, 3-glucanase from *Trichoderma koningii* induced by cell wall of *Rhizoctonia solani*. *Curr. Microbiol.* 52(2): 92-96.

Montenecourt, B. S. and Eveleigh, D. E. 1977. Preparation of mutants of *Trichoderma reesei* with enhanced cellulase production. *Appl. Environ. Microbiol.* 34(6): 777-782.

Moretti, M ., Bocchini-Martins, D. A., Silva, R. D., Rodrigues, A., Sette, L. D., and Gomes, E. 2012. Selection of thermophilic and thermotolerant fungi for the production of cellulases and xylanases under solid-state fermentation. *Brazilian J. Microbiol.* 43(3): 1062-1071.

Moustafa, A. F. and Abdel-Azeem, A. M. 2008. *Thielavia gigaspora*, a new thermotolerant ascomycetes from Egypt. *Microbiol. Res.* 163:441–444.

Mukherjee, P. K., Upadhyay, J. P., and Mukhopadhyay, A. N. 1989. Biological control of *Pythium* damping-off of cauliflower by *Trichoderma harzianum. J. Biological control* 3: 119-124.

Mustafa, A., Khan, M. A., Inam-ul-Haq, M., Pervez, M. A., and Umar, U. 2009. Usefulness of different culture media for in vitro evaluation of *Trichoderma* spp. against seed borne fungi of economic importance. *Pak. J. Phytopathol,* 21(1): 83-88.

Muthukumar, A., Eswaran, A., and Sanjeevkumas, K. 2011. Exploitation of *Trichoderma* species on the growth of *Pythium aphanidermatum* in chilli. *Brazilian J. Microbiol.* 42(4): 1598-1607.

Nagar, S. 2017. Studies on bio-control and plant growth promoting efficacy of *Trichoderma* strains in chickpea (*Cicer arietinum* L.). Doctoral dissertation, Rvskvv, Gwalior, 160p.

Nallathambi, P., Umamaheswari, C., Thakore, B. B. L., and More, T. A. 2009. Post-harvest management of ber (*Ziziphus mauritiana* Lamk) fruit rot

238

(*Alternaria alternata* Fr. Keissler) using *Trichoderma* species, fungicides and their combinations. *Crop Prot.* 28(6): 525-532.

Narayanan, S., Lata, K. P., and Kotasthane, A. S. 2006. Genetic relatedness among *Trichoderma* isolates inhibiting a pathogenic fungi *Rhizoctonia solani*. *Afr. J. Biotechnol.* 5(8): 580-584.

Nayani, S., Mayak, S., and Glick, B. R. 1998. Effect of plant growth-promoting rhizobacteria on senescence of flower petals. *Indian J. Exp. Biol.* 36: 836-839.

Nuzum, C. 1989. A simple method for the preservation of some non-sporing fungi. *Australasian Plant Pathol.* 18(4): 104-105.

Oerke, E. C. 2006. Crop losses to pests. *J. Agric. Sci.* 144(1): 31-43.

Okoth, S. A., Roimen, H., Mutsotso, B., Muya, E., Kahindi, J., Owino, J. O., and Okoth, P. 2007. Land use systems and distribution of *Trichoderma* species in Embu region, Kenya. *Trop. Subtrop. Agroecosystems* 7(2): 105-122.

Ortiz-Castro, R., Contreras-Cornejo, H. A., Macias-Rodríguez, L., and Lopez-Bucio, J. 2009. The role of microbial signals in plant growth and development. *Plant Signal Behav.* 4(8): 701-712.

Padder, B. A. and Sharma, P. N. 2011. *In vitro* and *in vivo* antagonism of biocontrol agents against *Colletotrichum lindemuthianum* causing bean anthracnose. *Arch. Phytopathol. Plant Prot.* 44(10): 961-969.

Pandya, J. R. 2010. Isolation, identification, evaluation and mass production of native *Trichoderma* sp. Doctoral dissertation, Navsari Agricultural University, Navsari, 107p.

Papavizas, G. C. 1981. Survival of *Trichoderma harzianum* in soil and in pea and bean rhizospheres. *Phytopathol.* 72(1): 121-125.

Papavizas, G. C. 1985. *Trichoderma* and *Gliocladium*: biology, ecology, and potential for biocontrol. *Annu. Rev Phytopathol.* 23(1): 23-54.

Park, M. S., Seo, G. S., Lee, K. H., Bae, K. S., and Yu, S. H. 2005. Morphological and cultural characteristics of *Trichoderma* spp. associated with green mold of oyster mushroom in Korea. *Plant Pathol. J.* 21(3): 221-228.

Parkinson, D., Gray, T. R., and Williams, S. T. 1971. *Methods for study-ing the ecology of soil micro-organisms.* Oxford, Blackwell Scientific Publications, New Jersey. 116 p.

Parmar, H. J., Bodar, N. P., Lakhani, H. N., Patel, S.V., Umrania, V. V., and Hassan, M. M. 2015. Production of lytic enzymes by *Trichoderma* strains during in vitro antagonism with *Sclerotium rolfsii*, the causal agent of stem rot of groundnut. *Afr. J. Microbiol. Res.* 9(6): 365-372.

Paul, J. S., Tiwari, K. L., and Jadhav, S. K. 2015. Long term preservation of commercial important fungi in glycerol at 4^0C. *Int. J. Biol. Chem.* 9(2): 79-85.

Perkins, D. D. 1963. Use of a helper strain in *Neurospora crassa* to maintain stocks of uvs-4 and uvs-5, which deteriorate unless sheltered in heterokaryons. *Fungal Genet. Newslett.* 40:66.

Poosapati, S., Ravulapalli, P. D., Tippirishetty, N., Vishwanathaswamy, D. K., and Chunduri, S. 2014. Selection of high temperature and salinity tolerant

240

Trichoderma isolates with antagonistic activity against *Sclerotium rolfsii.* *SpringerPlus*, 3(1): 641.

Pounder, J. I. and Bowman, B. J. 1999. Storage of Aconidial Strains of *Neurospora crassa* by Freezing at -80^{0}C. *Fungal Genet Rep.* 46(1): 32-33.

Prakash, M. G., Gopal, K. V., Anandaraj, M., and Sarma, Y. R. 1999. Evaluation of substrates for mass multiplication of fungal biocontrol agents *Trichoderma harzianum* and *T. virens.* *J. Spices Aromat. Crops* 8(2): 207-210.

Premchandra, G. S., Saneoka, H., and Ogata, S. 1990. Cell membrane stability an indicator of drought tolerance as affected by applied nitrogen in soybean. *J. Agric. Sci.* 115: 1332-1343.

Presta, A. and Stillman, M. J. 1997. Incorporation of copper into the yeast *Saccharomyces cerevisiae* Identification of Cu (I)- Methallothionein in intact yeast cells. *J. Inorg. Biochem.* 66: 231–240.

Przybylowicz, P. and Donohue, J. 1988. *Shiitake Grower's Handbook.* Kendall/Hunt Publishing Co., Dubuque, 217p.

Purkayastha, R. P. and Bhattacharyya, B. 1982. Antagonism of micro-organisms from jute phyllosphere towards *Colletotrichum corchori.* *Trans. Br. Mycol. Soc.* 78(3): 509-513.

Rahman, A., Begum, M. F., Rahman, M., Bari, M. A., Illias, G. N. M., and Alam, M. F. 2011. Isolation and identification of *Trichoderma* species from different habitats and their use for bioconversion of solid waste. *Turkish J. Biol.* 35(2): 183-194.

Rai, S. 2017. Genomic diversity of antagonistic *Trichoderma* species against phytopatogen of *Lycopersicon Esculentum* Mill. Doctoral dissertation, Dept. of Microbiology, Sam higginbottom university of agriculture, technology and science, 216p.

Rashmi, K., Sinha, B., Pramesh, K. and Devi, P. S., 2017. Native *Trichoderma* spp. for the Management of Stem Rot of Groundnut Caused by *Sclerotium rolfsii* Sacc in Manipur, India. *Int. J. Curr. Microbiol. App. Sci.* 6(10): 1343-1351.

Rasool, S., Hameed, A., Azooz, M. M., Siddiqi, T. O., and Ahmad, P. 2013. *Ecophysiology and responses of plants under salt stress.* Springer, New York, pp.1-24.

Raut, I., Oancea, F., Trinidad, A. B. L. S., Calin, M., Constantinescu-aruxandei, D., Doni, M. B., Arsene, M. L., Vasilescu, G., Şesan, T. E., and Jecu, L. 2017. Evaluation of *Trichoderma* spp. as a biocontrol agent against *Phytophthora parasitica. Sci. Bull. Ser. Biotechnol.* 21: 179-182.

Rawat, L., Singh, Y., Shukla, N., and Kumar, J. 2013. Salinity tolerant *Trichoderma harzianum* reinforces NaCl tolerance and reduces population dynamics of *Fusarium oxysporum* f. sp. *ciceri* in chickpea (*Cicer arietinum* L.) under salt stress conditions. *Arch. Phytopathol. Plant Prot.* 46(12): 1442-1467.

Rawat, L., Bisht, T. S., Kukreti, A., and Prasad, M. 2016. Bioprospecting drought tolerant *Trichoderma harzianum* isolates promote growth and delay the onset of drought responses in wheat (*Triticum aestivum* L.). *Mol. Soil Biol.* 7: 102- 108.

242

Reetha, S., Bhuvaneswari, G., Selvakumar, G., Thamizhiniyan, P., and Pathmavathi, M. 2014. Effect of temperature and pH on growth of fungi *Trichoderma harzianum*. *J. Chem. Biol. Phys. Sci.* 4: 3287-92.

Resende, M. I. P., Jakoby, I. C. M. C., dos Santos, L. C. R., Soares, M. A., Pereira, F. A. D. I., Souchie, E. L., and Silva, F. G. 2014. Phosphate solubilization and phytohormone production by endophytic and rhizosphere *Trichoderma* isolates of guanandi (*Calophyllum brasiliense* Cambess). *Afr. J. Microbiol. Res.* 8(27): 2616-2623.

Riddell, R. W. 1950. Permanent stained mycological preparations obtained by slide culture. *Mycologia* 42(2): 265-270.

Rifai, M. A. 1969. A revision of the genus *Trichoderma. Mycological Papers.* 116: 1-56.

Rini, C. R. and Sulochana, K. K. 2007. Management of seedling rot of chilli (*Capsicum annuum* L.) using *Trichoderma* spp. and fluorescent pseudomonads (*Pseudomonas fluorescens*). *J. Trop. Agric.* 44: 79-82.

Rodriguez-Kabana, R. 1969. Enzymatic interactions of *Sclerotium rolfsii* and *Trichoderma viride* in mixed soil culture. *Phytopathol.* 4(3): 112- 116.

Rojo, F. G., Reynoso, M. M., Ferez, M., Chulze, S. N., and Torres, A. M. 2007. Biological control by *Trichoderma* species of *Fusarium solani* causing peanut brown root rot under field conditions. *Crop Prot.* 26(4): 549-555.

Romero, A. O. 2007. Technological development to control green mold attack (*Trichoderma* spp.) for commercial cultivation of edible fungi (*Pleurotus ostreatus* and *Lentinula edodes*) in Mexico. *Afr. J. Agric. Res.* 7(34): 4724-4730.

Ross, C. W. and Salisbury, F. B. 1992. Hormones and growth regulators: Cytokinins, ethylene, abscissic acid, and other compounds. *Plant Physiol.* 382-407.

Rousk, J., Brookes, P. C., and Baath, E. 2009. Soil bacterial and fungal communities across a pH gradient in an arable soil. *Appl. Environ. Microbiol.* 4: 1340-1351

Ruijter, G. J. G., Bax, M., Patel, H., Flitter, S. J., van de Vondervoort, P. J. I., De Vries R. P., Van Kuyk, P. A., and Visser, J. 2003. Mannitol is required for stress tolerance in *Aspergillus niger* conidiospores. *Eukaryot. Cell* 4:690–698.

Ryu, D. D. Y. and Mandels, M. 1980. Cellulases: biosynthesis and applications. *Enzyme Microbial Technol.* 2: 91–102.

Sab, J., Nagaraja, A., and Nagamma, G. 2014. Efficacy of bio-pesticides against *Sclerotium rolfsii* sacc. causing collar rot of chickpea (*Cicer arietinum* L.). *Bioscan.* 9(1): 335-339.

Sadasivam, S. and Manickam, A. 2008. *Biochemical methods* (3rd Ed.). New Age International Publishers, New Delhi, India, pp 43-47.

Saju, K. A., Anandaraj, M., and Sarma, Y. R. 2002. On farm production of *Trichoderma harzianum* using organic matter. *Indian Phytopathol.* 55(3): 277-281.

Samuels, G. J., Dodd, S. L., Gams, W., Castlebury, L. A., and Petrini, O. 2002. *Trichoderma* species associated with the green mold epidemic of commercially grown *Agaricus bisporus*. *Mycologia* 94(1): 146-170.

244

Santos, V. L. and Linardi, V. R. 2004. Biodegradation of phenol by a filamentous fungi isolated from industrial effluents identification and degradation potential. *Process. Biochem.* 39: 1001-1006.

Sarkar, S., Narayanan, P., Divakaran, A., Balamurugan, A., and Premkumar, R. 2010. The *in vitro* effect of certain fungicides, insecticides, and biopesticides on mycelial growth in the biocontrol fungus *Trichoderma harzianum. Turkish J. Biol.* 34(4): 399-403.

Savitha, M. J. and Sriram, S., 2015. Morphological and molecular identification of *Trichoderma* isolates with biocontrol potential against *Phytophthora* blight in red pepper. *Pest Manag. Hortic. Ecosyst.* 21(2): 194-202.

Sekhar, Y. C., Ahammed, S. K., Prasad, T. N. V. K. V., and Devi, R. S. J. 2017. Identification of *Trichoderma* species based on morphological characters isolated from rhizosphere of groundnut (*Arachis hypogaea* L.). *Int. J. Sci. Environ. Technol.* 6(3): 2056 – 2063.

Shah, S., Nasreen, S., and Sheikh, P. A. 2012. Cultural and Morphological Characterization of *Trichoderma* spp. Associated with Green Mold Disease of *Pleurotus* spp. in Kashmir. *Res. J. Microbiol.* 7: 139-144.

Shakeri, J. and Foster, H. A. 2007. Proteolytic activity and antibiotic production by *Trichoderma harzianum* in relation to pathogenicity to insects. *Enzyme Microbial Technol.* 40(4): 961-968.

Sharma, K. K. and Singh, U. S. 2014. Cultural and morphological characterization of rhizospheric isolates of fungal antagonist *Trichoderma. J. Appl. Nat. Sci.* 6(2): 451-456.

Shukla, N., Awasthi, R. P., Rawat, L., and Kumar, J. 2012. Biochemical and physiological responses of rice (*Oryza sativa* L.) as influenced by *Trichoderma harzianum* under drought stress. *Plant Physiol. Biochem.* 54: 78-88.

Singh, K. 2016. Screening of indigenous *Trichoderma* isolates for abiotic stress and evaluation against collar rot of lentil. Doctoral dissertation, Department of Plant Pathology, BAU, Sabour, 210p.

Singh, A., Shahid, M., Srivastava, M., Pandey, S., Sharma, A., and Kumar, V. 2014. Optimal physical parameters for growth of *Trichoderma* species at varying pH, temperature and agitation. *Virol. Mycol.* 3(1): 1-7.

Smith, V. L., Wilcox, W. F., and Harman, G. E. 1990. Potential for biological control of *Phytophthora* root and crown rots of apple by *Trichoderma* and *Gliocladium* spp. *Phytopathol.* 80(9): 880-885.

Smitha, C., Finosh, G. T., Rajesh, R., and Abraham, P. K. 2014. Induction of hydrolytic enzymes of phytopathogenic fungi in response to *Trichoderma viride* influence biocontrol activity. *Int. J. Curr. Microbiol. App. Sci.* 3(9): 1207-1217.

Soliman, M. S., Shalabi, H. G., and Campbell, W. F. 1994. Interaction of salinity, nitrogen, and phosphorus fertilization on wheat. *J. plant Nutr.* 17(7): 1163-1173.

Sonavane, P. and Venkataravanappa. 2017. Compatibility Studies of *Trichoderma harzianum* isolate with fungicides used against soil borne disease in Coorg mandarin-pepper-coffee plantations. *Int. J. Curr. Microbiol. App. Sci.* 6(8): 346-354.

Sreedevi, B., Charitha Devi, M., and Saigopal, D. V. R. 2011. Isolation and screening of effective *Trichoderma* spp. against the root rot pathogen *Macrophomina phaseolina. J. Agric. Technol.* 7(3): 623-635.

Stocco, M., Mónaco, C., and Cordo, C. 2010. A comparison of preservation methods for *Trichoderma harzianum* cultures. *Revista iberoamericana de micologia*, 27(4): 213-215.

Sundaramoorthy, S. and Balabaskar, P. 2013. Biocontrol efficacy of *Trichoderma* spp. against wilt of tomato caused by *Fusarium oxysporum* f. sp. *lycopersici. J. Appl. Biol. Biotechnol.* 1(03): 36-40.

Sutton, D. A., Fothergill, A. W., and Rinaldi, M. G. 1998. *Guide to Clinically Significant Fungi* (1st Ed.). Williams & Wilkins, Baltimore, 471 p.

Tapwal, A., Kumar, R., Gautam, N., and Pandey, S. 2012. Compatibility of *Trichoderma viride* for selected fungicides and botanicals. *Int. J. Plant Pathol.* 3(2): 89-94.

Thangavelu, R., Palaniswami, A., and Velazhahan, R. 2004. Mass production of *Trichoderma harzianum* for managing *Fusarium* wilt of banana. *Agric. Ecosyst. Environ.* 103(1): 259-263.

Tomar, A., Prasad, L., Mishra, A., and Sagar, S. 2017. Antagonistic Action of *Trichoderma* isolates against *Fusarium oxysporum* f. sp. *lycopersci. Int. J. Curr. Microbiol. App. Sci.* 6(2): 258-265.

Tsuchida, O., Yamagata, Y., Ishizuka, T., Arai, T., Yamada, J., Takeuchi, M., and Ichishima, E. 1986. An alkaline proteinase of an alkalophilic *Bacillus* sp. *Curr. Microbiol.* 14:7.

Upadhyay, J. P. and Mukhopadhyay, A. N. 1986. Biological control of *Sclerotium rolfsii* by *Trichoderma harzianum* in sugarbeet. *Int. J. Pest Manag.* 32(3): 215-220.

Upadhyay, R. S. and Rai, B. 1979. Ecological survey of Indian soil fungi with special reference to *Aspergillus*, *Peniciilium* and *Trichoderma*. *Rev. Ecol. Biol. of Soil* 16: 39-49.

Vinale, F., Sivasithamparam, K., Ghisalberti, E. L., and Lorito, M. 2008. *Trichoderma*-plant-pathogen interatctions. *Soil Biol. Biochem.* 40: 1–10.

Vincent, J. M. 1927. Distortion of fungal hyphae in the presence of certain inhibitors. *Nat.* 59:850.

Viterbo, A., Landau, U., Kim, S., Chernin, L., and Chet, I. 2010. Characterization of ACC deaminase from the biocontrol and plant growth-promoting agent *Trichoderma asperellum* T203. *FEMS Microbiol. Lett.* 305(1): 42-48.

Vithya, S. 2017. Evaluation of abiotic stress tolerant PGPR and *Trichoderma* sp. for growth and disease management in black pepper (*Piper nigrum* L.). MSc(Ag) thesis, Kerala Agricultural University, Thrissur, 113p.

Volesky, B., May, H., and Holan, Z. R. 1993. Cadmium biosorption by *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* 41: 826–829.

Wang, C., Knill, E., Glick, B. R., and Defago, G. 2000. Effect of transferring 1-aminocyclopropane-1 carboxylic acid (ACC) deaminase genes into *Pseudomonas fluorescens* strain CHA0 and its gacA derivative CHA96 on their growth-promoting and disease-suppressive capacities. *Can. J. Microbiol.* 46:898–907.

Waskale, T. 2017. MSc (Ag) thesis. Studies on bioefficacy of *Trichoderma* strains against *Fusarium oxysporum* f. sp. *ciceri* and their compatibility with fungicides, Rajmata Vijayaraje Scindia Krishi Vishwa Vidyalaya,Gwalior. 59p.

Wen, Z., Liao, W., and Chen, S. 2005. Production of cellulase by *Trichoderma reesei* from dairy manure, *Bioresour. Technol.* 96: 491–499.

Wessels, J. G. H. 1986. Cell wall synthesis in apical hyphal growth. *Int. Rev. Cytol.* 104: 37–79.

Wheeler, B. E. J. 1969. An Introduction to plant disease and fungi, John Wiley. *Phytopathol.* 22: 837- 845.

Widden, P. and Hsu, D. 1987. Competition between *Trichoderma* species: effects of temperature and litter type. *Soil Biol. Biochem.* 19(1): 89-93.

Wilson P. S., Ketola, E. O., Ahvenniemi, . P. M., Lehtonen, M. J., and Valkonen, J. P. T. 2008. Dynamics of soilborne *Rhizoctonia solani* in the presence of *Trichoderma harzianum*: effects on stem canker, black scurf and progeny tubers of potato. *Plant Pathol.* 57(1): 152–161.

Windham, M.T., Elad, Y., and Baker, R. 1986. A mechanism for increased plant growth induced by *Trichoderma* spp. *Phytopathol.* 76: 518–521.

Yaquab, F. and Shahzad, S. 2005. *In vitro* evaluation of microbial antagonists against *Sclerotium rolfsii. Pakist. J. Bot.* 37(4): 1033.

Zhang, J., Howell, C. R., and Starr, J. L. 1996. Suppression of *Fusarium* colonization of cotton roots and *Fusarium* wilt by seed treatments with

249

Gliocladium virens and *Bacillus subtilis*. *Biocontrol Sci. Technol.* 6: 175-187.

Zhang, F., Chen, C., Zhang, F., Gao, L., Liu, J., Chen, L., Fan, X., Liu, C., Zhang, K., He, Y., and Chen, C. 2017. *Trichoderma harzianum* containing 1-aminocyclopropane-1-carboxylate deaminase and chitinase improved growth and diminished adverse effect caused by *Fusarium oxysporum* in soybean. *J. Plant Physiol.* 210: 84-94.

Appendices

APPENDIX- I

Composition of media used for serial dilution

1. *Trichoderma* selective medium (TSM) (Elad *et al., 1981*)

Glucose	: 3.0 g
NH_4NO_3	: 1.0 g
KCl	: 0.15 g
K_2HPO_4	: 1.0 g
$MgSO_4. 7H_2O$	: 0.5 g
Rose Bengal	: 150 mg
Agar	: 15 g
Distilled water	: 1000 ml

2. Malt extract agar (MEA)

Malt extract	: 20 g
Agar	: 20 g
Distilled water	: 1000 ml

3. Oat meal extract agar (OEA)

Oat meal	: 60 g
Agar	: 12.5 g
Distilled water	: 1000 ml

4. Potato dextrose agar (PDA)

Potato	: 200 g
Dextrose	: 20 g
Agar	: 20g
Distilled water	: 1000 ml

5. Special nutrient agar (SNA) (Harman *et al.,* 1991)

KH_2PO_4	: 1.0 g
$MgSO_4. 7H_2O$	: 0.5 g
KCl	: 0.2 g
Glucose	:0.2 g
Sucrose	: 1.0 g
Agar	: 20.0 g
Distilled water	: 1000 ml

APPENDIX II

Composition of media used in enzyme assay

1. Carboxy methyl cellulose medium (CMC)

Sodium nitrate	: 2.0 g
Dipotassium phosphate	: 1.0 g
Magnesium sulphate	: 0.5 g
Potassium chloride	: 0.5 g
Carboxy methyl cellulose	: 2.0 g
Peptone	: 0.02 g
Agar	: 17.0 g
Distilled water	: 1000 ml

2. Casein broth

Glucose	: 15 g
Yeast extract	: 5 g
$CaCl_2$	: 2 g
Distilled water	: 1000 ml

APPENDIX III

Composition of media used for ACC deaminase assay

1. Dworkin and Foster salts minimal medium (Dworkin and Foster, 1958)

Glucose	: 2.0 g
Gluconic acid	: 2.0 g
Citric acid	: 2.0 g
KH_2PO4	: 4.0 g
Na_2HPO4	: 6.0 g
MgSO4.7H2O	: 0.2 g

Micro nutrient solution (10 ml solution)

$CaCl_2$	: 200 mg
$FeSO_4.7H2O$	: 200 mg
H_3BO_3	: 15 mg
$ZnSO_4.7H2O$	: 20 mg
Na_2MoO_4	: 10 mg
KI	: 10 mg
NaBr	: 10 mg
$MnCl_2$	: 10 mg
$COCl_2$	: 5 mg
$CuCl_2$	: 5 mg
$AlCl_3$	: 2 mg
$NiSO_4$	: 2 mg
Distilled water	: 1000 ml
ACC	: 3 mM

Exploitation of abiotic stress tolerant strains of *Trichoderma* spp. for the management of soil borne fungal pathogens

By

STELLA DONCY P.P.

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ABSTRACT OF THE THESIS

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ABSTRACT

Soil borne phytopathogenic fungi are known to cause severe yield loss of several crops. To mitigate these crop ailments, farmers mostly rely on chemical methods of disease control such as fungicides and other pesticides which are deleterious to the environment. To date, biological control is an ecofriendly approach for the effective management of crop diseases. The fungi belonging to the genus *Trichoderma* are one among the most exploited biocontrol agents in the field of agriculture. However, the performance of *Trichoderma* spp. gets reduced when it is exposed to abiotic stressed conditions such as drought, high temperature, salinity, acidity and fungicides. Hence, this study was proposed to identify and exploit stress tolerant isolates of *Trichoderma* spp. with antagonistic potential in Kerala.

Intensive soil sampling surveys were conducted across different stressed ecosystems of Kerala *viz.,* Palakkad, Alappuzha, Vytilla, Kumarakom, Wayanad and Thrissur for the isolation and enumeration of native *Trichoderma* spp. A total of 24 isolates were obtained from 52 soil samples collected from different locations. Based on the number of *Trichoderma* spp. obtained from each district, they were serially numbered and abbreviated according to the name of the location. Accordingly, PAT 1 to PAT 6 represents number of isolates of *Trichoderma* spp. from Palakkad district, ALT 1 to ALT 3 from Alappuzha, VYT 1 and VYT 2 from Ernakulam district, KUT 1 from Kottayam district, WAT 1 to WAT 8 from Wayanad and THT 1 to THT 4 from Thrissur district. Cultural and morphological identification of these isolates were carried out under *in vitro* conditions.

Isolates of *Trichoderma* spp. were subjected to *in vitro* screening for abiotic stress tolerance such as high temperature, drought, acidity, salinity and also to test their sensitivity towards copper fungicides. The isolates PAT6 and WAT2 were found as thermotolerant, VYT2 and ALT 1 as drought tolerant, ALT 3 and ALT 1 as acid tolerant and saline tolerant and the isolates ALT1, ALT3 and PAT 1 as copper fungicide tolerant. The selected six isolates were

further subjected to biochemical tests and the study showed that the isolates VYT 2, ALT 3 and ALT 1 showed highest cellulase, β- 1, 3 glucanase and protease activity. Likewise, isolates PAT 1, ALT 1 and ALT 3 were found as best producers of ACC deaminase and PAT 1 and ALT 1 as the best cytokinin producer.

The best performing isolates (ALT 1, ALT 3, PAT 1, PAT 6 and VYT 2) after enzyme study were subjected to dual culture experiment with five major soil borne pathogens to test their antagonistic potential. The isolates ALT 3, ALT 1, PAT 6 and PAT 1 showed more than 70 per cent inhibition of *R. solani* whereas, isolates ALT1 and VYT 2 showed only 57.78 and 55 per cent inhibition of *S. rolfsii* respectively. However, no significant difference was noticed among the isolates when grown against *F. solani*. Cent per cent inhibition of *P. aphanidermatum* was noticed with *Trichoderma* isolates PAT 1 and ALT 1. All five isolates showed 100 per cent inhibition on the growth of pathogen, *P. capsici*. Among the five, four isolates *viz.,* ALT 1, ALT 3, PAT 1 and PAT 6 with best antagonistic potential were subjected to molecular characterization at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram and the isolates showed homology with the nucleotide sequence of *Trichoderma asperellum*.

A pot culture experiment was laid with the selected isolates *viz.,* ALT 1, ALT 3, PAT 1 and PAT 6 to test the growth promotion of cowpea and biocontrol efficacy against *Rhizoctonia solani*. It was observed that the isolate PAT 6 coated seeds showed 100 per cent germination and also recorded better biometric characters and yield. Moreover, the lowest per cent disease incidence of 11.11 per cent was only recorded with both the isolates ALT 3 and PAT 6.

Thus, the study has enlightened our knowledge on the existence of abiotic stress tolerant isolates of *T. asperellum* which can be employed in future for the biocontrol of soil borne pathogens in such conditions.